Suppression of Apoptosis by Non-Genotoxic Carcinogens.

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medicine.

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Alison Claire Bayly

School of Biological Sciences

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abstract.

Peroxisome proliferators are a diverse group of non-genotoxic rodent liver carcinogens that exert their effects without directly damaging or interacting with DNA. Non-genotoxic carcinogens cannot be identified on the basis of their structure or their effects on DNA. In order to assess the potential human hazard posed by these chemicals, the mechanism(s) responsible for their hepatocarcinogenicity must be established. A number of mechanisms have been proposed to explain the carcinogenicity of the peroxisome proliferators. One novel hypothesis is that peroxisome proliferators and other non-genotoxic carcinogens may both suppress the normal process of apoptosis in the liver and elevate mitosis, facilitating the survival and proliferation of damaged or potentially tumourigenic cells.

In this thesis, the ability of peroxisome proliferators to suppress apoptosis has been examined *in vitro* using primary cultures of rat hepatocytes and the Reuber hepatoma cell line Fao. Following a systematic assessment of a range of hepatoma cell lines, Fao was validated as being a suitable model system for this purpose: Fao cells were found to be well differentiated, were easy to manipulate and responded to peroxisome proliferators.

The peroxisome proliferators nafenopin and Wy-14,643 reversibly maintained the viability of primary cultures of rat hepatocytes which otherwise degenerated within 8 days of their establishment. Maintenance of hepatocyte viability was associated with a significant reduction in hepatocyte apoptosis. Apoptosis could be induced in primary hepatocyte and Fao cultures by treatment with the physiological negative growth regulator TGFβ_1, which has been implicated in the control of hepatocyte apoptosis *in vivo*. Co-addition of nafenopin or Wy-14,643 reduced TGFβ_1-induced hepatocyte and Fao apoptosis significantly. In contrast nafenopin had no effect on Fao apoptosis induced by the DNA damaging agents etoposide and hydroxyurea. Furthermore, nafenopin had no apparent effect on the level of expression of Bcl-2, a known suppressor of drug-induced apoptosis in a number of other cell systems. These results imply that peroxisome proliferators do not suppress apoptosis regardless of the death stimulus and that they may interfere specifically with the TGFβ_1 signalling pathway.

Maintenance of hepatocyte viability by nafenopin was not associated with a detectable change in the level of expression of $TGF\beta_1$ mRNA nor by a change in the mRNA for the mannose-6-phosphate receptor, which is believed to mediate the transport of $TGF\beta_1$ into hepatocytes from neighbouring cells. While these investigations into the molecular mechanism(s) by which peroxisome proliferators suppress apoptosis were not conclusive, the results presented in this thesis provide a solid foundation on which detailed mechanistic studies can be based in the future.

Declaration.

I declare that this thesis is my own composition. No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this, or any other university or other institute of learning.

Notes on Copyright.

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This thesis is dedicated with love to the memory of my grandparents.

Abbreviations.

AFB1	Aflatoxin B1
AIDS	Acquired immune deficiency syndrome
AO	Acridine orange
ATA	Aurintricarboxylic acid
ATP	Adenosine triphosphate
BD	Becton Dickenson
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
$[Ca^{2+}]$	Intracellular calcium
CAGE	Conventional agarose gel electrophoresis
cAMP	Cyclic-AMP
CCl_4	Carbon tetrachloride
CD3 (4,8,36)	Cluster determinant 3 (4,8,36)
CDK	Cyclin dependent kinase
C. Elegans	*Caenorhabditis elegans*
CML	Chronic Myeloid Leukaemia
CTL	Central Toxicology Laboratory
2,4-D	Dichlorophenoxyacetic acid
DAG	Diacylglycerol
ddH_2O	Double distilled water
DEHP	Dehydroepiandrosterone
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modification of Eagles Medium
DMF	Dimethylfluoride
DMN	Dimethylnitrosamine
DMSO	Dimethylsulphoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(b-aminoethyl)N' tetraacetic acid

EGF	Epidermal growth factor
FCM	Flow cytometry
FCS	Foetal calf serum
FIGE	Field inversion gel electrophoresis
GGT	Gammaglutamyl transpeptidase
GST-P	Placental glutathione S-transferase
Ham's F12	Ham's Nutrient Mixture F12
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor
Ho258	Hoechst 33258
HRE	Hormone response element
H_2O_2	Hydrogen peroxide
ICE	Interleukin 1-β-converting enzyme
Ig	Immunoglobulin
IL1-β	Interleukin 1-β
IL-3 (2,4,6)	Interleukin-3 (2,4,6)
IP_3	Inositol 1,4,5-triphosphate
kb	Kilo base
kD	Kilo Dalton
M-6-PR	Mannose-6-phosphate receptor
min.	Minute(s)
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NGF	Nerve growth factor
pADPRp	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
PPBP	Peroxisome proliferator binding protein

PPAR	Peroxisome proliferator activated receptor
pRb	Retinoblastoma protein
prICE	Protease resembling ICE
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SGP-2	Sulphated glycoprotein 2
SV40	Simian vacuolating virus 40
TB	Trypan blue
TβR-1(II)	TGFβ receptor I (II)
TBS	Tris-buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TdT	Terminal deoxynucleotidyl transferase
TPA	12-O-Tetradecanoylphorbol-13-acetate
TPE	Tris-phosphate-EDTA
tTgase	Tissue transglutaminase
TGFβ (α)	Transforming growth factor β (α)
TNFα	Tumour necrosis factor α
TRPM-2	Testosterone repressed prostate message 2
UDS	Unscheduled DNA synthesis
UV	Ultra violet
wt	Wild type

Chapter 1.

General Introduction.

1. General Introduction.

Until relatively recently, it was assumed that the majority of chemical carcinogens exert their effects by damaging DNA (Ames *et al.*, 1973). However, a group of chemicals exist which do not directly interact with or damage DNA, but which cause cancer in experimental animals (Ashby and Tennant, 1991). How do such non-genotoxic carcinogens contribute to the development of cancer? This question is one of fundamental importance in cancer research and forms the basis of the work described in this thesis. The theory under investigation here is that non-genotoxic carcinogens may suppress the normal process of cell death by apoptosis, allowing the anomalous survival of damaged or potentially tumourigenic cells. This theory has been examined using one group of non-genotoxic rodent liver carcinogens: the peroxisome proliferators.

1.1 Carcinogenesis.

It is widely accepted that carcinogenesis is a multi-stage process, the three main stages being initiation, promotion and progression (Pitot, 1993). Initiation is the induction of irreversible and heritable genetic changes, which can occur spontaneously or as a result of exposure to ionising radiation, ultraviolet (UV) light or genotoxic chemicals (Farber and Cameron, 1980; Pitot, 1993). These changes, which usually take the form of simple mutations, transversions, transitions or deletions in DNA, are not in themselves sufficient for carcinogenesis, but must be 'fixed' into the genome. Indeed, the majority of initiated cells do not progress to a neoplastic state but remain quiescent unless exposed to a promoting agent (Pitot, 1993). Promotion is the selective amplification of initiated cells and is thought to involve cell proliferation and / or suppression of differentiation or cell death (Bursch *et al.*, 1992; Pitot, 1993). Such focal proliferation leads to the development of nodules, papillomas or altered cell foci, whose persistence is dependent on continuous exposure to the promoting agent. This stage is reversible and withdrawal of tumour promoters often leads to regression of promoted lesions. The third, irreversible stage in cancer development is termed progression and involves further genetic changes leading to malignant growth (Pitot, 1993). A fourth and less clearly defined stage is the subsequent invasion of cancer cells

into other tissues. It is typically this metastasis that is ultimately lethal to the patient (reviewed by Farber and Cameron, 1980).

1.2 Genotoxic and Non-Genotoxic Carcinogenesis.

Neoplasia occurs as a result of mutations in two classes of genes (Solomon *et al.*, 1991). Proto-oncogenes are activated by dominant genetic mutations and the expression of these activated, mutant genes leads to perturbed regulation of cell proliferation, cell differentiation and / or cell death (Bishop, 1991; Yarnold, 1991). In contrast, alterations to tumour suppressor genes cause a loss of normal gene function, releasing cells from the growth constraints normally placed upon them (Weinberg, 1991; Patterson, 1992). Mutations of proto-oncogenes and tumour suppressor genes represent the initiation event of carcinogenesis and may be inherited, as occurs in diseases such as retinoblastoma, Wilms tumour (Knudsen, 1986) and some forms of breast cancer (Porter *et al.*, 1993; Vogelstein and Kinzler, 1994), or alternatively may arise from exposure to genotoxic carcinogens, which directly interact with and damage DNA (Purchase, 1994). The mutagenicity and therefore the carcinogenic potential of such genotoxic agents can be assessed using tests such as the Ames *Salmonella* test (Ames *et al.*, 1975).

Recent studies have demonstrated that certain carcinogens exert their effects without any direct interaction with DNA (reviewed by Ashby and Tennant, 1991; Purchase, 1994). These non-genotoxic carcinogens are thought to act by potentiating the survival and clonal expansion of preneoplastic cells (Melnick, 1992; Purchase, 1994). An important feature of non-genotoxic carcinogens is that, although they may damage DNA indirectly, they do not need to be present at the time of mutation (Ashby, 1992). In rodents, the most common site of action of non-genotoxic carcinogens is the liver, although the skin and kidney can often be affected (Green, 1991). Since non-genotoxic carcinogens are not positive in tests such as the Ames test and cannot often be identified on the basis of their chemical structure, the risk they pose to humans is often difficult to assess (Green, 1991; Ashby, 1992).

Non-genotoxic carcinogens can be divided into a number of classes on the basis of their mode of action. Excess production of trophic hormones, including thyroid stimulating hormone, oestrogens and progestogens stimulates hyperplasia and ultimately

neoplasia in an apparently tissue- and species-specific manner (Purchase, 1994). Other chemicals, including saccharin and 1,2 dichlorobenzene, induce mitogenesis as a compensatory response to their prolonged cytotoxicity (Anderson, 1990; Grasso *et al.*, 1991). The enhanced rate of cell division stimulated by these chemicals is thought to 'fix' potentially carcinogenic mutations into a cell population (Cohen and Ellwein, 1990). Non-genotoxic carcinogenesis is often receptor-mediated (Green, 1991; Poellinger *et al.*, 1992). For example, the most potent non-genotoxic carcinogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) activates the intracellular dioxin receptor (Whitlock, 1993). A distinct group of chemicals called peroxisome proliferators activate another member of the steroid hormone receptor superfamily, termed the peroxisome proliferator-activated receptor (PPAR; Issemann and Green, 1990). The mechanism of carcinogenicity of this latter group of carcinogens is examined in this thesis.

1.3 Peroxisome Proliferators.

Peroxisome proliferators form a diverse group of chemicals that includes industrial phthalate ester plasticisers, such as di(2-ethyl-hexyl) phthalate (DEHP, Lake *et al.*, 1975), herbicides, including 2,4-dichlorophenoxyacetic acid (2,4-D, Vainio *et al.*, 1983) and hypolipidaemic drugs, which are used widely in the treatment of coronary heart disease (Reddy *et al.*, 1980; Reddy and Lalwani, 1983). Examples of hypolipidaemic drugs are clofibrate and its structural analogues which include nafenopin and methylclofenapate and compounds that are structurally unrelated to clofibrate such as Wy-14,643 and BR-931. Peroxisome proliferation can also be induced by a number of nutritional conditions such as a high fat diet or vitamin E deficiency and can occur as a result of hypothermia (Reddy and Lalwani, 1983). Although peroxisome proliferators are structurally diverse, selected examples of their structures are given in Fig. 1.1.

Peroxisome proliferators are so called because they stimulate a dramatic increase in the number of peroxisomes in hepatocytes (Reddy and Lalwani, 1983). Peroxisomes are single, membrane-bound cytoplasmic organelles which are distributed widely in most eukaryotic cells and which have roles in cell respiration, gluconeogenesis, purine catabolism, thermogenesis and most importantly lipid metabolism (Reddy and Lalwani, 1983). Indeed, over half the enzymes present in the mammalian peroxisome are involved

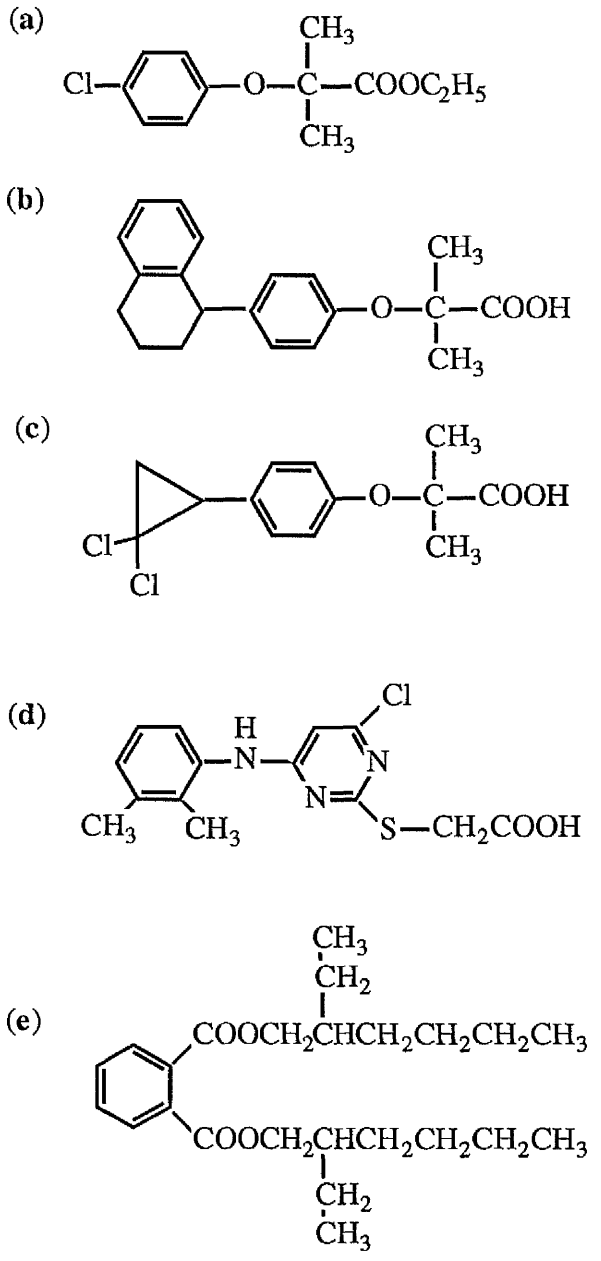


Figure 1.1. Chemical Structures of Selected Peroxisome Proliferators. (from Reddy and Rao, 1986). **(a)** clofibrate (ethyl-α-*p*-chlorophenoxyisobutyrate); **(b)** nafenopin (2-methyl-2[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy] propionic acid); **(c)** ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)phenoxy]2-methyl propionic acid); **(d)** Wy-14,643 ([4-chloro-6-(2,3-xylidino)2-pyrimidinylthio] acetic acid); **(e)** DEHP (di(2-ethylhexyl)phthalate).

in the metabolism of fatty acids (Mannaerts and Van-Veldhoven, 1990). The activation of fatty acids prior to their metabolism is mediated by a long chain acyl-CoA synthetase, with the result that long-chain fatty acids are preferentially activated and metabolised (Reddy and Lalwani, 1983; Mannaerts and Van-Veldhoven, 1990). Activated fatty acids are then oxidised, firstly by acyl-CoA oxidase and then by other enzymes of β-oxidation, yielding acetyl-CoA, shortened acyl-CoA and hydrogen peroxide (H_2O_2) as a by-product. Carnitine acyl-transferases convert acetyl-CoA and acyl-CoA to carnitine esters which are then further oxidised in the mitochondria and H_2O_2 is decomposed to oxygen and water by catalase, a marker enzyme for peroxisomes (De Duve and Baudheim, 1966; Van den Bosch *et al.*, 1992).

1.3.1 Hepatic Responses to Peroxisome Proliferators.

The cellular responses to peroxisome proliferators have been characterised most extensively in the rodent liver and these are discussed below. Evidence of a response to peroxisome proliferators in non-rodent systems is discussed on page 28. Under normal physiological conditions, the hepatocytes of rodents contain few peroxisomes, with the ratio of peroxisomes to mitochondria being approximately 1:8 (Reddy and Lalwani, 1983). However, following treatment with a peroxisome proliferator there is a dramatic increase in peroxisome number, size and structure, the extent of which is dependent on the peroxisome proliferator and the dose and duration of exposure to the chemical (Cohen and Grasso, 1981; Reddy and Lalwani, 1983). For example, an oral dose of 500 mg/kg of the hypolipidaemic drug clofibrate to CFE rats stimulates a 10-fold increase in the number of hepatocyte peroxisomes (Svoboda *et al.*, 1967), while a 100 mg/kg dose of nafenopin stimulates a doubling of peroxisomes and a 6-fold increase in the size of the peroxisomal compartment (Staubli *et al.*, 1977). The increase in peroxisomal number can occur within as little as 24 hours of treatment and usually reaches a steady state level after about 14 days (Hess *et al.*, 1965; Svoboda *et al.*, 1967).

Peroxisome proliferation is accompanied by liver enlargement, which results from both hepatocyte hypertrophy and hyperplasia (Moody *et al.*, 1977). Such hepatomegaly is a characteristic response of laboratory animals to xenobiotics and is termed 'additive growth' to distinguish it from the liver growth that occurs during

development or regeneration after injury (Schulte-Hermann, 1974). As seen with the effect on peroxisomal number, the extent of liver hyperplasia in response to peroxisome proliferators is compound-dependent. Furthermore, doses of different compounds that produce equivalent levels of peroxisome proliferation can stimulate varying degrees of hepatomegaly and hyperplasia (Moody *et al.*, 1992). For example the peroxisome proliferators nafenopin and Wy-14,643 induce far more extensive hyperplasia than clofibrate and DEHP (Moody and Reddy, 1978). The increase in liver size and weight seen in response to peroxisome proliferators is associated not only with peroxisome proliferation but also with proliferation of the smooth endoplasmic reticulum and stimulation of replicative DNA synthesis (Reddy *et al.*, 1979; Cohen and Grasso, 1981).

Ultimately, chronic exposure of rodents to peroxisome proliferators results in the development of liver tumours (Reddy *et al.*, 1980). Again, the efficiency of different peroxisome proliferators in inducing liver tumours is variable. Administration of Wy-14,643 to Fischer rats at 0.1% $^{w}/_{w}$ in the diet for 16 months led to 100% incidence of hepatocellular carcinomas in animals that survived the course of the treatment (Reddy *et al.*, 1979). Similarly, chronic administration of nafenopin at a level of 0.1% $^{w}/_{w}$ in the diet for 25 months led to 11/15 Fischer rats developing hepatocellular carcinomas (Reddy and Rao, 1977). In contrast, administration of the weaker carcinogen DEHP at a dietary concentration of 1.2% $^{w}/_{w}$ for 2 years led to only a 10% incidence of liver tumours (Kluwe *et al.*, 1982). A summary of carcinogenicity studies conducted with selected peroxisome proliferators is given in Table 1.1.

In addition to stimulating a variety of morphological changes in liver cells, peroxisome proliferators also trigger the induction of a number of liver enzymes (Orton and Parker, 1982; Moody *et al.*, 1992). This induction is commonly used as a marker for the biological effects of peroxisome proliferators. A consistent response of hepatocytes to peroxisome proliferators is the induction of peroxisomal enzymes of β–oxidation, including acyl-CoA oxidase and peroxisomal bifunctional enzyme (Lazarow and De Duve, 1976; Lazarow, 1977), and carnitine acetyltransferases (Moody and Reddy, 1974). The induction of these enzymes arises from an elevation in mRNA translation, mRNA content and gene transcription (Reddy and Lalwani, 1983; Chatterjee *et al.*, 1987). The induction of peroxisomal β-oxidation by the peroxisome proliferators bezafibrate and

Compound	Species	Sex	Dietary Level (%)	Duration (months)	No. of Animals Initial	Effective*	Animals with Tumours (%)	References
Clofibrate	F-344 rats	Male	0.5	24-28	15	11	10 (91%)	Reddy and Quereshi, 1979
Nafenopin	F-344 rats	Male	0.1	18-25	15	15	12 (80%)	Reddy and Rao, 1977
Nafenopin	CS mice	Male	0.1 (0.05†)	12 (8†)	9	9	9 (100%)	Reddy *et al.*, 1986
Wy-14,643	F-344 rats	Male	0.1	16	15	15	15 (100%)	Reddy *et al.*, 1979
BR-931	F-344 rats	Male	0.2	16	20	20	20 (100%)	Reddy *et al.*, 1980

Table 1.1. Incidence of Liver Tumours in Rats and Mice Fed Hypolipidaemic Peroxisome Proliferators.

(adapted from Reddy and Lalwani, 1983).

* Number of animals alive at onset of first tumour.

† Nafenopin was administered at 0.1% $^{w}/_{w}$ for 12 months followed by 0.05% $^{w}/_{w}$ for a further 8 months.

clofibrate has been shown to be related to the induction of liver fatty acid binding protein, which is thought to be responsible for the intracellular transport of fatty acids and for the protection of hepatocytes from the toxicity of high levels of fatty acids (Brandes *et al.*, 1990).

Peroxisome proliferators are also transcriptional activators of an isozyme of cytochrome P450, namely cytochrome P4504A1 (Orton and Parker, 1982; Moody *et al.*, 1992). This microsomal enzyme is responsible for the ω-hydroxylation of medium and long chain fatty acids. A close association exists between the induction of cytochrome P4504A1 and the peroxisomal enzymes of β-oxidation (Sharma *et al.*, 1988). The induction of cytochrome P4504A1 is thought to be an early event following administration of peroxisome proliferators, which may actually precede the process of peroxisome proliferation itself. It has been proposed that ω-hydroxy fatty acids, derived from the activity of cytochrome P4504A1, are metabolised further to long chain dicarboxylic acids and it is these which act as the stimulus for peroxisome proliferation (Sharma *et al.*, 1988; Gibson *et al.*, 1990).

The *in vivo* potency of a particular peroxisome proliferator appears to be determined, at least in part, by the rate at which the chemical is absorbed, its distribution pattern and rate of excretion (Schulte-Hermann, 1974; Reddy and Lalwani 1983). However, the exact mechanism(s) by which peroxisome proliferators exert their many effects remain to be established. In a number of different biological systems, the mitogenic response of a cell to growth factors is mediated by a cascade of signals, transduced from the cell membrane to the intracellular target(s) of the growth factor (Cohen, 1992). These signals typically involve changes in intracellular calcium ($[Ca^{2+}]$), activation of second messengers such as protein kinase C (PKC) and elevated transcription of cellular proto-oncogenes (Bieri, 1993). A number of studies indicate that these responses may also be involved in the response of hepatocytes to peroxisome proliferators.

1.3.1.1 Effect on Calcium and Inositol Phosphate Cascades.

The effect of the peroxisome proliferator nafenopin on $[Ca^{2+}]$ and the inositol phosphate cascade has been investigated (Ochsner *et al.*, 1990). An immediate and

sustained, concentration-dependent increase in [Ca^{2+}] was detected in primary hepatocytes after addition of nafenopin at mitogenic doses. This was followed by an increased rate of efflux of Ca^{2+} from the cells. The increase in [Ca^{2+}] was only slightly affected by a depletion of extracellular Ca^{2+} concentrations, indicating that nafenopin mobilises Ca^{2+} mainly from intracellular stores (Ochsner *et al.*, 1990). Other agents that stimulate DNA synthesis in the liver, including vasopressin and angiotensin II, also mobilise Ca^{2+} in liver cells via a mechanism involving the second messenger inositol 1,4,5-triphosphate (IP_3; Michalopoulos, 1990). It appears that while nafenopin mobilises Ca^{2+} via a mechanism independent of IP_3, it can impair the IP_3 cascade induced by other liver mitogens (Ochsner *et al.*, 1990). It is not yet clear whether peroxisome proliferators such as nafenopin directly inhibit phosphatidylinositol kinases or whether the elevation in [Ca^{2+}] induced by nafenopin impairs the inositol phosphate cascade via a negative feedback mechanism. However, since Ca^{2+} mobilisation is involved in lipid metabolism, it is possible that some of the effects of nafenopin, and perhaps other peroxisome proliferators on lipid metabolism are mediated via an increase in [Ca^{2+}] (Ochsner *et al.*, 1990).

1.3.1.2 Activation of Protein Kinase C.

The term PKC encompasses a family of closely related Ca^{2+}-dependent serine threonine kinases which are activated by diacylglycerol (DAG), one of the products of receptor-mediated inositol phospholipid metabolism (Huang, 1989). PKC activation has been implicated in the regulation of a number of cellular processes, including proliferation and differentiation (Huang, 1989; Nelsestuen and Bazzi, 1991; Grunicke and Uberall, 1992). A number of tumour promoters, including the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), mimic the effects of DAG, increasing the affinity of PKC for Ca^{2+} and thus increasing the activity of the enzyme at equivalent [Ca^{2+}] (Bieri, 1993).

Several peroxisome proliferators are activated to acyl-CoA thioesters by the microsomal enzyme acyl-CoA synthetase and it has been proposed that these acyl-CoA thioesters are the pharmacologically active form of the chemicals (Bronfman *et al.*, 1986). While peroxisome proliferators themselves do not activate PKC, acyl-CoA

thioesters of the peroxisome proliferators clofibric acid, nafenopin, ciprofibrate, bezafibrate and tibric acid, have been shown to stimulate rat liver and brain PKC activity (Bronfman *et al.*, 1989). A role for PKC in the response of hepatocytes to peroxisome proliferators is also supported by the finding that inhibitors of PKC activity suppressed clofibrate-induced enzyme induction (Watanabe *et al.*, 1992) and the induction of DNA synthesis by nafenopin (Bieri *et al.*, 1991). While these findings support a role for PKC in peroxisome proliferator activity it is not yet apparent which isoforms of PKC are involved.

1.3.2 Response of Other Organs to Peroxisome Proliferators.

The most dramatic response of rodents to peroxisome proliferators is seen in the parenchymal cells of the liver. However, some peroxisome proliferation has also been detected in the renal cortex of mice treated with the peroxisome proliferator methylclofenapate (Reddy *et al.*, 1975). Furthermore, induction of enzymes of peroxisomal β-oxidation has been demonstrated in cells of the renal cortex following treatment with BR-931, Wy-14,643 and fenofibrate (Lalwani and Reddy, 1981). A slight elevation in peroxisomal number was also detected in the myocardial cells of rats following treatment with both bezafibrate and clofibrate (Fahimi *et al.*, 1980). However, although peroxisome proliferator-induced metastases have been detected in the lung and pancreas of experimental animals (Reddy and Lalwani, 1983), there is no evidence that peroxisome proliferators cause the development of tumours in these tissues.

1.3.3 Species-Specific Action of Peroxisome Proliferators.

Pronounced species differences in response to peroxisome proliferators are apparent. Indeed, while the effects of peroxisome proliferators on the rodent liver have been well characterised, evidence of a response to peroxisome proliferators in non-rodent systems is far less forthcoming. Reddy *et al.*, (1984) detected both peroxisome proliferation and increased levels of peroxisomal enzymes in the livers of cats, chickens, pigeons, rhesus monkeys and cynomolgus monkeys exposed to the peroxisome proliferator ciprofibrate. Peroxisome proliferation and an associated induction of the enzymes of peroxisomal β-oxidation have also been detected in the amphibian *Rana*

esculenta following administration of clofibrate (Ciolek and Dauca, 1991). However other studies, both *in vivo* and *in vitro*, have highlighted marked species differences in response to a range of peroxisome proliferators (Lake *et al.*, 1989; Foxworthy *et al.*, 1990), with enzyme induction occurring in the absence of peroxisome proliferation. Clofibrate induced hepatomegaly in beagle dogs but induced only a slight increase in liver weight in Rhesus monkeys after a 3 month treatment at a dietary level of 2% $^{w}/_{w}$. In contrast fenofibrate had no effect in either animal (Platt and Thorp, 1966; reviewed in Cohen and Grasso, 1981).

There is little evidence of peroxisome proliferation occurring in the human system (Hanefeld *et al.*, 1980; Parzefall *et al.*, 1990), a fact that has led some authors to propose that peroxisome proliferators are unlikely to pose a risk to humans (Cohen and Grasso, 1981; Purchase, 1994). However it is important to note that, since the molecular processes involved in the pathway of response to peroxisome proliferators have not yet been fully established in the rodent system, it is not yet possible to determine whether peroxisome proliferation and cancer are causally linked. Thus, until the multiple molecular actions of peroxisome proliferators are more fully understood, the risk posed by these rodent carcinogens to man cannot be fully assessed.

1.3.4 Proposed Mechanisms of Hepatocarcinogenicity of Peroxisome Proliferators.

1.3.4.1 Oxidative Stress.

The close association between peroxisome proliferation and rodent liver carcinogenesis has led to the suggestion that these two phenomena may be causally linked (Reddy *et al.*, 1980; Reddy and Rao, 1986; Popp *et al.*, 1989). Despite reports that peroxisome proliferators are not directly mutagenic (Warren *et al.*, 1980; Von Daniken *et al.*, 1981), the high incidence of liver tumours induced by these chemicals has led to the proposal that peroxisome proliferators must initiate, at least indirectly, some form of DNA damage (Reddy and Rao, 1986).

Peroxisome proliferation is accompanied by the induction of enzymes of the peroxisomal β-oxidation system whose activity results in the production of H_2O_2 (Reddy and Lalwani, 1983). Under normal conditions H_2O_2 is subsequently decomposed by catalase to oxygen and water (Van den Bosch *et al.*, 1992). However, the increase in

activity of β-oxidation enzymes (~20 fold) following peroxisome proliferation is not matched by the increase in catalase activity (< 2-fold) (Reddy and Rao, 1989). Indeed, some authors have reported that following treatment with a peroxisome proliferator, hepatocytes actually exhibit lower relative catalase activity per peroxisome than under normal circumstances (Bendayan and Reddy, 1982; Moody *et al.*, 1991). The consequent imbalance in rates of H_2O_2 production and degradation is thought to result in the production of excess H_2O_2, which diffuses out from hepatocyte peroxisomes into the cytosol. Interaction of H_2O_2 with Fe^{2+} can lead to the generation of highly reactive hydroxyl radicals, capable of lipid peroxidation (Loeb *et al.*, 1988). It is proposed that products of lipid peroxidation, such as lipofuscin, are responsible for inducing single strand breaks in DNA (Reddy and Rao, 1989; Reddy, 1990). In support of this theory, a 2-fold increase in levels of the adduct 8-hydroxydeoxyguanosine, which forms following free radical damage to DNA, has been detected in the livers of rats treated with a number of peroxisome proliferators, including clofibrate, DEHP and simfibrate (Kasai *et al.*, 1989; Takagi *et al.*, 1990). DNA alterations have also been detected by ^{32}P post-labelling in the livers of rats fed ciprofibrate and Wy-14,643 in a long term study (Randerath *et al.*, 1991).

Oxidative stress is not universally accepted as the mechanism of hepatocarcinogenicity of the peroxisome proliferators. Tamura *et al.* (1990) suggest that H_2O_2 leaking into the cytosol would be rapidly decomposed and could not play a role in hepatocarcinogenesis. It has been demonstrated that, although the rate of H_2O_2 leakage from hepatic peroxisomes is increased 2-4 fold following treatment with a variety of peroxisome proliferators, the concentration of total intracellular H_2O_2 does not increase comparably (Tamura *et al.*, 1990). It is also important to note that, although lipofuscin accumulation has been proposed to account for the carcinogenicity of the peroxisome proliferators, the extent of lipofuscin accumulation does not always correlate with the ability of a particular chemical to induce liver tumours (Marsman *et al.*, 1992). Further evidence against the oxidative stress hypothesis has arisen from the use of an assay for unscheduled DNA synthesis (UDS), which provides an indirect measurement of carcinogen-induced DNA damage. No UDS was detected in rats treated with the peroxisome proliferators Wy-14,643, nafenopin, BR-931 or DEHP (Cattley *et al.*, 1986).

Despite the fact that peroxisome proliferators have long been regarded as non-genotoxic chemicals, contradictory results have emerged recently demonstrating that certain peroxisome proliferators can actually exert genotoxic effects in primary cultures of rat and human hepatocytes (Reisenbichler and Eckl, 1993; Hwang *et al.*, 1993). The peroxisome proliferators nafenopin and ciprofibrate induced a significant number of sister chromatid exchanges at doses above 30 μM, after only 3 hours of treatment. Higher concentrations or longer exposure times resulted in chromosomal aberrations and the formation of micronuclei (Reisenbichler and Eckl, 1993). The number of sister chromatid exchanges, micronuclei and chromosome aberrations occurring in rat hepatocyte cultures in response to the peroxisome proliferator Wy-14,643, increased in a similarly dose-dependent manner, although these changes only occurred after a 48 hour treatment (Hwang *et al.*, 1993). This delay in DNA damage is proposed to occur either because peroxisome proliferators are only weak genotoxins or because they require other events to occur prior to the onset of DNA damage, such as induction of peroxisomes or the activation of specific receptors (Hwang *et al.*, 1993). Interestingly, in both cases, a significant correlation was reported between the number of sister chromatid exchanges and the extent of peroxisome proliferation, an observation that could be explained by the oxidative stress theory outlined above (Reddy, 1990). However, a less dramatic increase in micronuclei was detected in primary cultures of human hepatocytes exposed to Wy-14,643 and the damage did not appear to correlate with peroxisome proliferation (Hwang *et al.*, 1993). The results of these studies would suggest that peroxisome proliferators may function not only as tumour promoters but as complete carcinogens, albeit with only a weak capacity for the initiation of DNA damage. However since no genotoxic activity of peroxisome proliferators has been reported *in vivo*, this hypothesis remains to be validated.

1.3.4.2 Tumour Promotion by Sustained Hyperplasia.

It has been proposed that the potency of a hepatocarcinogen is related to its ability to induce hepatocyte proliferation (Cayama *et al.*, 1978; Lewis and Swenberg, 1982). Indeed, a number of studies have demonstrated that a genotoxic carcinogen is more potent if administered at a time when cells are rapidly dividing (Frei and Harsono,

1967; Pound and McGuire, 1978; Schulte-Hermann *et al.*, 1981). While the majority of chemical carcinogens do not stimulate DNA synthesis directly, regenerative hyperplasia often occurs in order to compensate for the toxic effects of the chemicals (Michalopoulos *et al.*, 1987). Peroxisome proliferators however, exhibit only minimal acute toxicity and therefore the DNA synthesis they induce is termed additive or augmentative hyperplasia (Schulte-Hermann, 1974; Michalopoulos *et al.*, 1987). During initiation, sustained hyperplasia is thought to 'fix' mutations into a cell population by transforming chemically-induced DNA lesions into permanent somatic mutations before they can be repaired (Lewis and Swenberg, 1982; Butterworth *et al.*, 1987). It may also be that the altered structure of replicating DNA facilitates the formation of chemical-DNA adducts, resulting in increased sensitivity to a carcinogen (Butterworth *et al.*, 1987). Enhanced levels of cell division are also important in the promotion stage of tumorigenesis, providing a stimulus for the clonal expansion of previously initiated cells (Farber and Cameron, 1980).

The ability of peroxisome proliferators and other non-genotoxic hepatocarcinogens to promote the development of chemically-induced lesions has been demonstrated clearly (Peraino *et al.*, 1975; Pitot *et al.*, 1980; Kraupp-Grasl *et al.*, 1990). For example, in one study, exposure of rats to nafenopin following an initiating dose of the carcinogen, aflatoxin B1 (AFB1), led to a higher incidence of liver adenoma and carcinoma than seen following exposure to AFB1 alone (Kraupp-Grasl *et al.*, 1990). What is perhaps less apparent is how peroxisome proliferators contribute to the development of liver tumours in the absence of a chemical mutagen. One explanation is that peroxisome proliferators may promote the development of preneoplastic cells that have arisen spontaneously (Schulte-Hermann *et al.*, 1983). In support of this hypothesis is the observation that aged mice and rats, which are likely to have accumulated genetic lesions throughout their lifetime, are more susceptible than young animals to hepatocarcinogenesis induced by the peroxisome proliferator nafenopin (Kraupp-Grasl *et al.*, 1991). This enhanced susceptibility of aged rats to tumour promotion by nafenopin was found to be unrelated to enhanced levels of oxidative DNA damage (Huber *et al.*, 1991).

The carcinogenicity of the peroxisome proliferators is reported to correlate more with their mitogenicity than with their ability to stimulate peroxisome proliferation (Marsman *et al.*, 1988). The peroxisome proliferators DEHP and Wy-14,643 differ markedly in their carcinogenic potential (see page 24). However, the increase in peroxisomal number induced by these two chemicals was found to be equivalent after 52 weeks of treatment and peroxisomal enzyme activity only differed by 25%. In contrast, a 10-fold increase in replicative DNA synthesis was detected in the livers of rats treated with Wy-14,643 compared to those treated with DEHP, indicating that it is the mitogenic response elicited by the peroxisome proliferators which is responsible for their carcinogenicity (Marsman *et al.*, 1988).

A number of studies involving chronic exposure to the peroxisome proliferators ciprofibrate, nafenopin, clofibric acid and LY171883 have failed to demonstrate a sustained increase in replicative DNA synthesis in the liver (Yeldandi *et al.*, 1989; Eacho *et al.*, 1991). In addition, it has been reported that peroxisome proliferators administered alone are unable to stimulate an increase in the number of preneoplastic liver foci to the same extent as compensatory liver regeneration (Columbano *et al.*, 1987). Thus, although sustained hepatocellular replication may be crucial to hepatocarcinogenesis induced by many peroxisome proliferators, the extent of cell replication induced by a particular chemical may not necessarily represent an accurate reflection of its carcinogenicity (Marsman *et al.*, 1992).

1.3.4.3 Promotion of Specific Preneoplastic Foci by Peroxisome Proliferators.

Classical tumour promoters such as phenobarbital and cyproterone acetate have been shown to facilitate the development of preneoplastic foci to tumours by selective stimulation of the growth of specific subtypes of liver foci. These foci can be characterised phenotypically by the expression of particular markers such as gammaglutamyltranspeptidase (GGT) and placental glutathione S-transferase (GST-P, Schulte-Hermann *et al.*, 1990). Initiation-promotion experiments using AFB1 and the peroxisome proliferators nafenopin, Wy-14,643 and DEHP indicate that peroxisome proliferators selectively stimulate the growth of pre-neoplastic liver foci that differ phenotypically to those stimulated by other liver tumour promoters (Kraupp-Grasl *et al.*,

1990). The foci amplified by these peroxisome proliferators were described as weakly basophilic (Kraupp-Grasl *et al.*, 1990), with only weak staining for GGT and GST-P expression (Grasl-Kraupp *et al.*, 1993a). This phenotype resembles that seen in adenomas and carcinomas induced by nafenopin, indicating that the foci are likely to be the precursors for tumours induced by this compound (Grasl-Kraupp *et al.*, 1993a). It has been proposed that these foci have a selective growth advantage in the presence of nafenopin, which is the result of a hyper-responsiveness to both enzyme induction and stimulation of DNA synthesis (Grasl-Kraupp *et al.*, 1993b).

1.3.4.4 Receptor-Mediated Hepatocarcinogenesis.

The specificity of the response of hepatocytes to peroxisome proliferators is maintained even by hepatocytes which are not in their usual environment. It has been demonstrated that rat hepatocytes transplanted into the anterior chamber of the eye still undergo peroxisome proliferation, accompanied by the induction of peroxisomal enzymes, in response to the peroxisome proliferator clofibrate (Rao *et al.*, 1986). Several authors have suggested that the tissue- and species-specific responses to peroxisome proliferators can be explained by the existence in hepatocytes of a specific receptor that is activated by this class of non-genotoxic carcinogens. The existence of a receptor for peroxisome proliferators was first demonstrated following the isolation of a 70 kilo Dalton (kD) binding protein from rat liver using a nafenopin-affinity column (Lalwani *et al.*, 1987). This receptor was termed the peroxisome proliferator binding protein (PPBP, Lalwani *et al.*, 1987). PPBP was subsequently shown to be identical to HSC72, a member of the HSP70 family of heat shock proteins (Alvares *et al.*, 1990). The ubiquitous expression of HSC72 in cells, together with the finding that certain peroxisome proliferators, including the potent hypolipidaemic drug Wy-14,643, do not bind to this protein, now suggests that this protein is unlikely to mediate the effects of peroxisome proliferators (Green, 1992a).

An alternative receptor, termed PPAR, has been cloned from mouse liver cells (Issemann and Green, 1990). PPAR is a member of the steroid hormone nuclear receptor superfamily that includes receptors for thyroid hormone β, vitamin D and retinoic acid α (Issemann and Green, 1990). Since the identification of mouse PPAR, it has become

apparent that PPAR is not a single receptor, but a family of closely related receptor subtypes. Three PPAR subtypes, termed α, β and γ, have been isolated from *Xenopus laevis* (Dreyer *et al.*, 1993), the α form being homologous to mouse and rat PPAR. Another mouse receptor, homologous to *Xenopus* PPARγ has also been isolated (Zhu *et al.*, 1993). In addition, two receptors, NUC1 and hPPAR have been isolated from humans (Schmidt *et al.*, 1992; Sher *et al.*, 1993). The existence of more than one receptor for peroxisome proliferators may facilitate multiple signalling pathways, providing an explanation for the variety of cellular responses to these chemicals.

Nuclear hormone receptors are intracellular and bind with high affinity to their ligands. In common with other nuclear hormone receptors, PPAR appears to require an interaction with the retinoid X receptor (RXR) for efficient DNA binding (Gearing *et al.*, 1993). Furthermore the ligand for RXR, 9-cis retinoic acid, enhances the activity of PPAR (Issemann *et al.*, 1993). In this heterodimeric state, nuclear hormone receptors recognise motifs upstream of their target genes called hormone response elements (HREs) and, by binding to these motifs, act as transcriptional enhancers or silencers (Green, 1992a). PPAR has been shown to bind to an HRE 5' of the acyl-CoA oxidase gene, whose transcription is increased during peroxisome proliferation (Tugwood *et al.*, 1992). Furthermore, the PPAR-RXR heterodimer has been shown to activate an HRE upstream of the peroxisomal bifunctional enzyme gene, whose activity and expression is also up-regulated during peroxisome proliferation (Bardot *et al.*, 1993).

A good correlation has been demonstrated between the ability of a particular peroxisome proliferator to activate PPAR and its capacity to induce peroxisome proliferation (Green 1992a). For example, the potent peroxisome proliferator Wy-14,643 has a higher affinity for PPAR than the much weaker agent MEHP (a primary metabolite of the steroid, DEHP). Further evidence that PPAR mediates the effects of the peroxisome proliferators comes from the observation that the highest expression of PPAR is found in the liver, in which the greatest response to peroxisome proliferators has been detected. Lower levels of expression are found in less responsive tissues such as the kidney, heart and brown adipose tissue (Issemann and Green, 1990). The expression of PPAR in these different organs also correlates well with the pattern of

acyl-CoA oxidase induction that follows exposure to a peroxisome proliferator (Beck *et al.*, 1992).

Direct binding of peroxisome proliferators to PPAR has not been demonstrated and the physiological ligand for PPAR has yet to be identified. One possibility is that peroxisome proliferators modulate PPAR indirectly by inducing the production of the natural PPAR ligand via a perturbation of lipid metabolism (Green 1992b). It has been suggested that peroxisome proliferators may bind to liver fatty acid binding protein, displacing fatty acids which are then free to activate PPAR (Issemann *et al.*, 1992). The theory that fatty acids or a cholesterol metabolite represent the natural ligand for PPAR is further supported by the fact that a high fat diet (Nilsson *et al.*, 1986), the steroid DEHP (Frenkel *et al.*, 1990) and fatty acids themselves (Intrasuksri and Feller, 1991; Keller *et al.*, 1993) can all induce peroxisome proliferation in hepatocytes.

Since PPAR appears to mediate at least some of the effects of peroxisome proliferators, it is not unreasonable to assume that this receptor may also play a role in their hepatocarcinogenicity (Green, 1992a). If the oxidative stress hypothesis is correct, the ability of PPAR to induce acyl-CoA oxidase expression would be a critical stage in the carcinogenic process. Alternatively, PPAR may alter the expression of specific genes involved in cell growth or differentiation (Green, 1992b). Elevated levels of the proto-oncogenes *c-myc* and *c-Ha-ras* have been detected in rat liver following administration of the peroxisome proliferators clofibrate, ciprofibrate (Cherkaoui-Malki *et al.*, 1990; Hegi *et al.*, 1993) and BR-931 (Hsieh *et al.*, 1991). Interestingly a decrease in epidermal growth factor (EGF) receptor transcripts (Hsieh *et al.*, 1991) and EGF receptor binding (Gupta *et al.*, 1988) has also been demonstrated following administration of peroxisome proliferators to rats, mirroring changes seen during prolonged exposure to tumour promoters (Tsai and Michalopoulos, 1991) and following partial hepatectomy in rat liver (Meyer and Jirtle, 1989). For example, chronic exposure of rats to the tumour promoter phenobarbital promotes the development of hepatocellular carcinoma (Preat *et al.*, 1987). Hepatocytes from such chronically-treated rats have a reduced proliferative capacity, which has been attributed in part to a decrease in EGF receptor expression (Eckl *et al.*, 1988). It has been proposed that preneoplastic cells are refractive to the anti-proliferative effects of phenobarbital and therefore have a growth advantage over normal

cells. Thus clonal expansion of putative preneoplastic hepatocytes can occur (Jirtle and Meyer, 1991).

1.3.4.5 Suppression of Apoptosis by Peroxisome Proliferators.

Sustained cell proliferation alone may not be sufficient to explain the development of liver tumours following administration of peroxisome proliferators (Melnick, 1992). Studies with a number of peroxisome proliferators, including ciprofibrate, clofibric acid and nafenopin, have failed to demonstrate a persistent increase in replicative DNA synthesis in response to the chemicals (Yeldandi *et al.*, 1989; Eacho *et al.*, 1991). A novel and exciting hypothesis proposed recently is that non-genotoxic carcinogens may contribute to tumour promotion by inhibiting cell death by apoptosis (Bursch *et al.*, 1992), a process described in more detail in section 1.4 (page 38). Evidence in support of this hypothesis has been obtained from studies *in vivo*: A number of non-genotoxic carcinogens and tumour promoters, including cyproterone acetate (Schulte-Hermann *et al.*, 1980) and phenobarbital (Schulte-Hermann, 1974), induce massive hyperplasia in the liver. Withdrawal of these chemicals results in regression of the liver with a concomitant increase in cell death by apoptosis (Bursch *et al.*, 1984). This liver regression is reversible and can be inhibited by re-administration of the relevant chemical (Bursch *et al.*, 1984). Interestingly, the apoptosis that follows withdrawal of cyproterone acetate from the liver can also be inhibited by administration of the peroxisome proliferator nafenopin (Bursch *et al.*, 1986). Recent reports that the peroxisome proliferators clofibrate, Wy-14,643 and ETYA can protect preadipocytes from apoptosis induced by retinoic acid treatment (Chawla and Lazar, 1994), and that clofibrate and DEHP protect against paracetamol-induced hepatotoxicity in mice (Nicholls-Grzemski *et al.*, 1992), provide further evidence for the protective effects of the peroxisome proliferators. In light of these results it has been proposed that peroxisome proliferators, in common with other non-genotoxic carcinogens, may act to delay or inhibit the onset of apoptotic death, which would otherwise result in the removal of damaged or potentially tumourigenic cells from the liver (Bursch *et al.*, 1992). The survival of such preneoplastic cells could allow their integration into a cell population where further mutations would lead to the development of tumours.

Conclusive evidence of a role for apoptosis in peroxisome proliferator-induced hepatocarcinogenesis has proved elusive. Studies of the effects of peroxisome proliferators on apoptosis have been hindered by a number of factors. The extent to which the molecular markers of apoptosis, established predominantly in the immature rat thymocyte (Wyllie, 1980), are applicable to the liver remains to be established. Furthermore, studies in the liver demonstrate that, even when liver regression is at its peak following withdrawal of liver mitogens such as cyproterone acetate, the number of cells exhibiting a morphology typical of apoptosis constitute only 1-2% of the total cell number (Bursch *et al.*, 1984; Bursch *et al.*, 1986). This can be explained by the fact that apoptotic cells *in vivo* are rapidly recognised and phagocytosed by neighbouring cells (Wyllie *et al.*, 1980). In contrast to necrosis, apoptosis occurs in single cells scattered throughout a tissue and only a very small percentage of apoptotic cells are recognisable at any one time. In light of these problems, this thesis has examined the effect of peroxisome proliferators on liver cell apoptosis using *in vitro* cell systems which are more easy to manipulate and which facilitate more detailed experimentation.

1.4 Cell Death.

Cell death has been described historically as an uncontrolled, degenerative response to injury or disease and as such has generated little scientific interest. However, the last two decades have witnessed a significant change in perspective and cell death is now recognised, somewhat paradoxically, as being essential to life, with critical roles in development, immunology, neurobiology, metamorphosis and tissue homeostasis (reviewed in Wyllie, 1993; Schwartzman and Cidlowski, 1993). From the earliest stages of development onwards, it is essential that damaged, senescent or unwanted cells are eliminated in a controlled and specific manner. The fashioning of limbs and organs during embryogenesis requires not only the production of new cells but also the deletion of those that are redundant (Hammar and Mottet, 1971), while the development of a fully competent immune system requires the systematic removal of potentially lethal self-reactive B- and T-lymphocytes which would otherwise attack the body's own tissue (MacDonald and Lees, 1990; Cohen *et al.*, 1992). Furthermore, maintenance of adult tissue homeostasis is dependent on a tightly regulated balance between cell gain through

cell division, and cell loss by terminal differentiation or cell death (Dive and Wyllie, 1993). While much attention has been focused on understanding the mechanisms involved in proliferation and differentiation, the significance of cell death in this equation has only recently been fully recognised. It is well established that cells can be killed by exposure to a variety of noxious chemicals, but a more novel concept is that cells can actively participate in their own self-destruction or suicide. Cell death can now be classified into one of two broad categories, that differ both morphologically and biochemically. Necrosis is a metabolically passive form of cell death which is almost invariably the result of a major perturbation in the cellular environment (Trump *et al.*, 1981), while apoptosis is an active, controlled and specific form of cell death which appears to be under the internal control of the cell involved (Wyllie *et al.*, 1980).

1.4.1 Necrosis.

Necrosis or 'accidental cell death' occurs almost exclusively as a result of gross deviation from normal environmental or physiological conditions (reviewed in Wyllie *et al.*, 1980; Trump *et al.*, 1981; Dive and Wyllie 1993). Cells become necrotic in response to hypoxia (Jennings *et al.*, 1975), complement attack (Hawkins *et al.*, 1972), pH or temperature extremes (Buckley, 1972) and exposure to toxic chemicals (McLean *et al.*, 1965). Tumours also frequently contain zones of necrotic tissue which usually develop as a result of hypoxia (Wyllie, 1985). These diverse stimuli alter the ionic homeostasis of a cell dramatically, either by direct attack on the plasma membrane or by interference with mitochondrial ATP production, causing the failure of ATP-dependent ion pumps (Wyllie *et al.*, 1980; Bowen and Bowen, 1990). The resulting increase in plasma membrane permeability allows loss of K^+ from the cell and an influx of Ca^{2+}, Na^+ and water. The elevation in $[Ca^{2+}]$ is proposed to be a crucial event in necrotic cell death, causing not only mitochondrial damage, but also alterations to the cytoskeleton and activation of Ca^{2+}-dependent phospholipases, which are responsible for widespread and lethal disruption of membrane proteins (Trump *et al.*, 1981; Dive and Wyllie, 1993).

Necrosis falls morphologically into reversible and irreversible stages (reviewed in Sandritter and Riede, 1975; Wyllie *et al.*, 1980; Trump *et al.*, 1981). Early changes include cellular swelling and dilation of the endoplasmic reticulum and mitochondria,

caused by water redistribution, and blebbing of the plasma membrane. At this stage a return to normal physiological conditions would allow the recovery of the cell. However, in the presence of persistent cellular insults, a series of rapid and irreversible changes lead ultimately to the death and disintegration of the cell. High amplitude swelling of the mitochondria is accompanied by clumping and flocculation of nuclear chromatin and Ca^{2+}-activated phospholipases cause the lethal disruption of plasma and mitochondrial membranes. Lysosomal damage and the subsequent release of proteolytic lysosomal enzymes ensures the final degradation of the cell. Necrosis typically affects contiguous sheets of cells *in vivo* and the release of lysosomal enzymes from degenerating cells causes exudative inflammation in surrounding viable tissue. This inflammation is a necessary response to extensive tissue injury which permits the clearing of cell debris from the damaged area (Cohen, 1993). However since such a response is disruptive and can often lead to scarring, necrosis is not a teleologically appropriate mode of cell death in situations where cell death must occur without damage to surrounding tissue structure or function.

1.4.2 Apoptosis.

The term 'apoptosis' was introduced in 1972 to describe the physiological death of mammalian cells (Kerr *et al.*, 1972). In contrast to necrosis, apoptosis affects single cells scattered throughout healthy tissue (Wyllie *et al.*, 1980). Apoptotic cells *in vivo* are rapidly recognised and phagocytosed by macrophages or neighbouring cells prior to plasma membrane breakdown, preventing the release into the extracellular space of proteolytic enzymes which cause tissue inflammation (Duvall and Wyllie, 1986). The absence of an inflammatory response is a crucial feature of apoptosis, since it facilitates the deletion of cells with minimal disruption to surrounding viable tissue architecture.

In mammalian tissues, apoptosis accounts for the cell loss seen during embryonic tissue modelling, tissue atrophy following hormone ablation and normal tissue turnover (reviewed in Duvall and Wyllie, 1986; Gerschenson and Rotello, 1992; Wyllie, 1993). Examples include the removal of interdigital cells from a solid limb paddle during digit formation (Hammar and Mottet, 1971), regression of the prostate following castration (Kerr and Searle, 1973) and the death of mature neutrophils (Savill *et al.*, 1989).

Apoptosis is also essential in the immune system for the deletion of self-reactive B- and T- lymphocytes (MacDonald and Lees, 1990) and is the mode of cell death in cell-mediated immune killing (Cohen *et al.*, 1985). In the liver, apoptosis serves as a mechanism for the removal of unwanted and excessive cells produced in response to chemically-induced tissue damage (Tessitore *et al.*, 1989).

Apoptosis is often equated with programmed cell death. However, the term apoptosis was introduced initially to describe a morphological and biochemical phenomenon rather than a genetic program. The term 'programmed cell death' has historically been applied to the mode of cell death seen in a number of invertebrate systems, where specific cells are programmed to die at a particular time during development with exquisite accuracy. For example, the nematode *Caenorhabditis elegans* (*C. elegans*) has 1090 somatic cells, 131 of which die at specific and pre-determined stages of development by a mechanism which has morphological similarities to mammalian apoptosis (Ellis *et al.*, 1991). The disappearance of a tadpole's tail and the removal of insect larval tissue during metamorphosis are also programmed events, under the control of the hormones thyroxine and ecdysone respectively (Bowen and Bowen, 1990). Until very recently, these invertebrate systems were the only true examples of cell death occurring as a programmed event. However, similar examples have now emerged in the mammalian system, including the involution of breast tissue that occurs after weaning (Walker *et al.*, 1989), and follicular atresia during the menstrual cycle (Hughes and Gorospe, 1991). The identification of a number of genes implicated in mammalian apoptosis has provided further evidence that apoptosis is a genetically programmed event. Details of these genes and their potential roles in the cell death pathway are discussed on pages 60-74.

Since cell death by apoptosis is such an integral part of normal cell development and tissue homeostasis, abnormalities in its regulation are likely to contribute to the pathogenesis of a number of diseases. The premature death of neurones is associated with a number of neurological degenerative disorders such as Alzheimer's and Parkinson's disease (Carson and Ribeiro, 1993) and the inappropriate deletion of T-cells by apoptosis has been implicated in the development of AIDS (Ameison and Capron, 1991; Gougeon and Montagnier, 1993). The effects of inhibition of cell death can be

equally devastating. A defect in the mechanism of deletion of self-reactive B- and T-lymphocytes can pre-dispose to auto-immune disorders (Kroemer and Martinez, 1994) and perhaps most pertinent to this thesis, suppression of cell death has been associated with the development of cancer (Bursch *et al.*, 1992). It is interesting to speculate that in healthy tissue, apoptosis may normally serve as a mechanism for the deletion of cells which have sustained potentially tumourigenic lesions. Inhibition of cell death could thus contribute to the development of cancer by potentiating the anomalous survival of such preneoplastic cells, long enough to allow the acquisition of further transforming mutations.

1.4.2.1 Morphology of Apoptosis.

The morphological features of apoptosis are highly distinctive and occur with a considerable degree of fidelity, regardless of cell type (reviewed in Wyllie *et al.*, 1980; Duvall and Wyllie, 1986; Wyllie, 1993). However, it is not yet apparent which of these changes is causally related to the cell death process. A cell undergoing apoptosis shrinks and separates from its neighbours, losing any specialised areas of cell membrane such as junctional complexes and microvilli, although the plasma membrane itself remains intact. The cytoplasm contracts, causing crowding of intact organelles and dilation and some vesiculation of the endoplasmic reticulum occurs. The fusion of such vesicles with the plasma membrane gives the plasma membrane a pitted appearance when viewed by scanning electron microscopy. Concomitant changes occur in the nucleus and these are the most conspicuous features of apoptosis. The nuclear chromatin condenses into dense compact masses which may coalesce into a crescentic cap lining the nuclear membrane. The nucleolus fragments and invaginations of the nuclear membrane may divide the nucleus. Characteristic ruffling or blebbing of the cell surface also occurs, followed by an explosion of the cell into membrane-bound fragments or apoptotic bodies. *In vivo*, apoptotic cells and bodies are rapidly recognised and phagocytosed by macrophages or neighbouring viable cells (Savill *et al.*, 1993). The ultimate degradation of the apoptotic cell is then carried out by enzymes from the phagocyte's lysosomes. *In vitro* however, in the absence of phagocytosis, the final stages of cell degeneration are associated with a

loss of plasma membrane integrity, a process that is often referred to, somewhat confusingly, as 'secondary necrosis' (Schwartzman and Cidlowski, 1993).

1.4.2.2 Biochemistry of Apoptosis.

Although the morphological features of apoptosis have been fairly well characterised, a complete understanding of the biochemical and molecular mechanisms that underlie these changes has proved somewhat elusive. Studies have been hindered by the asynchronous nature of apoptosis and the small number of apoptotic cells that exist within a tissue at any one time. One exception is the immature thymus gland. Immature thymocytes are extremely sensitive to the induction of apoptosis by glucocorticoid hormones and other agents (Wyllie *et al.*, 1980; McConkey *et al.*, 1988a; Raffray *et al.*, 1993). Furthermore, the death of thymocytes following exposure to such agents is more synchronous than in many epithelial cell systems, providing a sufficient quantity of apoptotic cells for biochemical analysis. For this reason, current knowledge of the biochemistry of apoptosis is derived mainly from studies involving immature thymocytes or other cells of haemopoietic origin which maintain this susceptibility to undergo apoptosis (Wyllie, 1980; McConkey *et al.*, 1989a; Williams and Smith, 1990). It remains to be established whether biochemical features of apoptosis identified in these haemopoietic cells are universal to all other cell and tissue types.

1.4.2.2.1 Non-Random Cleavage of DNA.

The most extensively studied biochemical event in apoptosis to date has been the non-random cleavage of nuclear DNA. When the DNA of apoptotic immature rat thymocytes is separated by conventional agarose gel electrophoresis (CAGE), a characteristic banding pattern or 'ladder' is obtained, similar to that produced after digestion of DNA with micrococcal nuclease (Wyllie, 1980) but quite distinct from the smear that is indicative of the non-specific DNA degradation seen in necrosis. The definitive pattern of DNA fragmentation seen during apoptosis arises as a result of the normal conformation of nuclear DNA. Within the nucleus, lengths of DNA are tightly wound around a core of histone proteins, forming a series of clusters called nucleosomes, joined by more loosely associated lengths of linker DNA (Arends *et al.*,

1990). DNA surrounding the histones is inaccessible to the action of endonucleases and therefore only the linker DNA joining adjacent nucleosomes is exposed to such enzymes. Consequently, the activation of an endogenous endonuclease during apoptosis results in the production of double stranded DNA fragments, consisting of integer multiples of approximately 180-200 base pairs, which appear as discrete bands on agarose gels (Wyllie, 1980). DNA is only degraded further if the histone proteins are digested by proteases, as occurs in necrosis, exposing all the DNA to endonuclease activity and producing the spectrum of DNA fragments detected as a smear on agarose gels.

Internucleosomal DNA fragmentation has been detected in the majority of apoptotic systems analysed and has until recently been regarded as the most significant biochemical hallmark of apoptotic cell death. It is unknown whether the endonuclease(s) responsible for this internucleosomal DNA fragmentation is activated or synthesised *de novo* in response to an apoptotic stimulus. A number of candidate enzymes have been proposed, including DNase I (Peitsch *et al.*, 1993a), DNase II (Barry and Eastman, 1993) and an 18kD nuclease called NUC 18 (Gaido and Cidlowski, 1991). In the nematode *C. elegans,* an endonuclease called *nuc 1* is responsible for DNA fragmentation (Ellis and Horvitz, 1986). Some controversy exists as to the biochemical properties of the mammalian endonuclease(s): the endonuclease initially identified in apoptotic thymocytes was reported to be a Ca^{2+}- and Mg^{2+}-dependent neutral enzyme, whose activity was inhibited by Zn^{2+} (Cohen and Duke, 1984; McConkey *et al.*, 1989a; Giannakis *et al.*, 1991). In contrast, the endonuclease isolated by Barry and Eastman (1993) from apoptotic Chinese Hamster Ovary cells is Ca^{2+}- and Mg^{2+}- independent and is activated at an intracellular pH of less than 7. There has also been some contention over whether fragmentation occurs via single or double strand breaks. The majority of reports agree that internucleosomal fragmentation occurs via double stranded DNA cleavage (Wyllie, 1980; Arends *et al.*, 1990). However, it has also been proposed that a similar pattern of DNA fragmentation could be caused by single stranded cuts in DNA (Peitsch *et al.*, 1993b).

Chromatin cleavage in immature thymocytes becomes evident after only 1 hour of exposure to glucocorticoids and correlates with the onset of chromatin condensation

(Wyllie, 1980). This finding has lead to speculation that the two phenomena are causally related (Wyllie *et al.*, 1984; Arends *et al.*, 1990). Moreover DNA fragmentation has been proposed to be a critical event in the cell death process. Support for this hypothesis comes from the observation that aurintricarboxylic acid (ATA), an inhibitor of endonuclease enzymes, can protect immature thymocytes from apoptosis induced by glucocorticoid hormones (McConkey *et al.*, 1989b). ATA has also been shown to exert a protective effect over sympathetic neurons deprived of nerve growth factor (NGF; Batistatou and Greene, 1991) and haemopoietic cells deprived of interleukin-3 (IL-3, Crompton, 1991). However, ATA has a range of pharmacological activities and therefore its ability to suppress apoptosis is not necessarily evidence that endonuclease activation is essential to the cell death process (Mogil *et al.*, 1994). Certainly in the *C. elegans* system, the genes responsible for cell death are quite distinct from and act independently of the gene responsible for DNA fragmentation (Ellis and Horvitz, 1986). Indeed, endonuclease activation occurs in *C. elegans* after phagocytosis has occurred. Mutations of the *nuc-1* gene prevent this DNA degradation but do not prevent the death of the cell.

The reliability of the DNA 'ladder' as a universal biochemical marker of apoptosis has been brought into question recently by the emergence of an increasing number of systems in which morphological features of apoptosis occur in the absence of internucleosomal DNA fragmentation (Boe *et al.*, 1991; Oberhammer *et al.*, 1993a; Oberhammer *et al.*, 1993b; Zakeri *et al.*, 1993; Mogil *et al.*, 1994). Such a failure to demonstrate internucleosomal DNA fragmentation may in some cases simply be the result of technical limitations or the very small number of apoptotic cells available for analysis. For example it has been stated recently that primary rat hepatocytes, induced to enter apoptosis by transforming growth factor β_1 (TGFβ_1), exhibit chromatin condensation without any associated internucleosomal DNA fragmentation (Oberhammer *et al.*, 1993a). This finding contrasts with earlier reports demonstrating the occurrence of DNA fragmentation in apoptotic hepatocytes (Shinagawa *et al.*, 1991; Sanchez *et al.*, 1992).

It has been proposed that, although chromatin condensation probably requires DNA cleavage, it is unlikely that the fragments would need to be as small as those

produced by internucleosomal digestion (Walker *et al.*, 1993). Apoptosis induced in thymocytes and lymphocytes by the topoisomerase inhibitors teniposide and etoposide has been shown to be associated with a two-stage process of DNA fragmentation. Initially, interaction of the drugs with topoisomerase II causes cleavage of DNA into high molecular weight fragments of 300 or 50 kilo bases (kb) which can be separated by field inversion gel electrophoresis of DNA (FIGE, Walker *et al.*, 1991). A similar pattern of high molecular weight DNA fragmentation has since been observed in a number of other model systems (Oberhammer *et al.*, 1993b; Brown *et al.*, 1993). This high molecular weight fragmentation is believed to represent the release of loops and rosettes of chromatin from the nuclear matrix (Filipski *et al.*, 1990; Walker *et al.*, 1991) and may sometimes be followed by the internucleosomal DNA fragmentation more frequently associated with apoptosis (Walker *et al.*, 1993). It has been demonstrated recently in thymocytes that the initial cleavage of DNA to 300 and 50 kb fragments is dependent on Mg^{2+} and that the formation of 50 kb fragments is also facilitated by Ca^{2+} (Sun and Cohen, 1994). As mentioned above, internucleosomal DNA fragmentation in apoptotic thymocytes is inhibited by Zn^{2+}. However, subsequent studies have revealed that Zn^{2+} does not inhibit apoptosis completely, allowing the formation of high molecular weight fragments of DNA and some clumping of heterochromatin (Cohen *et al.*, 1992; Brown *et al.*, 1993). A hypothesis now gaining considerable support is that high order fragmentation of DNA is an early and essential step which must represent a 'point of no return' in apoptosis, while internucleosomal DNA fragmentation occurs later and is not critical to the cell death process (Brown *et al.*, 1993; Oberhammer *et al.*, 1993b). This hypothesis is supported by the recent finding that serine protease inhibitors prevent internucleosomal DNA cleavage in immature thymocytes but do not prevent the formation of high molecular weight DNA fragments, nor the ultimate death of the cells by apoptosis (Weaver *et al.*, 1993). Further doubt has been cast on the importance of DNA fragmentation in apoptosis by a recent and somewhat controversial report that cytoplasts from two fibroblast cell lines undergo the shrinkage and membrane blebbing typical of apoptosis in the absence of a nucleus altogether (Jacobson *et al.*, 1994). On the basis of this finding it has been proposed that the nucleus is not essential for apoptosis but that cytoplasmic events orchestrate the apoptotic program (Jacobson *et al.*, 1994).

However, it is difficult to reconcile how a process under such strict control as apoptosis could occur without some form of nuclear signalling pathway.

Assimilation of all the current data gives an unclear picture of the importance of internucleosomal DNA fragmentation in apoptosis. One possibility is that chromatin cleavage aids cellular fragmentation and phagocytosis by decreasing the bulk of the nucleus (Arends *et al.*, 1990). An alternative suggestion is that DNA fragmentation is a protective phenomenon. Since apoptotic cells are often phagocytosed by neighbouring cells, DNA fragmentation may be necessary to prevent the transfer of potentially lethal genetic material to these viable cells (Arends *et al.*, 1990). Thus in some systems, including post-mitotic cells, internucleosomal DNA fragmentation may not be essential if there is little chance of foreign DNA being integrated into the phagocyte's genome (Zakeri *et al.*, 1993).

1.4.2.2.2 Nuclear Matrix Alterations.

Apoptosis has been described as an 'aberrant mitosis' (Ucker *et al.*, 1992). During mitosis, phosphorylation of nuclear lamin proteins leads to their solubilisation and consequently to disassembly of the nuclear lamina (Peter *et al.*, 1990). Similarly, changes to the nuclear lamina have been described in a variety of apoptotic systems, including cytotoxic T-cell-mediated cell death (Ucker *et al.*, 1992), glucocorticoid-treated thymocytes (Lazebnik *et al.*, 1993), MCF-7 breast carcinoma cells treated with tumour necrosis factor α (TNFα, Miller *et al.*, 1993) and renal carcinoma cells treated with camptothecin (Lazebnik *et al.*, 1993). It remains to be established whether this lamin disassembly during apoptosis is mediated by phosphorylation as occurs in mitosis (Lazebnik *et al.*, 1993). However, it has been proposed that nuclear lamin degradation may be a prerequisite for the DNA degradation that accompanies apoptosis, allowing the release of DNA from the nuclear matrix and thus exposing the DNA to the activity of endonucleases (Ucker *et al.*, 1992; Weaver *et al.*, 1993).

1.4.2.2.3 Requirement for Protein Synthesis?

In several cell types, apoptosis appears to be dependent on active protein synthesis. Thymocytes treated with glucocorticoids or ionising radiation and T-cell lines

or hybridomas deprived of the growth factor IL-2 can be protected from apoptosis by cycloheximide or actinomycin D, inhibitors of protein and RNA synthesis respectively (Wyllie *et al.*, 1984; Cohen and Duke, 1984). These findings have led to speculation that macromolecular synthesis is an absolute requirement of apoptosis, facilitating the production of novel 'death proteins'. However the protective effects of agents such as cycloheximide may be the result of alterations to other cellular processes by the inhibitor, or may represent a requirement for ongoing protein synthesis throughout apoptosis rather than the production of novel proteins (Martin, 1993). Indeed, in a number of cell systems including the human promyelocytic leukaemia cell line, HL-60 (Martin *et al.*, 1990), primary hepatocytes (Ledda-Columbano *et al.*, 1992) and rodent intestinal crypt cells (Ijiri and Potten, 1983), cycloheximide and actinomycin D actually induce apoptosis. These somewhat conflicting results may be explained by the existence of distinct apoptotic machinery within different cells. Those cells in which apoptosis can be inhibited by cycloheximide or actinomycin D may require the *de novo* production of particular proteins involved in the apoptotic process, such as the endonuclease. Alternatively, other cells may exist which already possess the effector molecules required for apoptosis but keep these repressed under normal circumstances by means of specific inhibitors. Suppression of protein synthesis in such systems would therefore induce apoptosis and these cells could be said to be 'primed' for apoptosis, having already synthesised the components of the apoptotic pathway. These opposing theories have been named the 'induction' and 'release' hypotheses respectively (Cohen, 1993). In some systems, such as cytotoxic T-lymphocytes, the presence of inhibitors of macromolecular inhibitors neither inhibits nor induces apoptosis, suggesting that the components of the apoptotic pathway are present constantly in these cells. This hypothesis has been named the 'transduction' hypothesis (Cohen, 1993).

1.4.2.2.4 Cell Surface Changes and Recognition of Apoptotic Cells.

Early membrane changes that occur during apoptosis include the loss of microvilli and intercellular contacts such as desmosomes and tight junctions, which are lost as the apoptotic cell rounds up and begins to detach from surrounding viable cells (Wyllie, 1993). Although there is also characteristic membrane blebbing, cells

undergoing apoptosis *in vivo* are distinguished from necrotic cells by the fact that membrane integrity is maintained throughout the cell death process (Wyllie *et al.*, 1980). A number of cell surface changes must occur to ensure, not only that the plasma membrane remains intact, but that the dying cell can be recognised rapidly by macrophages or neighbouring cells prior to phagocytosis.

Transglutaminases are a group of Ca^{2+}- dependent enzymes which catalyse the formation of ε(γ-glutamyl)lysine cross-links between proteins, or the incorporation of amines into proteins (Folk, 1980). Such post-translational modifications confer on proteins resistance against physical and chemical attack. The general biological function of transglutaminases appears to be the protection of cell and tissue integrity. Plasma factor XIIIa catalyses the polymerisation of fibrin during blood coagulation (Lorand, 1972) and 'epidermal' transglutaminase is responsible for the formation of a cornified envelope beneath the plasma membrane of terminally differentiated keratinocytes (Thatcher and Rice, 1985). A third transglutaminase, termed 'tissue' transglutaminase (tTgase), is proposed to be involved in apoptosis (Fesus *et al.*, 1987; Knight *et al.*, 1991).

Administration of mitogens such as lead nitrate or EGF to rats results in liver hyperplasia. Withdrawal of these compounds is followed by liver involution and an associated elevation in the incidence of apoptosis. An increased level of tTgase mRNA and protein has been detected during this wave of apoptosis (Fesus *et al.*, 1987). tTgase expression results in the formation within apoptotic hepatocytes of a highly cross-linked protein shell, rendering the cells impervious to detergents, urea and other chaotropic agents (Fesus *et al.*, 1989). The substrate for the formation of this shell is reportedly similar to the protein involucrin, a precursor for cornified envelopes of terminally differentiated keratinocytes (Tarcsa *et al.*, 1992). The resulting insolubility of apoptotic cells provides a method for their isolation from tissue (Fesus *et al.*, 1989). Further experiments using neonatal rat hepatocytes have demonstrated that the level of tTgase expression is directly related to the rate of apoptosis (Piacentini *et al.*, 1991).

Degradation of apoptotic cells is accompanied by the accumulation of ε(γ-glutamyl)lysine isodipeptide in the blood and in tissue culture fluid of primary cells (Fesus *et al.*, 1991). This isodipeptide is the end-product of proteins previously cross-linked by transglutaminases. Its presence in blood plasma following the regression of

liver hyperplasia is interpreted as further evidence of a role for transglutaminases in the program of apoptosis. Moreover, a reduced tTgase activity associated with a decrease in the rate of apoptosis has been implicated in metastatic tumour progression (Knight *et al.*, 1991).

Transglutaminase activity contributes to maintenance of plasma membrane integrity in cells undergoing apoptosis. A phenomenon of equal importance is the rapid phagocytosis of these dying cells. Phagocytosis may be mediated by macrophages which are 'professional phagocytes', or by other cell types such as neighbouring epithelial cells in healthy tissue or adjacent tumour cells in tumours (Duvall and Wyllie, 1986). The rapid engulfment of dying cells prevents the potentially lethal release of nuclear and lysosomal material into the extracellular space. How does a phagocyte distinguish an apoptotic cell from one which is viable? In the nematode *C. elegans*, mutations of 7 different genes cause aberrations in the process of recognition and engulfment (Ellis *et al.*, 1991).These **c**ell **d**eath **a**bnormal genes have been placed into two groups, *ced 1, 6, 7, 8* and *ced 2, 5, 10*. Mutations in only one group do not affect phagocytosis adversely but if one gene from each group is mutated, phagocytosis is prevented. This indicates the existence of two parallel and partially redundant pathways for phagocytosis (Ellis *et al.*, 1991). While mammalian homologues of these genes have not yet been identified, it is likely that a number of proteins also co-operate to regulate the removal of apoptotic cells.

Lectins are proteins which recognise specific carbohydrate molecules. Binding of lectins on the surface of one cell to carbohydrates on the surface of another has been implicated as a method of cell adhesion (Albelda and Buck, 1990; Ozeki *et al.*, 1991). In some cell types, this interaction has been shown to be blocked by simple sugars recognised by lectins (Sharon and Lis, 1989). Lectin-carbohydrate interactions have been implicated as possible mechanism by which macrophages recognise apoptotic cells (Duvall *et al.*, 1985). It has been proposed that during apoptosis, glycan residues that are normally buried in the proximal regions of sugar side-chains of membrane glycoproteins, are exposed to macrophage lectins. Binding of mouse peritoneal macrophages to apoptotic thymocytes has been reported to be blocked *in vitro* by the simple sugars N-acetyl glucosamine and N,N'-diacetylchitobiose (Duvall *et al.*, 1985), suggesting that these sugars may interfere with the recognition system involved in the recognition of apoptotic cells. Phagocytosis of apoptotic rat hepatocytes has also been demonstrated to be mediated by sugar moieties, in particular the

asialoglycoprotein receptor (Dini *et al.*, 1985). However, direct expression of sugar moieties on the surface of apoptotic cells has yet to be demonstrated.

Another protein family implicated in the recognition of apoptotic cells is the integrin family of recognition proteins. Integrins recognise specific amino acid sequences (Albelda and Buck, 1990). In particular, the vitronectin receptor on macrophages recognises an Arg-Gly-Asp (RGD) tripeptide signal found in a number of matrix proteins including fibronectin, vitronectin and thrombospondin (Savill *et al.*, 1990). It has been proposed that this receptor is involved not only in the adhesion of cells to the extracellular matrix, but also in the recognition of apoptotic cells by macrophages. The recognition of ageing apoptotic neutrophils by macrophages *in vitro* has been reported to be to be determined by the level of expression on the macrophage of the $\alpha_v\beta_3$ vitronectin receptor (Savill *et al.*, 1990). Furthermore, the recognition of neutrophils by macrophages can be inhibited by monoclonal antibodies to the vitronectin receptor (Savill *et al.*, 1990). It has been proposed that a candidate ligand for the vitronectin receptor, thrombospondin, is secreted by macrophages, forming a molecular bridge between the thrombospondin binding moiety of the target apoptotic cell and the macrophage surface (Savill *et al.*, 1992). It appears that thrombospondin interacts not only with the vitronectin receptor but also with an 88kD monomer expressed by a number of cell types called CD36 (Savill *et al.*, 1993). Antibodies to CD36 can also inhibit the recognition of apoptotic neutrophils by macrophages.

An additional mechanism for the recognition of apoptotic cells involves changes in membrane phospholipids. In normal cells, the outer leaflet of the plasma membrane bi-layer consists of neutral phospholipids while the inner leaflet contains anionic phospholipids such as phosphatidylserine. Apoptotic thymocytes treated with dexamethasone, and a T-cell line deprived of IL-2, both exhibit increased phosphatidylserine expression compared to viable cells (Fadok *et al.*, 1992). Moreover, the uptake of apoptotic thymocytes by murine peritoneal macrophages is inhibited by liposomes containing phosphatidylserine, implicating phosphatidylserine recognition by macrophages as another signal for the removal of apoptotic cells.

The variety of mechanisms implicated in the recognition of apoptotic cells is not surprising considering the number of genes that regulate phagocytosis in *C. elegans*. It is not clear whether macrophages can utilise every recognition mechanism or are restricted to the

use of only one method. It is conceivable that the mechanisms employed may be species or cell-type dependent or may be determined by external influences such as the presence of cytokines at sites of inflammation (Savill *et al.*, 1993).

1.4.2.3 Detection and Quantitation of Apoptosis.

Studies of apoptosis have been hindered quite substantially by the limited number of methods available for the satisfactory detection and quantitation of apoptotic cells. Much of this difficulty arises from the asynchronous nature of apoptosis and the rapid detection and removal of apoptotic cells by phagocytes *in vivo* (Wyllie *et al.*, 1980). Similar problems arise *in vitro*, where, in the absence of phagocytosis, apoptotic cells rapidly undergo a process of secondary necrosis (Schwartzman and Cidlowski, 1993). As a result, even in tissues or cell cultures undergoing rapid apoptotic cell death, only a very small number of apoptotic cells are usually available at one time for analysis. An additional problem has arisen because certain features of apoptotic cells, such as oligonucleosomal DNA fragmentation, determined in thymocytes have not been detected in other cell types (Boe *et al.*, 1991; Oberhammer *et al.*, 1993b).

To date, morphological analysis, in particular detection of characteristic chromatin condensation patterns associated with apoptosis, remains the most reliable and universally accepted method of detection of apoptotic cells. Ideally this should be carried out at the level of electron microscopy. However, since preparation of samples for electron microscopy is a time-consuming and somewhat complicated procedure, this is not a suitable method for studying the kinetics of cell death. A more commonly used method, particularly in *in vitro* studies, is fluorescence microscopy of cells stained with fluorescent DNA-binding dyes such as acridine orange (AO, Gregory *et al.*, 1991) and Hoechst 33258 (Ho258, Oberhammer *et al.*, 1991). These dyes illuminate the condensed chromatin of apoptotic cells. Such staining methods have the advantage of being simple and fast, but quantitation of large numbers of cells using this method is tedious. Greater numbers of apoptotic cells can be analysed automatically by flow cytometry (FCM; reviewed in Darzynkiewicz *et al.*, 1992). The simultaneous analysis of several parameters, including changes in chromatin conformation and integrity, cell size and granularity, facilitates the distinction of apoptotic cells from those which are viable or

necrotic (Nicoletti *et al.*, 1991; Dive *et al.*, 1992a; Afanasyev *et al.*, 1993; Ormerod *et al.*, 1993; Gorczyca *et al.*, 1993). Typically, the decreased dye binding to DNA and the reduction in DNA content of apoptotic cells allows their separation as a hypodiploid peak below that seen with viable cells (Darzynkiewicz *et al.*, 1992). However, since this method has been reported to identify apoptotic bodies rather than intact cells (Afanasyev *et al.*, 1993), it is impossible to count absolute apoptotic cell numbers in many cell systems. Alternative assays using dual staining techniques, can correlate apoptotic cells with cell cycle status (Gorczyca *et al.*, 1993). FCM represents a powerful method for the quantitation of apoptosis, but since analysis is carried out on a stream of single cells, it is most suitable for cells in suspension.

A number of other methods for the detection of apoptotic cells are based on the fragmentation of DNA during apoptosis. Internucleosomal DNA fragmentation can be detected in a number of cell types undergoing apoptosis using CAGE followed by staining with the DNA intercalating dye ethidium bromide (Wyllie, 1980). The DNA from apoptotic cells appears as a 'ladder' of 180-200 base pair integer fragments. In contrast the DNA from viable cells remains as intact high molecular weight DNA, while necrotic cells yield a smear of DNA indicative of non-specific DNA fragmentation. Alternatively, DNA fragmentation assays involve separation of high molecular weight intact DNA from low molecular weight DNA fragments by centrifugation (Wyllie, 1980). A comparison of fragmented to intact DNA can be used as an indication of the extent of apoptosis. However since this method does not discriminate between the oligonucleosomal fragments produced in apoptosis and the non-specific low molecular weight fragments of DNA produced during necrosis, this method should not be used as sole evidence of the occurrence of apoptosis.

High order changes in chromatin structure are reported to precede oligonucleosomal DNA fragmentation and can be detected by FIGE (Walker *et al.*, 1991). Changes in the direction of voltage across an agarose gel allow the separation of large (>50 kb) fragments of DNA which are then visualised using ethidium bromide staining as described above.

The methods described have been used with varying degrees of success to study apoptosis *in vitro*, but are more difficult to apply to the *in vivo* situation. However, a

variety of methods are now emerging which can be used to detect apoptotic cells within a viable tissue section. DNA strand breaks associated with apoptosis, can be detected *in situ* by labelling the 3'-hydroxyl termini of DNA breaks with biotinilated deoxyuridine using either terminal deoxynucleotidyl transferase (TdT; TUNEL, Gavrieli *et al.,* 1992) or a DNA polymerase from *E. coli* (ISEL; Ansari *et al.,* 1993). In addition, with the discovery of new biochemical changes associated with apoptosis, the use of antibodies targeted to proteins thought to be up-regulated during apoptosis, such as tTgase may be of value in the future.

1.4.2.4 Intracellular Signalling in Apoptosis.

The marked similarities in apoptotic morphology of a wide variety of cell types suggest that the signalling pathway and mechanism of apoptosis may be common to all cell types or at least may have certain cardinal features. Little is understood clearly about the signals involved in apoptosis, there are even exceptions to those features that have been established. However, signalling events implicated in the induction of apoptosis include elevations in $[Ca^{2+}]$, activation of protein kinase A (PKA) and PKC, poly (ADP)-ribosylation and changes in gene expression.

1.4.2.4.1 Calcium.

The importance of calcium in the regulation of a variety of physiological processes, including cell differentiation, proliferation and metabolism, is well established (Orrenius *et al.*, 1989). In normal healthy cells, $[Ca^{2+}]$ is maintained at a considerably lower level than in the extracellular fluids, by the activity of ATP-dependent Ca^{2+} transport systems in the plasma membrane and Ca^{2+} sequestration systems, located primarily in the endoplasmic reticulum and mitochondria (Nicotera *et al.*, 1992). An ATP-dependent sequestration system has also been demonstrated in rat liver nuclei (Nicotera *et al.*, 1989). While rapid and transient increases in $[Ca^{2+}]$ are a normal and essential response to hormonal and / or neuronal stimulation, an uncontrolled and sustained elevation in $[Ca^{2+}]$ in the absence of additional mitogenic signals, is almost invariably associated with cell death (Orrenius *et al.*, 1989). A disruption in Ca^{2+} homeostasis is associated with necrotic cell death in ischaemia, neurotoxicity induced by

agents such as lead, cyanide and methyl mercury, liver toxicity in response to oxidative stress, cyanide and alkylating toxins and TCDD-induced thymic atrophy (reviewed in Orrenius *et al.*, 1989). The processes implicated in such Ca^{2+}-dependent cell killing include disruption to the cytoskeleton and mitochondria and uncontrolled activation of Ca^{2+}-dependent enzymes such as proteases, phospholipases and endonucleases (Nicotera *et al.*, 1992).

Elevations in $[Ca^{2+}]$ have also been implicated in the signalling pathway for apoptosis. A rapid and sustained elevation in $[Ca^{2+}]$ has been detected in immature thymocytes following treatment with glucocorticoid hormones (McConkey *et al.*, 1989b), TCDD (McConkey *et al.*, 1988a) and anti-CD3 antibody (McConkey *et al.*, 1989c). Similar increases have been detected in apoptotic hepatocytes exposed to oxidative stress (McConkey *et al.*, 1988b), TNFα-treated mammary adenocarcinoma cells (Bellomo *et al.*, 1992) and target cells of cytotoxic T-lymphocytes (Allbritton *et al.*, 1988). Evidence for the importance of Ca^{2+} in these cell death processes comes from the observation that inhibition of the increase in $[Ca^{2+}]$ by buffering Ca^{2+} with quin-2, chelation of extracellular Ca^{2+} using ethyleneglycol-bis(b-aminoethyl)N' tetraacetic acid (EGTA), or administration of calmodulin inhibitors, prevents the onset of cell death in these systems. Since apoptosis can also be prevented by incubation of these cells in Ca^{2+}-free medium, the source of the Ca^{2+} increase is likely to be extracellular. It is as yet unclear whether Ca^{2+} entry into the cell is mediated by existing Ca^{2+} channels. The Ca^{2+} channel blocker nifedipine can prevent the increase in $[Ca^{2+}]$ seen in apoptotic prostate cells (Martikainen and Isaacs, 1990). However, McConkey *et al.*, (1989b) have postulated that the Ca^{2+} influx seen in glucocorticoid-treated thymocytes is mediated by a heat-labile cytosolic factor, synthesised in response to glucocorticoid administration.

The role of Ca^{2+} in the apoptotic pathway remains to be fully established. One obvious proposal is that Ca^{2+} activates enzymes involved in apoptosis such as the endonuclease responsible for oligonucleosomal DNA fragmentation (see page 43) or tTgase (see page 48). McConkey *et al.* (1989b) have demonstrated that prevention of the Ca^{2+} increase in glucocorticoid-treated thymocytes inhibits the endonuclease and prevents apoptosis. However, since controversy currently surrounds the importance of internucleosomal DNA fragmentation in the apoptotic pathway, a universal role for Ca^{2+}

in apoptosis, based on endonuclease activation, cannot be supported. Moreover, results from a number of studies have failed to demonstrate a critical regulatory role for Ca^{2+} in apoptosis. Incubation of the T-cell derived CEM-C7 cells with dexamethasone in the absence of Ca^{2+} actually increased DNA fragmentation and slightly enhanced the lethal effect of dexamethasone (Bansal *et al.*, 1990). Similarly, [Ca^{2+}] chelation promoted the onset of apoptosis in ageing neutrophils (Whyte *et al.*, 1993), while calcium ionophores inhibited the onset of cell death in this system (Whyte *et al.*, 1993) and in the IL-3-dependent pre-B-cell lines BAF3 and FDCP-Mix (Rodriguez-Tarduchy *et al.*, 1992). In light of these conflicting results, it is not possible to propose a universal regulatory role for Ca^{2+} in apoptosis. A recent report concluded that Ca^{2+} influxes alone are unlikely to be sufficient to induce apoptosis (Duke *et al.*, 1994). It remains to be established whether such conflicting examples of the importance of Ca^{2+} in apoptosis represent cell type- and stimulus- dependent differences in the apoptotic pathway, or alternatively whether Ca^{2+} influxes are a consequence of rather than a trigger for apoptosis. Certainly, in a heterogeneous population of cells *in vitro*, it is possible that Ca^{2+} influxes occur predominantly as a result of the loss of membrane permeability that accompanies secondary necrosis.

1.4.2.4.2 Protein Kinase C.

There is accumulating evidence that PKC is involved in the modulation of apoptosis. However, this evidence is somewhat contradictory and details of the role of PKC in apoptosis require further clarification. To date, evidence of a regulatory role for PKC in apoptosis has been derived from studies involving PKC inhibitors and phorbol esters, which activate PKC directly (reviewed in Dive *et al.*, 1992b). The phorbol ester TPA has been shown to block DNA fragmentation and cell death induced in thymocytes by glucocorticoids and calcium ionophores (McConkey *et al.*, 1989d). Interestingly, treatment of thymocytes with the mitogen, concanavalin A, which operates via a mechanism involving PKC, causes a sustained increase in [Ca^{2+}] and cell proliferation. However, in the presence of a PKC inhibitor, H7 or sphingosine, the [Ca^{2+}] increase still occurs, but the cells undergo apoptosis (McConkey *et al.*, 1989d). Thus it appears that PKC activation blocks thymocyte apoptosis by diverting the Ca^{2+} signal from the cell

death pathway to one of proliferation. The protective effect of phorbol esters such as TPA has been reported in a number of other cell systems, including mouse fibroblast cells exposed to serum withdrawal or radiation (Tomei *et al.*, 1988), T-lymphocytes deprived of IL-2 (Rodriguez-Tarduchy and Abelardo, 1989), vascular endothelial cells deprived of serum (Araki *et al.*, 1990), leukaemic cells deprived of growth factors (Rajotte *et al.*, 1992) and B-cells treated with ionomycin (Vazquez *et al.*, 1991). TPA also protected cells from the Bursa of Fabricus of the chicken from spontaneous apoptosis (Asakawa *et al.*, 1993). The protective effect of phorbol ester in this and other cell systems was abolished by co-addition of inhibitors of PKC such as H7 or staurosporine. In direct contrast to these results, a number of reports exist demonstrating the induction of apoptosis by phorbol esters. TPA induced the onset of apoptosis in thymocytes, although somewhat confusingly conferred protection on these cells from ionophore-induced cell death (Kizaki *et al.*, 1989). Thymocyte apoptosis, induced by prostaglandin E_2 and isoproterenol, agents that elevate cyclic-AMP (cAMP), was also enhanced by co-addition of TPA (Suzuki *et al.*, 1991).

A number of possible factors could explain the conflicting nature of the results described above. The first concerns the nature of PKC activation by phorbol esters. Although initial exposure of cells to phorbol esters results in activation of PKC, this is followed by down-regulation of the enzyme as a result of proteolysis. As an example, short-term exposure of freshly isolated rat hepatocytes to TPA protects them from staurosporine-induced apoptosis. However, a longer exposure results, not in protection from cell death, but in the induction of apoptosis (Sanchez *et al.*, 1992). The extent of PKC down-regulation is dependent on the dose of phorbol ester and the cell type used. A second consideration is that the majority of PKC inhibitors used in these studies are not specific for PKC. Therefore the results obtained using PKC inhibitors may not only reflect the response of PKC but a range of other cellular processes. A further complication arises from the existence of at least ten isozymes of PKC which differ in their response to phorbol esters, PKC inhibitors and their requirement for growth factors (Knox *et al.*, 1993). A recent report demonstrates that the induction of apoptosis in stratified squamous epithelium of the tonsil is dependent on the differential expression of different PKC isozymes in these cells (Knox *et al.*, 1993). Thus, although PKC may play

a role in the regulation of apoptosis, it may well be that only certain isozymes are involved and that tissue differences exist.

1.4.2.4.3 Cyclic-AMP

cAMP is a second messenger molecule that activates the serine-threonine protein kinase, PKA. A role for cAMP in the signalling pathway of apoptosis has been demonstrated in studies of rodent palatal development, where epithelial cell death is believed to be mediated by cAMP (Pratt and Martin, 1975). Furthermore, agents that elevate intracellular cAMP, and analogues of cAMP have been demonstrated to induce apoptosis in isolated thymocytes (McConkey *et al.*, 1990a), murine lymphocytes (Dowd and Miesfeld, 1992) and in a myeloid leukaemia cell line (Lanotte *et al.*, 1991). In contrast elevations in cAMP have been shown to rescue sympathetic neurones from apoptosis induced by NGF deprivation (Edwards *et al.*, 1991). It is likely that the role of cAMP and PKA in apoptosis is dependent not only on cell type but also on the presence or absence of additional signalling events such as PKC activation (McConkey *et al.*, 1990b)

1.4.2.4.4 Poly (ADP-Ribose) Polymerase.

Poly (ADP-ribose) polymerase (pADPRp) mediates the cleavage of NAD^+ to nicotinamide and protein-linked ADP-ribose polymers (Ueda and Hayaishi, 1985). Over 95% of pADPRp activity occurs in the nucleus and is triggered by DNA strand breaks (Tsoponakis *et al.*, 1978; Benjamin and Gill, 1980a; Benjamin and Gill, 1980b). A number of proteins can be modified by pADPRp activity, including topoisomerase I and II, but the majority of ADP-ribose polymers are bound to pADPRp itself (Althaus and Richter, 1987). It has been recognised for some time that pADPRp activation promotes DNA excision repair (Creissen and Shall, 1982). A more recent report demonstrates that auto-modification of pADPRp facilitates the release of pADPRp from damaged DNA so that DNA repair enzymes can gain access to DNA single-strand breaks (Satoh and Lindahl, 1992).

The reactions catalysed by pADPRp consume NAD^+ and it has been proposed that in the presence of high levels of DNA damage, such as those seen during apoptosis,

this NAD^+ consumption leads to an associated and potentially lethal depletion of intracellular ATP (Carson *et al.*, 1986). In support of this theory, inhibitors of pADPRp have been shown to inhibit cytolytic-T-lymphocyte-induced cytotoxicity (Redegeld *et al.*, 1992) and can also prevent the decrease in NAD^+ that follows etoposide treatment of HL60 cells (Kaufmann *et al.*, 1993). However, a contrasting report indicates that pADPRp activation actually decreases during radiation-induced apoptosis of rat thymocytes (Nelipovich *et al.*, 1988). Moreover, inactivation of pADPRp has been demonstrated to induce apoptosis in a number of human tumour cell lines (Rice *et al.*, 1992) and inhibition of pADPRp augments dexamethasone-induced apoptosis of mouse lymphoma cells (Wielkens and Delfs, 1986).

It is not clear whether pADPRp has a protective role in apoptosis or whether its activity is ultimately the lethal event in the cell death process. It is difficult to reconcile results demonstrating that pADPRp activity is responsible for NAD^+ depletion with those that show decreasing pADPRp activity during apoptosis. One explanation for these differences has arisen from the recent finding that an early event in apoptosis induced in HL-60 cells by a variety of chemotherapeutic agents is the cleavage of pADPRp to an Mr ~85,000 fragment (Kaufmann *et al.*, 1993). In contrast to the intact molecule, this fragment is not activated by DNA strand breaks and its formation could provide an explanation for the decrease in pADPRp activity detected during radiation-induced thymocyte apoptosis. Recent studies using a cell free system have revealed that cleavage of pADPRp is mediated by a protease resembling interleukin 1β- (IL1-β-) converting enzyme (ICE; Lazebnik *et al.*, 1994; see page 66). It is proposed that cleavage of pADPRp during apoptosis may occur to ensure that the program of apoptosis is successful. Since the fragment of pADPRp is not activated by DNA strand breaks its activity cannot repair the DNA damage that accompanies apoptosis. Furthermore, it is proposed that a reduction in pADPRp activity is likely to reduce ATP consumption in the cell thereby releasing more energy for the completion of apoptosis (Lazebnik *et al.*, 1994).

1.4.2.5 Apoptosis: A Genetic Program.

As discussed earlier in this introduction (page 41) the term 'apoptosis' was first used to describe a morphological and biochemical phenomenon rather than a genetically controlled program. However, in the same way that proliferation and differentiation are gene-directed events, it is not unreasonable to assume that a process as fundamental and as highly regulated as apoptosis is under some form of genetic control. An increasing list of genes, implicated in the regulation of apoptosis, has emerged over recent years. The exact role played by each of these genes in the cell death pathway and the relationships between them, remain to be established.

The most comprehensive example of a genetic program of cell death is provided by the nematode *C. elegans* in which 131 of the cells produced die within an hour of birth. Genetic analyses have indicated that most programmed cell deaths in *C. elegans* involve the same mechanism and the same set of genes (Ellis and Horvitz, 1986). These genes have been identified by studying specific gene mutations that interfere with the normal program of cell death (reviewed in Ellis *et al.*, 1991; Driscoll *et al.*, 1992). Two genes of fundamental importance in the cell death process are *ced 3* and *ced 4* . Mutations in either of these genes prevent all programmed cell deaths and all 131 cells that would normally die, survive and go on to differentiate. The expression of *ced 3* and *ced 4* is negatively regulated by cell death specification genes, *ces 1* and *ces 2* and *egl-1* (**e**gg **l**aying **d**efective gene), whose roles are to determine which cells express the cell death program. *ced 3* and *ced 4* are also negatively regulated by the gene *ced 9* whose function is to protect those cells that should survive (Hengartner *et al.*, 1992). A loss of function mutation of *ced 9* leads to the death of the animal while a gain of function mutation prevents all cell deaths. This gain of function mutation is the result of a glycine-to-glutamate substitution in the open reading frame of *ced-9* (Hengartner and Horvitz, 1994a). Once a cell has died, its corpse is rapidly engulfed and degraded by neighbouring cells. Two sets of genes, have been identified which participate in the recognition and phagocytosis of dead cells (page 50). The degradation of cell corpses is carried out after phagocytosis.

The search is now on for mammalian counterparts to the genes identified in *C. elegans*.. The protein encoded by the gene *ced 9* shows sequence homology to the

mammalian proto-oncogene *bcl-2,* which can prevent apoptosis in a number of mammalian cell systems (Hengartner and Horvitz, 1994b; see page 70). Moreover, over-expression of *bcl-2* can mimic the protective effect of *ced 9* in *C. elegans*, and prevent the cell death that occurs as a result of a loss of function mutation of the *ced 9* gene (Hengartner and Horvitz, 1994b). Homology also exists between the *ced 3* gene and mammalian ICE (Yuan *et al.*, 1993; see page 66). An increasing number of other mammalian genes have been proposed as candidates for the regulation of apoptosis, although to date no other homologies have been found with the *C. elegans* system. The genes identified so far can be divided broadly into three classes: those that must be present for apoptosis to proceed, those whose expression is regulated during apoptosis and those that can prevent cell death. The major candidates in each group are discussed in more detail below.

1.4.2.5.1 Genes Implicated in the Regulation of Apoptosis.

1.4.2.5.1.1 *p53.*

The *p53* tumour suppressor gene encodes a nuclear phosphoprotein which acts as a transcription factor and sensor of DNA damage (Harris and Hollstein, 1993; Levine *et al.*, 1994). All cells express wild type (wt) p53 but since the protein only has a short half life, levels are low in normal cells (Zambetti and Levine, 1993). Following certain forms of DNA damage, post-translational stabilisation of wt p53 results in an increase in intracellular wt p53 expression (Lane, 1992). This over-expression of wt p53 protein leads to either growth arrest in G_1 (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992) or the induction of apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Fig. 1.2a). It is thought that by coupling DNA damage to growth arrest or apoptosis, *p53* acts as a cell cycle checkpoint between G_1 and S-phase, preventing the progression of cells with damaged DNA through the cell cycle (Lane, 1992). Growth arrest allows time for cells to repair DNA damage before mitosis. Alternatively, the induction of apoptosis provides a mechanism for the removal of cells whose damage is irreparable.

The mechanism by which *p53* mediates cell cycle arrest remains to be fully established. One important determinant may be the conformation of the p53 protein (Milner, 1994). It has been proposed that wt p53 exists in at least 3 different forms and

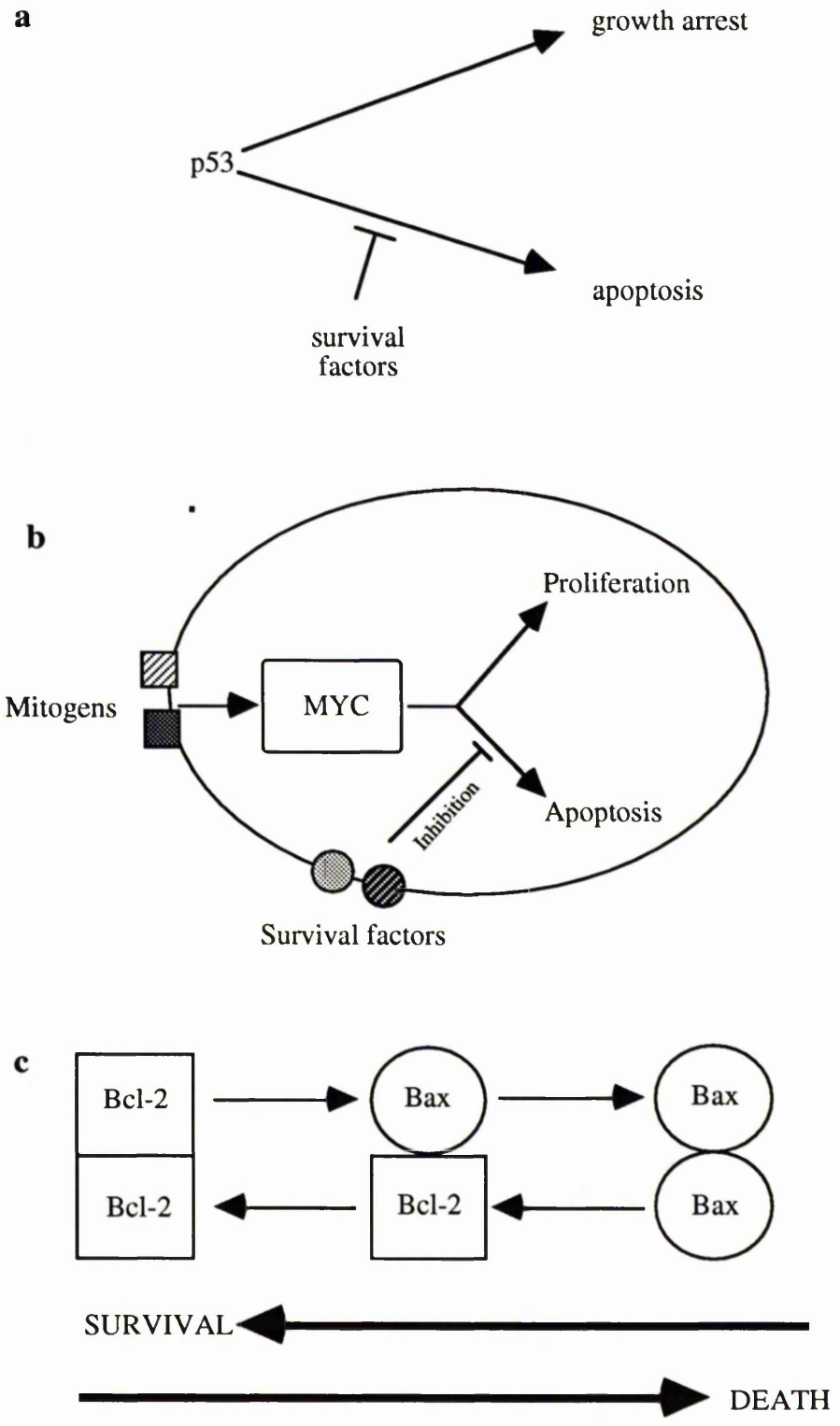


Figure 1.2. Models to explain current theories of the role of three genes in apoptosis.

(a) *p53* (from White, 1993);

(b) *c-myc* (from Evan *et al.*, 1994);

(c) *bcl-2* (from Oltvai *et al.*, 1993).

that each of these has a different role in cell cycle control (Milner, 1994). Another important factor in *p53*-induced growth arrest appears to be the DNA damage itself. It has been demonstrated that DNA strand breaks are essential for *p53*-induced growth arrest, whereas other forms of DNA damage do not invoke the same response (Nelson and Kastan, 1994). Much attention has also focused on the importance of genes whose transcription is dependent on *p53*. One such gene is *WAF1 /CIP1* (El-Deiry *et al.*, 1993; Harper *et al.*, 1993), a potent inhibitor of cyclin-dependent kinases. *WAF1* is expressed in the nucleus of T-cell lymphoma cells containing wt *p53* and is induced in these cells following exposure to ionising radiation or treatment with the chemotherapeutic agent adriamycin (El-Deiry *et al.*, 1994). However, *WAF1* is not induced in cells containing mutant *p53* or in cells induced to enter apoptosis via *p53*-independent mechanisms (El-Deiry *et al.*, 1994). It has been proposed that following DNA damage, wt *p53* induces *WAF1* transcription and that inhibition of cyclin-dependent kinases by *WAF1* causes cell cycle arrest (El-Deiry *et al.*, 1994). *p53* has also been shown to induce transcription of the gene *GADD45*, and a putative proto-oncogene, *mdm2* (Barak *et al.*, 1993). *GADD45* is up-regulated in response to DNA damage and its levels are highest in non-cycling cells (Fornace *et al.*, 1989). In contrast, *mdm2* is proposed to promote entry into S-phase (Levine *et al.*, 1994). Thus regulation of *GADD45* by *p53* may block the cell cycle at G_1, while the induction of *mdm2* allows cells to progress once DNA repair is complete (Levine *et al.*, 1994). In addition, at high levels, *mdm2* can bind to *p53* and prevent further transcriptional activation, providing a negative feedback mechanism for regulation of *p53* activity (Wu *et al.*, 1993).

The mechanism by which *p53* couples DNA damage to the apoptotic response is equally unclear. Since *p53* is induced only in response to DNA strand breaks, it is not surprising that *p53* has been shown to be important only in apoptosis induced by DNA damage (Lowe *et al.*, 1993; Clarke *et al.*, 1993). This has been demonstrated using mouse thymocytes. Thymocytes lacking wt *p53* are resistant to the effects of ionising radiation and the DNA damaging agent etoposide (Lowe *et al.*, 1993; Clarke *et al.*, 1993). However, the apoptotic death of immature thymocytes, induced by T-cell receptor engagement, treatment with glucocorticoids or calcium ionophore, can occur irrespective of the presence or absence of wt *p53* (Clarke *et al.*, 1993). Although it has been proposed

that genes induced by *p53* may mediate its effects on cell cycle progression, there is some controversy over the role of transcriptional activation by *p53* in apoptosis. A recent report has shown that the induction of apoptosis by DNA damaging agents is dependent on wt *p53* activity but is independent of new protein and RNA synthesis (Caelles *et al.*, 1994). The authors conclude that *p53* either represses the activity of genes required for cell survival or is directly involved itself in the apoptotic machinery or in DNA repair. In contrast it has been demonstrated that wt p53 has some control over the level of expression of mRNA encoding the Bax protein, and members of the Bcl-2 family which are implicated in the control of apoptosis (see page 72).

The absence of *p53*-induced cell cycle arrest and apoptosis could have potentially lethal consequences. A number of viruses, including simian vacuolating virus 40 (SV40) and adenovirus E1A and E1B inactivate the transcriptional activity of p53. This ability to block the normal activity of *p53* is likely to be highly beneficial to viruses, increasing the life-span of the infected cell and allowing the virus time to replicate (Lane *et al.*, 1994). Abrogation of the *p53* checkpoint is also likely to be an important determinant in the development of cancer. In the absence of a G_1/S checkpoint, cells that have accumulated DNA damage can continue to divide. This progression of cells with DNA damage into mitosis could contribute to the development of tumours (Kastan *et al.*, 1992). It is interesting that *p53* null mice develop normally to adulthood but are highly susceptible to the development of cancer (Donehower *et al.*, 1992). Furthermore, thymocytes and intestinal epithelial cells from these mice are resistant to radiation-induced apoptosis (Clarke *et al.*, 1993; Merritt *et al.*, 1994). The importance of wt *p53*-induced growth arrest may therefore explain the fact that the *p53* gene is one of the most commonly mutated genes in human tumours (Levine *et al*., 1991; Hollstein *et al.*, 1991).

1.4.2.5.1.2 *c-myc.*

The *c-myc* proto-oncogene is one of the immediate early response genes, whose expression is up-regulated rapidly after mitogenic stimulation of quiescent cells (Cole, 1986). However, in contrast to other immediate early response genes, which are induced only transiently in response to a mitogenic stimulus, *c-myc* is present continuously in proliferating cells (Evan *et al.*, 1992). While normal *c-myc* expression is thought to be

necessary for cell proliferation, deregulated *c-myc* expression has been linked to the induction of apoptosis in some cell systems. Withdrawal of growth factors from untransformed rodent fibroblasts results in rapid downregulation of c-Myc protein followed by growth arrest (Waters *et al.*, 1991). However, if *c-myc* is expressed constitutively in such growth factor-deprived cells, the cells cannot arrest and instead, undergo apoptosis (Evan *et al.*, 1992; Arends *et al.*, 1993). Similar results have been obtained using an IL-3-dependent myeloid cell line (Askew *et al.*, 1991). Deregulated *c-myc* expression has also been shown to increase the susceptibility of myeloid leukaemias to apoptosis induced by heat shock and chemotherapeutic agents (Lotem and Sachs, 1993).

The mechanism by which *c-myc* exerts its effects remain to be established. There is evidence that the protein encoded by *c-myc* is a transcription factor (Amati *et al.*, 1992). However, few targets for its activity have been defined. It has been demonstrated recently that c-Myc activity is dependent on dimerisation with a stable protein partner called Max (Amati *et al.*, 1994). Dimerisation of the two proteins occurs via a helix-loop-helix-leucine zipper. The importance of Max in c-Myc activity is highlighted by the fact that if the dimerisation domains are changed so that c-Myc no longer recognises Max, c-Myc cannot induce apoptosis (Amati *et al.*, 1994).

The involvement of *c-myc* in both proliferation and apoptosis has been explained by a 'dual signal' hypothesis. The basis of this hypothesis is that the components of the apoptotic pathway already exist in cells as a result of c-Myc expression. This is supported by the finding that untransformed fibroblasts expressing c-Myc can undergo apoptosis in the presence of the protein synthesis inhibitor cycloheximide (Evan *et al.*, 1992). It is proposed that when *c-myc* is switched on, the programs of both proliferation and apoptosis are initiated, but that under normal conditions apoptosis is suppressed by growth factors (Harrington *et al.*, 1994a; Fig. 1.2b, page 62). Growth factors identified as having the capacity to suppress *c-myc*-dependent apoptosis include insulin-like growth factors and platelet-derived growth factor (Harrington *et al.*, 1994b). Importantly, *c-myc*-induced cell death can also be inhibited by the product of the *bcl-2* proto-oncogene (see page 71).

Although the theory described above is attractive, conflicting evidence from other cell systems suggests that it may only apply to certain cell types. In contrast to the findings with fibroblasts, transfection of a T-lymphocytic cell line, CCRF CEM with *c-myc*, has been shown to inhibit, rather than induce, dexamethasone-induced apoptosis (Thulasi *et al.*, 1993). Moreover, it has been demonstrated in the CCRF CEM-C7 cell line and in HL60 leukaemic cells, that levels of *c-myc* and its protein product actually fall prior to the onset of apoptosis (Beere *et al.*, 1993; Wood *et al.*, 1994).

1.4.2.5.1.3 Interleukin 1-β-Converting Enzyme.

Cloning of the *ced-3* gene from *C. elegans*, and analysis of its protein product has revealed similarities with human *ICE* (Yuan *et al.*, 1993). *ICE* encodes a cysteine protease that cleaves the inactive precursor of IL1-β to the active form of the cytokine (Thornberry *et al.*, 1992). IL1-β is involved in mediating a variety of biological activities including inflammation, wound healing and hematopoiesis (Dinarello, 1991). A role for *ICE* in apoptosis has been demonstrated by the finding that over-expression of *ICE* in rat-1 fibroblasts results in the induction of apoptosis (Miura *et al.*, 1993). This effect is prevented by mutations in the active domain of *ICE* (Miura *et al.*, 1993). Interestingly, the death of fibroblasts over-expressing *ICE* can also be prevented by co-expression of the cowpox virus gene *crmA* (Miura *et al.*, 1993). This may reflect a role for *crmA* in prolonging the life span of the host after cowpox virus infection and provides further evidence of the ability of viruses to over-ride the apoptotic pathway (see page 64). In common with other mammalian genes involved in the regulation of apoptosis, *ICE*-induced cell death is also suppressed by Bcl-2 (Miura *et al.*, 1993).

It has been proposed that *ICE* functions as the mammalian homologue of *ced-3*. However, since there is as yet no evidence of *ICE* inducing apoptosis *in vivo*, the exact role played by the enzyme during mammalian cell death requires further clarification. It is known that the protein product of *ICE* consists of two subunits, P20 and P10. Although ICE must be proteolytically cleaved for enzyme activation (Thornberry *et al.*, 1992), both subunits are required for cell death (Miura *et al.*, 1993). However, the substrate acted on by ICE to induce cell death remains to be identified. Since not all cells have detectable amounts of IL1-β mRNA, it is possible that ICE has an additional

substrate to IL1-β, whose activation contributes to the initiation of apoptosis. A recent report demonstrates that a protease resembling ICE (prICE) which induces apoptosis in a cell free system, cleaves the nuclear enzyme pADPRp (Lazebnik *et al.,* 1994; see page 59). However ICE itself does not exert the same effect on pADPRp, suggesting that a family of ICE-like proteases may be involved in the mammalian pathway of apoptosis.

1.4.2.5.1.4 Fas.

The Fas antigen is a cell surface antigen that belongs to the TNF / NGF receptor superfamily (Itoh *et al.*, 1991). Fas is identical to the surface antigen APO-1 (Trauth *et al.*, 1989). Fas mRNA is expressed in a number of tissues including the liver, thymus, heart and ovary (Watanabe-Fukunga *et al.*, 1992) and by a variety of haemopoietic cells and cell lines (Trauth *et al.*, 1989; Drappa *et al.*, 1993; Nagata, 1994). All members of the TNF / NGF receptor family have a cysteine-rich extracellular domain, a transmembrane region and an internal tail. A 'death domain' on the internal tail of Fas appears to be responsible for signalling apoptosis (Itoh and Nagata, 1993). A role for Fas in the induction of apoptosis was first proposed after anti-Fas and anti-APO-1 antibodies were found to induce apoptosis in cells expressing the Fas antigen, such as murine T-cell lymphoma cells (Itoh *et al.*, 1991) and a human B lymphoma cell line (Trauth *et al.*, 1989). A role for Fas in the induction of apoptosis has also been demonstrated *in vivo* (Ogasawara *et al.*, 1993). Injection of anti-Fas antibodies is lethal to mice expressing wt Fas antigen (Ogasawara *et al.*, 1993). Liver sections from such mice exhibit areas of focal haemorrhage and necrosis. However these areas of necrosis are thought to be the result of massive amounts of hepatocyte apoptosis. It appears that the rate of apoptosis in the liver following anti-Fas administration is too rapid and widespread for all the apoptotic cells to be phagocytosed. Thus, somewhat unusually, apoptosis is followed by secondary degeneration of the cells, giving the impression of necrosis (Ogasawara *et al.*, 1993).

The Fas antigen may not always be responsible for the induction of apoptosis. Fas expression has been shown to induce proliferation rather than apoptosis in B cells from one chronic lymphocytic leukaemia (Mapara *et al.*, 1993). This proliferative response has been correlated with expression of *bcl-2* mRNA. Thus, while Fas

expression does appear to have a role in the apoptotic pathway, the response of cells to Fas may be determined by the presence of additional signalling events.

1.4.2.5.2 Genes that are Regulated During Apoptosis.

The expression of several genes is regulated during apoptosis. It remains to be established whether the induction of these genes is a causative or coincidental event in the apoptotic pathway.

1.4.2.5.2.1 *c-fos / c-jun.*

The *c-fos* and *c-jun* protooncogenes are immediate early response genes which code for transcription factors (Sheng and Greenberg, 1990). The products of the two genes form a heterodimer via a leucine zipper present in both proteins (Curran and Franza, 1988). The resulting heterodimer binds with high affinity to a consensus sequence on DNA, -TGACTCA-, thereby stimulating the transcription of nearby promoters (Curran and Franza, 1988). Like other immediate early response genes, *c-fos* and *c-jun* are up-regulated rapidly and transiently following extracellular stimulation (Curran and Franza, 1988). The two genes have also been implicated in the pathway of apoptosis.

Induction of *c-fos* and *c-jun* has been detected in IL-2 and IL-6-dependent cell lines induced to enter apoptosis by growth factor deprivation (Colotta *et al.*, 1992). This induction is immediate but transient: gene induction is seen 60 minutes after growth factor withdrawal, but after a further 60 minutes, levels are undetectable. The importance of *c-fos* and *c-jun* in the induction of apoptosis is highlighted by the finding that cells have an increased survival advantage following growth factor deprivation if *c-fos* and *c-jun* expression is reduced using anti-sense oligonucleotides (Colotta *et al.*, 1992).

Further evidence of a role for *c-fos* in apoptosis has been derived from experiments using a *fos*-LacZ transgenic mouse, in which expression of a detectable LacZ marker is driven by the regulatory element of *c-fos* (Smeyne *et al.*, 1993). Such studies indicate that during mouse embryogenesis apoptosis is preceded by *c-fos* expression. *c-fos* expression has also been shown to precede apoptosis in the regressing rat ventral prostate gland following castration (Buttyan *et al.*, 1988) and following

etoposide-treatment of the neuronal cell line, PC12 (Smeyne *et al.*, 1993). Furthermore, fibroblasts transformed with a vector expressing *c-fos* are approximately 10 times more sensitive to apoptosis induced by growth factor withdrawal than normal cells (Smeyne *et al.*, 1993). It remains to be established whether the induction of *c-fos* and *c-jun* in the early stages of apoptosis is an absolute requirement of the cell death pathway or merely reflects an adaptive stress response of a cell to toxic stimulation.

1.4.2.5.2.2 *TRPM-2.*

Testosterone repressed prostate message-2 (*TRPM-2*), also known as sulphated glycoprotein-2 (SGP-2) and clusterin, has been shown to be expressed in a number of tissues during apoptosis. Elevated levels of *TRPM-2* mRNA have been detected in regressing interdigital tissue during the development of rat limb buds, in prostatic rat epithelial cells following castration and in rat kidney cells following ureteral obstruction (Buttyan *et al.*, 1989). Accumulation of *TRPM-2* has also been detected in rat thymocytes, following an intraperitoneal injection of dexamethasone (Bettuzzi *et al.*, 1991), during the involution of the lactating breast (Strange *et al.*, 1992) and in regressing MCF-7 human breast tumours following oestrogen ablation (Kyprianou *et al.*, 1991). In this latter study, *TRPM-2* expression was found to coincide with enhanced expression of the transforming growth factor family of proteins which are potent growth inhibitors. Thus, the onset of apoptosis may be physiologically linked to suppression of proliferation in some systems.

Evidence of a role for *TRPM-2* in apoptosis is by no means conclusive. *TRPM-2* has been detected in the central nervous systems of humans and experimental animals (O'Bryan *et al.*, 1993). However, although *TRPM-2* levels are known to increase during neuronal development, no association has been demonstrated between *TRPM-2* expression and apoptosis in the developing mouse brain (O'Bryan *et al.*, 1993). Another study using human thymus, demonstrates that *TRPM-2* is actually expressed in different cells to those undergoing apoptosis (French *et al.*, 1992), raising the possibility that *TRPM-2* may be involved, not in the death pathway itself, but in the disposal of apoptotic cells following phagocytosis.

1.4.2.5.3 Genes that Suppress Apoptosis.

1.4.2.5.3.1 *bcl-2*.

The *bcl-2* proto-oncogene was first discovered as a result of its involvement in follicular lymphoma and other B-cell malignancies. In over 80% of cases, a t(14:18) translocation moves the *bcl-2* gene into juxtaposition with powerful promoter elements of the immunoglobulin (Ig) heavy chain locus (Tsujimoto *et al.*, 1985), resulting in deregulated *bcl-2* expression and over-production of Bcl-2 protein (Graninger *et al.*, 1987). The significance of this translocation can be seen in transgenic mice, bearing a *bcl-2*/Ig fusion gene. Such animals exhibit an expanded lymphoid compartment and extended B-cell survival (McDonnell *et al.*, 1989). These and subsequent experiments have implicated *bcl-2* as an inhibitor of apoptosis. Over-expression of *bcl-2* has been shown to suppress apoptosis induced by growth factor withdrawal in haemopoietic cells (Vaux *et al.*, 1988; Baffy *et al.*, 1993), neuronal PC12 cells (Mah *et al.*, 1993) and a cytotoxic T-cell line (Deng and Podack, 1993). The protective effect of *bcl-2* has also been reported during terminal differentiation of HL-60 myeloid leukaemia cells (Naumovski and Cleary, 1994).

The biochemical properties of Bcl-2 and the mechanism(s) by which its protective effect is exerted remain to be established. It was thought initially that Bcl-2 protein localises to the inner mitochondrial membrane (Hockenberry *et al.*, 1990). However, subsequent studies have established that Bcl-2 resides in the outer mitochondrial membrane (Nakai *et al.*, 1993) and membranes of other subcellular compartments such as the nuclear envelope and endoplasmic reticulum (Krajewski *et al.*, 1993). The recent finding that Bcl-2 also localises to chromosomes of mitotic nuclei (Lu *et al.*, 1994), indicates a potential involvement in the protection of mitotic cells from cell death by apoptosis.

Bcl-2 has been shown to interact with a number of different proteins. For example, the protein Bax shares extensive homologies with Bcl-2 and can form heterodimers with Bcl-2 (Oltvai *et al.*, 1993). In particular, Bcl-2 and Bax share two highly conserved regions, named BH1 and BH2 (**B**cl-2 **h**omology **1** and **2**). These two domains are now known to be involved in the formation of Bcl-2/Bax heterodimers and are essential for the protective effect of Bcl-2 (Yin *et al.*, 1994). However, in addition to

forming heterodimers, Bax can also form homodimers whose effects on apoptosis oppose that of Bcl-2. Over-expression of Bax has been found to accelerate apoptosis induced by growth factor withdrawal from an IL-3 dependent cell line and abrogate the protective effect of Bcl-2 (Oltvai *et al.*, 1993). Thus it appears that the decision between survival or death in response to an apoptotic stimulus is determined at least in part by the ratio of Bcl-2 to Bax (Oltvai *et al.*, 1993; Fig. 1.2c, page 62). If Bcl-2 is in excess, cells are protected from cell death whereas if Bax-Bax homodimers predominate, cells are susceptible to apoptosis (Korsmeyer *et al.*, 1993).

Another *bcl-2*-related gene that can regulate apoptosis independently of *bcl-2* is *bcl-x* (Boise *et al.*, 1993), which exists as two alternatively spliced mRNAs, *bcl-*x_L and *bcl-*x_S. Both negative and positive effects of *bcl-x* on apoptosis have been demonstrated. Stable transfection of *bcl-*x_L into an IL-3 dependent cell line has been shown to protect the cells from apoptosis induced by growth factor withdrawal to the same extent as *bcl-2*. In contrast, transfection of *bcl-*x_S into the same cells inhibited the protective effect of *bcl-2* (Boise *et al.*, 1993).

A role for *bcl-2* has been proposed in resistance of tumour cells to chemotherapeutic drugs. An enhanced survival advantage in the presence of *bcl-2* has been demonstrated in lymphoid cells exposed to DNA damaging agents (Walton *et al.*, 1993), and following exposure of neuroblastoma cells and a human leukaemic cell line to chemotherapeutic agents (Miyashita and Reed, 1993; Dole *et al.*, 1994). Furthermore human lymphoma cells, transfected with *bcl-2* have been shown to be resistant to apoptosis induced by inhibitors of thymidylate synthase (Fisher *et al.*, 1993). This protection was not mediated via classical resistance pathways such as altered drug targets or differences in levels of DNA damage (Fisher *et al.*, 1993) indicating that suppression of apoptosis by Bcl-2 and related proteins may represent a novel mechanism of drug resistance.

Interaction of *bcl-2* with other genes implicated in the control of apoptosis has been demonstrated. Transgenic mice carrying both *bcl-2* and *c-myc* exhibit enhanced hyperproliferation of pre-B and B cells and have been shown to be more susceptible to the development of tumours than single *bcl-2* or *c-myc* transgenic animals (Strasser *et al* 1990). In addition, Bcl-2 protein has been shown to inhibit apoptosis induced by growth

factor withdrawal and *c-myc* over-expression in Chinese Hamster Ovary cells (Bissonnette *et al.*, 1992) and in rat-1 fibroblasts (Wagner *et al.*, 1993). Under normal circumstances, *c-myc* over-expression provides cells with a growth advantage only in the presence of specific growth factors (Evan *et al.*, 1992, see page 65). It appears that co-expression of *bcl-2* abrogates this requirement for secondary survival signals so that cells which would normally undergo apoptosis in the absence of growth factors not only survive, but have the potential to undergo deregulated proliferation.

A relationship has also been demonstrated between expression of *bcl-2* , *bax* and the activity of *p53*. (see page 64) Transfection of murine leukaemia cells with a temperature sensitive *p53* has been found to induce a temperature-dependent decrease in *bcl-2* expression and a simultaneous elevation in *bax* expression (Miyashita *et al.*, 1994a). Down-regulation of *bcl-2* by a mutant form of *p53* has also been demonstrated in the MCF-7 breast cancer cell line (Haldar *et al.*, 1994). Moreover, mice deficient in *p53* have been shown to exhibit elevated Bcl-2 and depleted Bax protein levels in several tissue types (Miyashita *et al.*, 1994a). A negative response element has been identified in the *bcl-2* gene which is thought to mediate the down-regulation of *bcl-2* by *p53* (Miyashita *et al.*, 1994b). It is not yet apparent whether the response element meditates this transcriptional down-regulation directly. While it is hard to reconcile the identification of this response element with recent reports that *p53* does not act as a transcriptional regulator (Caelles *et al.*, 1994), the identification of this response element is further evidence of the ability of *p53* to exert its effects via regulation of the *bcl-2* family of genes. The rapid up-regulation of *bax* by *p53* is proposed to account for the speed of *p53*-induced apoptosis. $TGF\beta_1$-induced murine leukaemia cell death occurs more slowly than *p53* induced cell death. Although *bcl-2* expression is reduced following $TGF\beta_1$ administration, no associated increase in *bax* expression has been demonstrated (Selvakumaran *et al.*, 1994).

Some insight into the role of *bcl-2* has been gained from the study of mice deficient in *bcl-2* expression. Such animals have been shown to complete embryonic development. However, after birth these animals suffer retarded growth and premature death, associated with apoptotic involution of the thymus and spleen, polycystic kidney disease and hypopigmented hair (Veis *et al.*, 1993). These last two are symptomatic of a

defect in the redox pathway, suggesting that the Bcl-2 protein may exert its protective effect via an anti-oxidant pathway. This theory is further supported by the finding that over-expression of *bcl-2* can suppress lipid peroxidation while other known antioxidants can suppress apoptosis in an IL-3 dependent murine cell line (Hockenberry *et al.*, 1993).

1.4.2.5.3.2 *ras.*

A number of primary human neoplasms exhibit high levels of *ras* gene expression (Bos, 1989). Over-expression of the *ras* gene in rodent fibroblasts *in vitro* has been shown to confer metastatic activity on the cells (Spandidos and Wilkie 1984; Muschel *et al.*, 1986; Bradley *et al.*, 1986). In addition, early passage Chinese hamster ovary cells and aneuploid rat fibroblasts, transfected with human Ha-*ras* and inoculated into immunosuppressed mice, have been shown to develop aggressive properties including a high growth rate and metastatic potential (Wyllie *et al.*, 1987). The resulting tumours exhibited fewer apoptotic cells compared with tumours expressing only low levels of ras. The protective effect of the Ha-*ras* oncogene has been demonstrated further *in vitro*. Immortalised rat fibroblasts transfected with mutant Ha-*ras*, were seen to exhibit reduced apoptosis following serum withdrawal compared to controls (Arends *et al.*, 1993). Ha-*ras* activation has also been shown to be associated with a reduction in cell death of an IL-3 dependent mouse mast cell line (Andrejauskas and Moroni, 1989). Together these results implicate Ha-*ras* in the regulation of apoptosis and the rate of tumour development. Interestingly, a ras-related protein R-ras p23 has recently been shown to bind to Bcl-2. It has been proposed that R-ras may normally be involved in the signal transduction pathway of apoptosis but that if Bcl-2 is over-expressed, R-ras binds to Bcl-2 and is prevented from transmitting the apoptotic signal (Fernandez-Sarabia and Bischoff, 1993).

1.4.2.5.3.3 *Abl.*

The abelson (*abl*) oncogene is associated with chronic myeloid leukaemia, in which a chromosomal translocation results in the formation of a fusion gene, *bcr-abl*. The resulting gene product displays deregulated activity of the ABL protein tyrosine kinase, similar to that seen with the transforming oncogene, *v-abl* (Keliher *et al.*, 1990).

There is some evidence to suggest that haemopoietic cells from patients with chronic myeloid leukaemia (CML) have an enhanced survival potential *in vitro* following growth factor deprivation (Wang *et al.*, 1989). Experiments using an IL-3-dependent haemopoietic cell line, transfected with a temperature-sensitive *v-abl*, have demonstrated that v-ABL tyrosine kinase activity can suppress apoptosis induced by growth factor deprivation (Evans *et al.*, 1993a) and treatment with the chemotherapeutic agents melphalan and hydroxyurea (Chapman *et al.*, 1994). v-ABL activity does not affect the induction of DNA damage induced by these agents indicating that protection is exerted via a block in the coupling of damage to the apoptotic pathway (Chapman *et al.*, 1994).

1.4.2.6 Apoptosis in Tumours.

The progressive growth of tumours forms the basis of malignancy. The rate at which this growth occurs is determined by the comparative rates, within a tumour, of cell production through mitosis and cell loss through migration, exfoliation and cell death. (Wyllie, 1985). In the majority of experimental and human tumours, the rate of cell loss is high. Some of this cell loss can be accounted for by zones of necrosis that exist in poorly vascularised regions of a tumour where an insufficient blood and oxygen supply results in hypoxia (Denekamp *et al.*, 1982). However, high rates of cell loss are also observed in slow growing tumours such as basal cell carcinomas, where the incidence of necrosis is low, but the mitotic index is high (Kerr and Searle, 1972). This cell loss is attributed to apoptosis.

Apoptosis occurs widely in tumours and is reported to account for the majority of tumour cell loss that occurs (Sarraf and Bowen, 1988). Apoptotic cells have been detected in hepatic neoplasms, ascites tumours, sarcomas, small cell lung carcinoma and tumours of the colon, prostate and breast (Wyllie, 1985; Sarraf and Bowen, 1988; Cotter *et al.*, 1990). The reason for the existence of apoptotic cells within a tumour population requires clarification. Since tumour cells often exhibit a survival advantage over normal cells when confronted with mildly injurious stimuli such as hypoxia, it is unlikely that apoptosis in tumours occurs as a result of enhanced susceptibility of tumour cells to undergo apoptosis. An alternative proposal arises from the dependence of tumour cells on growth factors or hormones for survival. Hormone ablation has been demonstrated to

cause the regression of certain tumours (Cotter *et al.*, 1990; Kyprianou *et al.*, 1991), while apoptosis can be induced by other cytokines such as TNF (Robaye *et al.*, 1991). Thus, the high levels of apoptosis detected within a tumour cell population may occur as a result of changes in the concentration of particular cytokines or hormones in the tumour (Wyllie, 1985). Finally, it has been proposed that apoptosis occurs in a residual attempt by the host or the tumour itself at autoregulation.

1.4.2.7 Apoptosis and Cancer Chemotherapy.

Rates of apoptosis are enhanced during tumour regression (Kyprianou *et al.*, 1991) and apoptosis is induced in tumour cells by administration of chemotherapeutic agents (Barry *et al.*, 1990). Indeed the majority of cytotoxic agents used as anticancer agents including topoisomerase inhibitors (Walker *et al.*, 1991; Solary *et al.*, 1993), DNA cross-linking agents (Barry *et al.*, 1990; Fisher *et al.*, 1993; Evans and Dive, 1993) and antimetabolites (Dyson *et al.*, 1986) can induce apoptosis in sensitive cells (reviewed in Hickman, 1992). This is perhaps surprising considering the diversity of drugs currently available and suggests that sensitivity to cytotoxic drugs is determined not only by the drug-target interaction but also by the ability of a cell to engage an apoptotic response (Dive and Hickman, 1991; Hickman, 1992). Identification of the gene products that regulate apoptosis may help to shed some light on how a cytotoxic stimulus becomes coupled to an apoptotic response and why this response may not be elicited in some cases. Such knowledge would be of benefit in the identification of new targets for chemotherapeutic drugs, and in the treatment of some of the major human cancers which are at present resistant to the most potent chemotherapeutic agents.

1.5 Aims and Outline of this Thesis.

A fundamental question in cancer research is how agents, which do not directly interact with or damage DNA, contribute to the development of cancer. The peroxisome proliferators are one such group of non-genotoxic carcinogens, used in a variety of herbicides, plasticisers and hypolipidaemic drugs, and known to be carcinogenic to rodents (Reddy and Lalwani, 1983). An understanding of the mechanisms by which

these chemicals exert their carcinogenic effects is essential in order that the risk they pose to humans can be fully assessed.

One proposal is that peroxisome proliferators in common with other non-genotoxic carcinogens suppress the normal process of cell death by apoptosis in the liver, potentiating the survival of damaged or preneoplastic cells long enough for further transforming lesions to occur (Bursch *et al.*, 1992). To date this proposal has been investigated *in vivo*, where the rapid removal of apoptotic cells makes their quantification difficult. The aim of this thesis is to investigate the ability of peroxisome proliferators, in particular the hypolipidaemic drugs nafenopin and Wy-14,643, to suppress liver cell apoptosis *in vitro* using cell systems that are more amenable to studies of apoptosis than the *in vivo* situation and to investigate some of the molecular mechanisms by which this suppression could occur.

The thesis is divided into a number of chapters, each describing a different section of work. Chapter 2 (page 77-87) contains general methods used during the course of the investigation, while methods specific to one area of work are described in the relevant chapters. Chapter 3 (page 88-124) describes the work carried out to establish and validate a suitable *in vitro* hepatoma cell line for studying the effects of peroxisome proliferators on liver apoptosis. Since cell death and proliferation work in tandem to control liver homeostasis, the effects of nafenopin on liver cell proliferation are described in this chapter. In Chapter 4 (page 125-164) the hepatoma cell line has been used alongside primary rat hepatocytes to investigate the effect of peroxisome proliferators on spontaneous liver cell apoptosis. The results are compared with the effects on apoptosis induced by the DNA-damaging agents etoposide and hydroxyurea. Chapter 5 (page 165-199) describes the effects of peroxisome proliferators on TGFβ_1-induced liver cell apoptosis. In addition, this chapter outlines experiments carried out to investigate the molecular mechanisms by which the suppression of apoptosis by peroxisome proliferators could occur. A general discussion of the results presented in this thesis and the wider implications of these findings is given in Chapter 6 (page 200-209).

Chapter 2.

General Materials and Methods.

2. General Materials and Methods.

This chapter contains details of the general materials and methods used throughout this project. Materials and methods specific to Chapters 3, 4 and 5 are described in the appropriate chapter.

2.1 Materials.

2.1.1 Tissue Culture Reagents.

Fao hepatoma cells, derived from the Reuber H35-derived cell line H4IIEC3, were a gift from Dr Mary Weiss, Pasteur Institute, Paris and were later purchased from the European Animal Cell Culture Collection. $7800C_1$ and MH_1C_1 hepatoma cells, derived from the Morris hepatomas 7800C and 7795 respectively, were a gift from Professor Gautvik, Institute of Medical Biochemistry, Oslo. HTC cells, derived from the Morris hepatoma 7288C, were purchased from Flow, UK and RH1 cells were from the tissue culture cell bank at Zeneca Central Toxicology Laboratory (CTL), Alderley Park, UK.

William's E medium (William's medium) was from Gibco, UK and all other tissue culture media were from Imperial, UK. Foetal calf serum for hepatocyte culture was from Techgen, UK and for hepatoma cell culture, from Advanced Protein Products, UK. Trypsin was from Integra Biosciences, UK and all other tissue culture media reagents were from Sigma, UK, Flow, UK or Gibco, UK. Tissue culture plastics were from Nunc, UK or Costar, UK.

2.1.2 General Materials.

Nafenopin (2-methyl-2[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid was a gift from Ciba-Geigy, Basel, Switzerland. Wy-14,643 ([4-chloro-6-(2,3-xylidino)2-pyrimidinylthio] acetic acid) was purchased from ChemSyn, USA. Acridine orange, propidium iodide, and Hoechst 33258 were purchased from Molecular Probes Inc., Oregon, USA. Protogel polyacrylamide was from National Diagnostics, UK. and X-ray film was from Kodak, UK. All other chemicals were of analytical grade and were purchased from Sigma, UK, AnalR, or Aldridge, UK.

2.2 Methods.

2.2.1 Preparation of Primary Hepatocyte Suspensions.

Rat hepatocytes were isolated by Neil James and Gill Barber (Zeneca CTL) from 180-200g male Alderley Park rats (Wistar derived) by a two stage collagenase perfusion method (Seglen, 1973). Briefly, the livers of rats under terminal anaesthesia were perfused with collagenase (0.05%) and a hepatocyte suspension was obtained by passing digested livers through a 0.125 mm gauze. Cells were washed in ice-cold Hank's balanced salt solution, pelletted by centrifugation (8 x g, 10 min.) and the supernatant was removed by aspiration. This washing procedure was repeated 3 times to ensure that no excess collagenase remained in the cell suspension. Cells were counted using a haemocytometer and cell viability was determined by trypan blue (TB) exclusion (see page 81). Approximately 400 million cells were obtained per liver with a viability of ≥ 85%.

2.2.2 Culture of Primary Hepatocytes.

Freshly isolated hepatocytes were seeded at 1.8 x 10^6 / 25 cm^3 flask in William's medium (4 ml) supplemented with foetal calf serum (FCS, 10%, heat inactivated), insulin (10 μg/ml), hydrocortisone (0.1 μM), L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml; termed 'complete William's medium'). In order to aid attachment of hepatocytes, flasks were pre-coated with rat tail collagen as follows: rat tail collagen (200 μg/ml in acidified, sterile, double distilled water (ddH_20) pH 4.5) was added to each flask so that the base of the flask was covered. After 4 hours, collagen was removed by aspiration and the flasks were left to dry overnight in a sterile environment. Before addition of cells, the flasks were washed once with complete William's medium to remove any residual traces of acid. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed 4 hours and 24 hours after seeding and every 48 hours thereafter.

2.2.3 Culture of Hepatoma Cell Lines.

RH1 cells were cultured in Dulbecco's Modification of Eagles medium (DMEM) supplemented with FCS (10%) and HTC cells were cultured in the same medium further

supplemented with non-essential amino acids (1%). Fao and $7800C_1$ cells were cultured in Ham's Nutrient Mixture F12 (Ham's F12) supplemented with FCS (10%) or horse serum (15%) and FCS (2.5%) respectively. MH_1C_1 cells were cultured in Ham's Nutrient Mixture F10 (Ham's F10), supplemented with horse serum (15%) and FCS (5%). In addition all culture media contained L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 units/ml). Media containing these levels of serum and other supplements are referred to as 'complete'. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 and medium was changed every 48 hours.

Hepatoma cell lines were routinely seeded at 2 x 10^5-2 x 10^6 / 75 cm^3 flask in appropriate complete medium (10 ml) and were passaged every 5-7 days by trypsinisation. Medium was removed from the flasks by aspiration and monolayers were washed once with sterile phosphate buffered saline (PBS) at 37°C. PBS was removed from the flasks and cells were then washed briefly with a solution of trypsin (0.025%) and ethylenediaminetetraamine (EDTA, 0.01%) in PBS at 37°C. Following removal of the trypsin, cells were incubated at 37°C for 2-3 minutes (min.), until the cells had detached from the monolayer. Cells were then resuspended in appropriate complete medium (10 ml) and pelletted by centrifugation (200 x g, 5 min.) to remove any excess trypsin. The supernatant was removed, cells were resuspended in fresh medium and cell number and viability were determined by TB exclusion (see page 81). All cell lines were screened every 3 months for the presence of mycoplasma and were found to be negative.

2.2.4 Cryostorage of Hepatoma Cell Lines.

Early passage stocks of hepatoma cells were stored in liquid nitrogen. Cells from a confluent 75 cm^3 culture flask were removed from the monolayer by trypsinisation and were pelletted by centrifugation (200 x g, 5 min.). Cell pellets were resuspended in the appropriate medium (3 ml) containing dimethylsulphoxide (DMSO, 10%) and twice the usual serum concentration. Samples (1 ml) were aliquoted into cryostorage tubes and placed at 4°C for 1 hour. Cells were then transferred to the vapour phase of liquid nitrogen for 4 hours before being immersed in liquid nitrogen for storage.

Frozen samples were revived by fast thawing at 37°C and the entire contents of one vial placed in a 25 cm^3 flask. Appropriate medium (3 ml) was added slowly to the

cells to avoid cell lysis. Cultures were incubated in an atmosphere of 5% CO_2 at 37°C for 8 hours, after which time the medium was replaced with fresh medium to remove residual traces of DMSO from the cultures. Cultures were then maintained as described in section 2.2.3 (page 79). A new stock of hepatoma cells was revived when the passage number reached 25.

2.2.5 Charcoal-Stripping Serum.

In some experiments, cells were cultured in serum-free medium. However, cells would not form a monolayer if seeded in medium containing no serum. Therefore, in these experiments, cells were seeded in medium containing serum from which growth factors had been removed. FCS was stripped of growth factors using dextran-coated charcoal as follows: dextran (5 mg/ml) and acid-washed, activated charcoal (50 mg/ml) were mixed in ddH_2O (20 ml). The dextran-coated charcoal was then pelletted by centrifugation (300 x g, 10 min.) and the supernatant removed by aspiration. The charcoal was then added to FCS (100 ml) and heated to 55°C for 30 min. Charcoal was then separated from the FCS by centrifugation (300 x g, 10 min.) and the serum was transferred to another tube. The remaining charcoal was removed from the serum by sterile filtration (Cohen et al., 1993; Germain and Harbrioux, 1993).

2.2.6. Assessment of Cell Viability.

2.2.6.1 Trypan Blue Exclusion.

Equal volumes of cells in suspension and TB (0.3% in PBS) were mixed in an eppendorf tube and a sample (10 µl) loaded into one chamber of an Improved Neubauer haemocytometer. Viable cells were identified as those which excluded TB and appeared colourless. Non-viable cells with damaged plasma membranes allowed uptake of TB and therefore appeared blue. Duplicate counts of at least 150 cells were performed per data point and viable cell number was expressed as a percentage of the total number of cells counted.

2.2.6.2 Analysis of Primary Hepatocyte Nuclear Morphology.

The morphology of hepatocytes was examined by fluorescence microscopy of fixed hepatocyte monolayers stained with Hoechst 33258 (Ho258; adapted from Oberhammer *et al.*, 1991). Medium was removed from primary hepatocyte cultures by aspiration and monolayers were fixed for 5 min. in ice-cold methanol / acetic acid (2 ml of a 3:1 solution). Fixative was removed by aspiration and the flask bases were removed using a circular saw (Hobbycraft). Monolayers were stained with Ho258 (1 ml of a 5 μg / ml solution in ddH_2O) for 10 min. Monolayers were then washed once with ddH_2O and cells were mounted in a solution of citric acid (20 mM), di-sodium orthophosphate (50 mM) and glycerol (50%, pH 5.5) to achieve optimum fluorescence. Monolayers were examined at a wavelength of 350-460 nm using an Olympus BH2 microscope with fluorescence attachment. Ten randomly chosen fields, representing at least 800 cells, were analysed per data point. Apoptotic cells were identified as those with brightly stained, condensed or fragmented nuclei, and were expressed as a percentage of the total cell number counted. Viable cells were identified as those with diffusely stained, intact nuclei. The occasional cell undergoing mitosis was detected but such cells were readily distinguishable from apoptotic cells. Weakly stained cell ghosts were not counted. Photographs were taken using Zeiss optics on T-Max 400 film.

2.2.6.3 Analysis of Hepatoma Cell Nuclear Morphology.

The morphology of detached and attached hepatoma cells was examined by acridine orange (AO) staining and fluorescence microscopy (Gregory *et al.*, 1991). In order to isolate detached cells, the medium from hepatoma cultures was removed by aspiration and replaced with fresh medium. After 2 hours, medium was transferred to a universal tube and cells were pelletted by centrifugation (200 x g, 5 min.). Supernatant was removed by aspiration and the cell pellet was resuspended in fresh medium (10-50 μl, according to pellet size).

A sample of cell suspension (6 μl) was mixed on a glass slide with an equal volume of AO (10 μg/ml in PBS). The sample was covered with a glass cover slip and green fluorescence was detected at a wavelength of 500-525 nm using an Olympus BH2 microscope with fluorescence attachment. Apoptotic, viable and mitotic cells were

identified using the criteria described for primary hepatocytes (section 2.2.6.2, page 82). The number of apoptotic cells was expressed as a percentage of total cell number. Apoptotic cells were photographed using a BioRad MRC 600 confocal microscope. Cells were prepared for confocal microscopy by fixation in paraformaldehyde (4%) for 10 min. and slides were prepared as described above.

2.2.7 Analysis of DNA Integrity by Conventional Agarose Gel Electrophoresis.

The DNA from attached and detached hepatoma cells and primary hepatocytes was analysed by conventional agarose gel electrophoresis (CAGE; adapted from Smith *et al.*, 1989). Approximately 10^6 cells were pelletted by centrifugation (200 x g, 5 min.) and resuspended in 20 µl lysis buffer (EDTA (10 mM), Tris HCl (50 mM, pH 8) containing sodium lauryl sarkosinate (0.5%) and proteinase K (0.5 mg/ml)). Low gelling temperature agarose (10 µl of a 1% solution in 10 mM EDTA, pH 8) was melted at 70°C and added to the samples. Samples were then loaded into the dry wells of an agarose gel (2%) containing ethidium bromide (0.5 mg/ml). Low range molecular weight markers were loaded at one end of the gel. Samples were allowed to solidify for 5 min. before the gel was flooded with tris-phosphate-EDTA (TPE) running buffer. DNA was subjected to electrophoresis at 40 volts for 3 hours. The gel was then incubated at 37°C for 3 hours with TPE running buffer containing RNase A (0.1 mg/ml). DNA was visualised under UV illumination and gels were photographed on Kodak Polaroid 667 film.

2.2.8 Analysis of DNA Integrity by Field Inversion Gel Electrophoresis.

The DNA from primary hepatocytes and Fao hepatoma cells was prepared for analysis by field inversion gel electrophoresis (FIGE; Oberhammer *et al.*, 1993b). Approximately 2 x 10^6 cells were resuspended in lysis buffer (EDTA (0.1 M, pH 8) containing Tris HCl (0.01 M, pH 7.8) sodium chloride (NaCl, 0.02 M) and sodium dodecyl sulphate (SDS, 1%)). Low gelling temperature agarose (50 µl of a 1% solution in lysis buffer) was added to each sample. Samples were then transferred to a plug mould which was placed on ice for 30 min. Plugs were transferred to an eppendorf tube containing lysis buffer (1 ml containing 1% sodium lauryl sarcosylate and 0.1 mg/ml

proteinase K) and were incubated overnight at 37°C. Plugs were then stored in EDTA (500 mM) at 4°C until required.

FIGE was carried out using the method of Filipski *et al.* (1990). Plugs were loaded into an agarose gel (1.5%) and capped with low gelling temperature agarose. Molecular weight markers (50-1000 kb) were loaded at either end of the gel. Samples were left [illegible] the gel was flooded with running buffer (Tris acetate (10 mM, pH 8) and EDTA (1 mM)). DNA was subjected to electrophoresis at 150 volts with a ramping rate changing from T_1= 0.5 sec to T_2= 10 sec for the first 19 hours and from T'_1= 10 sec to T'_2= 60 sec for the following 19 hours with a forward to backward ratio of 3 at 4°C in a horizontal gel chamber using a model 200/20 power supply and Pulsewave switcher (Biorad). Following electrophoresis, the gel was stained with ethidium bromide (0.5 µg /ml) for 45 min. and then de-stained in ddH_2O for 60 min. The gel was incubated for a further 3 hours in running buffer containing RNase A (0.5 µg /ml) at 37°C. DNA was visualised under UV illumination and gels were photographed on Kodak Polaroid 667 film.

2.2.9 Treatment of Cells with Nafenopin and Wy-14,643.

Nafenopin and Wy-14,643 were added to the medium of primary hepatocyte and hepatoma cell cultures from a 20 mM stock dissolved in dimethylformamide (DMF). Control flasks were treated with DMF alone to the same final concentration of 0.25% $^{v}/_{v}$.

2.2.10 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.

2.2.10.1 Preparation of Samples.

Primary hepatocytes and hepatoma cells were gently scraped from the monolayer using a sterile cell scraper into the appropriate serum-free medium (10 ml). Cells were pelletted by centrifugation (200 x g, 5 min., 4°C) and the supernatant was removed by aspiration. Pellets were resuspended in ice cold PBS (1 ml), transferred to an eppendorf tube and pelletted by centrifugation (200 x g, 5 min., 4°C). The supernatant was removed and this washing procedure was repeated to remove any residual traces of serum from the culture medium that would otherwise interfere with subsequent protein assays.

Pellets were resuspended in ice cold EDTA-PBS (100 µl of a 1 mM solution) containing phenylmethylsulphonyl fluoride (PMSF, 1 µl from a saturated stock in isopropanol). Samples were sonicated using a Soniprep 150 sonicator fitted with a finger probe (2 x 15 second bursts) and centrifuged (9000 x g, 5 min., 4°C). This sonication and centrifugation procedure was repeated. Following the second centrifugation step, the supernatant was removed and transferred to a fresh eppendorf on ice. The protein content of the samples was analysed using a BioRad protein assay. Protein standards were made using bovine serum albumin (BSA) in ddH_2O and were assayed using a digital Series 2 UV spectrophotometer at 595 nm.

Samples were diluted to a final concentration of 4 µg/ml in ddH_2O and were then further diluted 1:1 in 2 x Laemmli sample buffer (see Appendix 1, page 211). Samples were boiled for 3 min. to denature the protein and were stored at -20°C prior to use.

2.2.10.2 SDS-Polyacrylamide Gel Electrophoresis.

Proteins were separated according to size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli and Favre, 1973). The composition of separating gels, stacking gels and solutions used during electrophoresis and western blotting are given in Appendix 1 (page 211).

Electrophoresis was carried out in a BioRad mini Protean II electrophoresis cell. The separating gel was poured between glass plates on a casting stand with 0.75 mm spacers. A gap of 2 cm was left at the top of the plate and the gel was overlaid with water-saturated butanol to prevent dehydration. After polymerisation of the separating gel, water-saturated butanol was removed, the gel was washed with ddH_2O and the stacking gel was poured on top. Wells were made using 0.75 mm combs. After polymerisation of the stacking gel, protein samples (15 µl / 30 µg protein) were loaded into each well alongside pre-stained low-range SDS-PAGE molecular weight markers (5 µl). Gels were flooded with SDS-PAGE running buffer and were subjected to electrophoresis at 100 V until samples had passed through the stacking gel, followed by 200 V through the separating gel. Use of two voltages ensured uniform separation of the proteins.

2.2.10.3 Western Blotting.

Proteins were transferred by electrophoresis from the separating gel onto nitrocellulose filters using a BioRad mini Protean II blotting apparatus (Towbin *et al.*, 1979). A piece of nitrocellulose (Hybond C) and two pieces of filter paper (Whatmann 3 mm) were cut to the approximate size of the gel. The gel, nitrocellulose and filter paper were pre-soaked in transfer buffer. The gel and nitrocellulose were then sandwiched between the filter paper and pre-soaked sponges and proteins were transferred at 50 V for 1 hour in transfer buffer. Nitrocellulose filters were stored in Saran wrap at -20°C prior to protein detection.

2.2.10.4 Protein Detection.

Filters were blocked overnight, unless otherwise stated, at 4°C in Tris-buffered saline (TBS) containing Marvel milk powder (2% $^{w}/_{v}$) to prevent non-specific binding of antibodies. Filters were incubated at room temperature on a shaking platform in TBS containing Tween-20 (0.5%) and Marvel (2% $^{w}/_{v}$, TMT) and the appropriate primary antibody, for 1-2 hours. Dilutions of specific antibodies and incubation times are given in the appropriate chapters. Unbound primary antibody was washed from the filter using TMT (4 x 15 min.). Filters were then incubated for one hour in TMT containing the appropriate secondary antibody, conjugated to horseradish peroxidase. Details of the secondary antibodies used are given in the appropriate chapters. Filters were washed again in TMT (4 x 15 min.) and labelled proteins were detected using an enhanced chemiluminescence (ECL) detection system which involves a catalytic light-emitting reaction by the peroxidase-conjugated secondary antibody. Filters were immersed in the reaction solution, covered in Saran wrap and the luminescent reaction product was detected by exposure to X-ray film. Following protein detection, filters were stained in Ponceaus solution to ensure even protein loading in each lane.

2.2.11 Statistical Analysis of Results.

Cell counts were performed in duplicate and all experiments, with the exception of preliminary investigations, were performed three times. The mean (x) and standard error (se) from these results were then calculated. The statistical significance of the

difference between control and test results was determined using the students t-test and the Wilcoxon-Mann-Whitney test (C. Rose, Syntex Development Research, personal communication).

Chapter 3.

Establishment of an *In Vitro* Model for Peroxisome Proliferator Action.

3.1 Introduction.

3.1.1 The Need for *In Vitro* Models of Peroxisome Proliferator Action.

To date, progress in elucidating the molecular mechanisms of action of the peroxisome proliferators has been hindered severely by a lack of appropriate *in vitro* models that can be readily manipulated. The development of an *in vitro* model for studies of peroxisome proliferator action would facilitate *in vitro* tests for peroxisome proliferation (Bieri *et al.*, 1990), transfection assays for studies of peroxisome proliferator-induced enzyme induction and gene expression (Osumi *et al.*, 1990) and species comparisons of peroxisome proliferation (Mitchell *et al.*, 1984). For the purpose of this thesis, the development of an *in vitro* model of peroxisome proliferator action was a prerequisite for examining the effect of these agents on hepatocyte apoptosis. Prior to the initiation of this thesis, knowledge of the effects of peroxisome proliferators on liver apoptosis was derived from studies carried out *in vivo* (Bursch *et al.*, 1986). However, the rapid recognition and engulfment of apoptotic hepatocytes by macrophages and adjacent hepatocytes has hampered accurate quantitation of liver apoptosis *in vivo*. The development of an *in vitro* cell system that responds to peroxisome proliferators and maintains the ability to undergo apoptosis would facilitate more detailed analysis of the effects of peroxisome proliferators on liver apoptosis and the molecular mechanisms by which such effects may be mediated.

3.1.2 Current *In Vitro* Systems for Studies of Peroxisome Proliferator Action.

3.1.2.1 Primary Hepatocytes.

It is possible to reproduce some of the effects of peroxisome proliferators *in vitro* using primary cultures of rat hepatocytes. One effect, peroxisome proliferation, has been detected in primary hepatocyte cultures treated with, for example, nafenopin (Bieri *et al.*, 1984) and ciprofibrate (Thangada *et al.*, 1989). Moreover, the induction of peroxisomal enzymes of β-oxidation has been detected in rat hepatocyte cultures treated with ciprofibrate (Thangada *et al.*, 1989), nafenopin (Bieri *et al.*, 1984), bezafibrate (Foxworthy *et al.*, 1990), clofibric acid (Mitchell *et al.*, 1984) and perfluorinated fatty acid analogues (Intrasuksri and Feller, 1991). Furthermore, induction of the microsomal

enzyme cytochrome P4504A1 has been detected in rat hepatocytes in response to nafenopin (Muakkassah-Kelly *et al.*, 1987) and clofibric acid (Bars *et al.*, 1989).

Although primary rat hepatocytes respond to peroxisome proliferators *in vitro,* they have only limited use for detailed analysis of the molecular mechanisms of peroxisome proliferator-induced hepatocarcinogenesis. Within only a few days of isolation, primary hepatocytes undergo a process of so-called 'dedifferentiation' or 'redifferentiation' in which differentiated hepatocyte function is lost and the cells revert towards a foetal phenotype (Sirica *et al.*, 1979; Guguen-Guillouzo and Guillouzo, 1983). The rate of transcription of genes encoding liver-specific enzymes such as albumin and cytochrome P4504A1 decreases dramatically (Paine *et al.*, 1979; Steward *et al.*, 1985; Zaret *et al.*, 1988). Loss of cytochrome P450 expression is a particular problem in toxicological investigations since metabolism of a variety of chemical carcinogens by members of the cytochrome P450 enzyme family often determines their toxicity and hepatocarcinogenicity (Paine *et al.*, 1979). An additional problem is that hepatocytes rapidly lose viability following their isolation (Meredith, 1988).

A number of methods have been employed to increase the life-span of hepatocytes in primary culture and to maintain differentiated hepatocyte function. These methods are all based on replacing factors thought to be required *in vivo* by hepatocytes for survival. Successful methods include supplementing culture media with hormones (Dich *et al.*, 1988), differentiation-inducing agents such as DMSO (Muakkassah-Kelly *et al.*, 1987) and nicotinamide (Paine *et al.*, 1979) and modification of extracellular matrix components in the substratum on which hepatocytes are cultured (Sudhakaran *et al.*, 1986; Bissell *et al.*, 1987). In addition, hepatocyte function has been promoted by culturing hepatocytes as co-cultures with other liver epithelial cells (James *et al.*, 1992) or as a spheroid, in which hepatocytes exist as a 3-dimensional aggregate surrounded by extracellular matrix components (Tong *et al.*, 1990; Takezawa *et al.*, 1992; Roberts and Soames, 1993). Despite the success of such methods in extending the life-span of hepatocytes *in vitro*, loss of differentiated hepatocyte function is not prevented but merely delayed. Furthermore, generation of primary hepatocytes is time-consuming. Taken together these factors necessitate the development of a cell line model for use in

this thesis in the examination of the ability of peroxisome proliferators to suppress liver cell apoptosis.

3.1.2.2 Hepatoma Cell Lines.

Cell lines have the advantage of being readily available and, since they are generally easier to manipulate in culture than primary cells, they permit a greater diversity of experimentation than primary hepatocytes. However, care must be taken in the choice of cell line for investigations of this type, since the majority of liver cell lines currently available are derived from hepatomas and many appear to maintain few functions characteristic of differentiated hepatocytes. One such example is the poorly differentiated hepatoma cell line HTC, derived from the Morris hepatoma 7288C (Thompson *et al.*, 1966). More useful cell lines for modelling hepatocyte activity and function are those that exhibit fewer deviations from the normal hepatocyte. A number of these so-called 'minimal deviation' hepatomas exist, including MH_1C_1, derived from Morris hepatoma 7795 (Richardson *et al.*, 1969), $7800C_1$, derived from Morris hepatoma 7800C (Richardson *et al.*, 1973) and the Fao hepatoma cell line, derived from the Reuber H35-derived cell line H4IIEC3 (Deschatrette and Weiss, 1974). It has been established that these cell lines secrete albumin (Richardson *et al.*, 1969; Schiaffonati *et al.*, 1991), have stable peroxisomal β-oxidase activity (Spydevold and Bremer, 1989; Brocard *et al.*, 1993) and exhibit other markers of differentiated liver function. For example, $7800C_1$ cells express enzymes of the urea cycle (Richardson *et al.*, 1973), and can induce tyrosine aminotransferase expression in response to hydrocortisone (Richardson *et al.*, 1973), while the Fao cell line has been shown to express phenobarbital-inducible forms of cytochrome P450 (Wiebel *et al.*, 1984; Corcos and Weiss, 1988).

Of more relevance to this thesis is the response of these cell lines to peroxisome proliferators. Induction of enzymes of peroxisomal β-oxidation has been reported in $7800C_1$ and MH_1C_1 cells treated with tetradecylthioacetic acid and fatty acid analogues (Spydevold and Bremer, 1989; Sorensen *et al.*, 1993). This induction was the result of both increased mRNA transcription and stabilisation (Sorensen *et al.*, 1993). Induction of peroxisomal enzymes of β-oxidation has also been detected following ciprofibrate treatment of the H4IIEC3 cell line, from which the Fao cell line was derived (Osumi *et*

al., 1990) and in Fao cells themselves (Brocard *et al.*, 1993). However, a comparative study of the suitability of hepatoma cells for studying the pleiotropic effects of peroxisome proliferators has not been conducted.

The use of cell lines derived from tumours as a model for normal cells *in vivo* is often criticised. Cells derived from a tumour are transformed and therefore are likely to demonstrate phenotypic differences compared to normal cells. This is particularly true in the case of cell lines derived from rapidly growing hepatomas, since entry into the cell cycle is often associated with loss of differentiated function (Bucher, 1987). In order to overcome this problem, efforts are being made to establish an immortalised hepatocyte cell line, that is stable in culture, maintains differentiated function but is not transformed. To date, the most successful experiments carried out to immortalise hepatocytes have been carried out using SV40 infection of adult rat hepatocytes (Isom *et al.*, 1980; Woodworth *et al.*, 1986). In contrast to normal, differentiated hepatocytes, these immortalised cells are capable of proliferation, yet retain a differentiated phenotype with high tyrosine aminotransferase expression and inducible cytochrome P450 enzymes (Bayad *et al.*, 1991). A similar technique has been used successfully to immortalise mouse hepatocytes (Zaret *et al.*, 1988). Since hepatocytes have a low transfection efficiency, an alternative approach has been the creation of a transgenic mouse harbouring a large tumour (large 'T') antigen, encoding sequences of SV40 (Paul *et al.*, 1988). Non-tumourigenic cell lines maintaining high levels of differentiated function have been established from this transgenic mouse (Paul *et al.*, 1988; Hohne *et al.*, 1990). However, preliminary experiments carried out at Zeneca CTL while this thesis was in its early stages suggested that transfection of hepatocytes with large 'T' antigen was not a successful approach for the creation of a well differentiated cell line. While hepatocytes were capable of proliferation following transfection, this occurred at the expense of differentiated function (Ruth Roberts, Zeneca CTL, personal communication). Thus, although immortalised cell lines are likely to be a powerful tool in future toxicological investigations, it was not possible to utilise such cells in this thesis.

3.1.3 Aims of Chapter.

The ultimate aim of this thesis is to investigate the ability of peroxisome proliferators to suppress hepatocyte apoptosis and to examine the molecular mechanism(s) by which such suppression may occur. Ideally, the effects of peroxisome proliferators on hepatocyte apoptosis should be investigated *in vitro* using primary hepatocytes, since they are not transformed and most closely resemble hepatocytes *in vivo*. However, difficulties in the production and culture of primary hepatocytes preclude their use in some types of experiments. While hepatoma cells may be phenotypically different to hepatocytes, they have the advantage of being readily available and more amenable to experimentation than primary cells.

The aim of this chapter is to validate and characterise a suitable hepatoma cell line for investigations of the mechanism of action of the peroxisome proliferators. An ideal cell line for this purpose should express markers of differentiated liver function, be responsive to peroxisome proliferators and be easy to manipulate in culture. The first section of this chapter describes experiments conducted to examine these characteristics in five rat hepatoma cell lines. The cell lines are the four already described, HTC, $7800C_1$, MH_1C_1 and Fao, and an additional cell line, RH1, obtained from the cell bank at Zeneca CTL. While the majority of the work described was conducted by myself, expression of markers of differentiated liver function by each of the five cell lines was examined by my co-worker, Nicola French at Zeneca CTL. The results of these experiments are highly pertinent to this chapter and are therefore outlined, with acknowledgements, alongside more detailed descriptions of results obtained from my own experiments. This chapter also describes in detail the characteristics of the chosen hepatoma cell line, Fao. Since the aim of this thesis is to use a hepatoma cell line in tandem with primary rat hepatocytes, the characteristics of these two cell types are also compared.

Maintenance of liver homeostasis is determined by a tightly regulated balance between cell gain, in the form of proliferation and cell loss, in the form of cell death. A disruption to either side of this equation could contribute to tumour promotion. Thus, although the main focus of this thesis is the effect of peroxisome proliferators on liver

apoptosis, the proliferative response of the chosen hepatoma cell line to peroxisome proliferators has been examined.

3.2 Methods.

Throughout this chapter, culture of primary rat hepatocytes and hepatoma cells, treatment with nafenopin, cell counts and viability measurements were carried out as described in the general methods section (pages 77-87).

3.2.1 Determination of the Growth Kinetics of Hepatoma Cell Lines in the Presence and Absence of Nafenopin.

Hepatoma cells were seeded in 25 cm^3 flasks containing the appropriate complete medium (4 ml). The density at which cells were seeded per flask was dependent on cell type as follows: RH1 and HTC: 5 x 10^4; Fao: 1 x 10^5; $7800C_1$ and MH_1C_1: 2 x 10^5. After 24 hours, medium was removed by aspiration and replaced with that containing nafenopin (50 μM) or DMF alone (0.25% $^v/_v$). At 24 hour intervals over the following 8-12 days, cells were removed from the monolayer by trypsinisation, resuspended in fresh medium (2 ml) and cell number and viability were determined by TB exclusion.

3.2.2 Determination of Constitutive and Nafenopin-Induced Expression of Albumin, PPAR and Cytochrome P4504A1 by Hepatoma Cells and Primary Hepatocytes.

Hepatoma cells were seeded at 5 x 10^5-2 x 10^6 / 75 cm^3 flask in the appropriate complete medium (10 ml). After 24 hours, medium was removed by aspiration and replaced with fresh medium containing nafenopin (50 μM) or DMF alone (0.25% $^v/_v$). In experiments conducted by Nicola French, cells were grown in the presence of nafenopin or DMF alone for 48 hours. In my experiments, Fao cells were grown in the presence of nafenopin or DMF alone for 5 days. Fresh medium containing nafenopin or DMF was replaced at 48 hour intervals. After the appropriate time, protein samples were made from hepatoma cell monolayers as described in the general methods section (page 84).

Primary hepatocytes were isolated and seeded as described (see page 78). After 24 hours, medium was removed and replaced with fresh medium containing either nafenopin (50 μM) or DMF alone (0.25% $^v/_v$). Fresh medium containing nafenopin or DMF was replaced in the flasks every 24 hours. Protein samples were made from freshly

isolated and 1-day old hepatocytes, and from nafenopin and DMF-treated hepatocyte monolayers 2 and 4 days after seeding as described in the general methods section (page 84). SDS-PAGE and western blotting were carried out as described (page 85).

Albumin expression in hepatoma cells and primary hepatocytes was detected by incubation of nitrocellulose filters with a monoclonal sheep anti-albumin primary antibody (Serotec), diluted to 1:5000, followed by antibody localisation using a horseradish peroxidase-linked rabbit anti-sheep secondary antibody (Dako), diluted to 1:10 000.

PPAR expression in hepatoma cells was detected by incubation of nitrocellulose filters with a polyclonal rabbit anti-PPAR primary antibody (gift from Stephen Green, Zeneca CTL), diluted to 1:10 000 followed by antibody localisation using a horseradish peroxidase-linked donkey anti-rabbit secondary antibody (Amersham), diluted to 1:8000.

Cytochrome P4504A1 expression in hepatoma cells and primary hepatocytes was detected by incubation of nitrocellulose filters with a monoclonal sheep anti-P4504A1 (Bains *et al.*, 1985) antibody diluted to 1:10 000 followed by antibody localisation using a horseradish peroxidase-linked rabbit anti-sheep secondary antibody (Dako) diluted to 1:10 000.

Laser densitometry of autoradiographs was performed to provide an approximate quantitation of the level of expression of markers of differentiated liver function in hepatoma cells and primary hepatocytes.

3.2.3 Determination of the Effect of Nafenopin on the Growth of Fao Cells in Reduced Serum.

Fao cells were seeded at 1 x 10^5 / 25 cm^3 flask in complete Ham's F12 medium (4 ml). After 24 hours, medium was removed by aspiration and replaced with that containing FCS (2% $^v/_v$) and nafenopin (50 μM) or DMF alone (0.25% $^v/_v$). Fresh medium containing nafenopin or DMF was replaced every 48 hours. Cell number and viability were determined at 24-48 hour intervals over the following 12 days. The effect of nafenopin on Fao cell number was also examined in the absence of serum. Fao cells were seeded at 3 x 10^5 cells / 25cm^3 flask in Ham's F12 medium (4 ml) supplemented

with charcoal-stripped FCS (10% $^{v}/_{v}$). After 24 hours, medium was removed by aspiration and monolayers were washed with serum-free Ham's F12 medium to remove residual traces of serum. Medium was replaced with that containing BSA (0.2%) and either nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Cell number and viability were assessed by TB exclusion at 24-48 hour intervals over the following 7 days.

3.2.4 Determination of the Effect of Nafenopin on the Cell Cycle Distribution of Fao Cells.

The cell cycle distribution of Fao cells in the presence of nafenopin or DMF alone was examined by single parameter FCM. Fao cells were seeded at 1 x 10^6 / 75 cm^3 flask in Ham's F12 medium (10 ml) containing charcoal-stripped FCS (10%). After 24 hours, medium was removed by aspiration and replaced with serum-free Ham's F12 medium containing BSA (0.2%) and either nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Control cells were cultured in complete Ham's F12 medium. After a further 48 hours, cells were removed from the monolayer by trypsinisation and resuspended in fresh medium (2 ml). Cells were counted and 5 x 10^5 cells were transferred to a universal tube. Cells were pelletted by centrifugation (200 x g, 5 min.). The supernatant was removed by aspiration and cell pellets were fixed in paraformaldehyde (0.1% in PBS) containing Triton X-100 (0.1%). Propidium iodide (PI, 20 μl of a 2.5 mg/ml solution in PBS) was added to each cell sample 5 min. prior to analysis. Cell cycle analysis of serum control and appropriately-treated serum starved Fao cells was performed using a Becton Dickenson (BD) FACS Vantage Cytometer connected to an HP Consort 32 computer (BD, Palo Alto, CA, USA). 10 000 cells were analysed with respect to red fluorescence using an argon laser (150 mW) set to excite at 488 nm. Data were analysed as single parameter frequency histograms using Lysis II software (BD) and the Cell Fit program.

3.2.5 Determination of the Effect of Nafenopin on S-Phase in Fao Cells.

The effect of nafenopin on S-phase in Fao cells was examined by dual parameter FCM (McNally and Wilson, 1986). Fao cells were seeded and treated with nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) as described above in section 3.2.4. Bromodeoxyuridine

(BrdU, BD; 10 µl of a 1 mM solution in PBS) was added directly to the culture medium 20 hours after serum withdrawal and addition of nafenopin and cells were incubated for at 37°C. After 30 min., cells were removed from the monolayer by trypsinisation, resuspended in serum-free Ham's F12 medium (5 ml) and counted. Cells were pelletted by centrifugation (200 x g, 5 min.), resuspended in PBS containing 1% BSA (1 ml) and transferred to an eppendorf tube. Cells were pelleted by centrifugation (500 x g, 10 min.) and the supernatant removed by aspiration. The cell pellet was resuspended in normal saline (100 µl) which was then added rapidly with vortexing to ethanol (1 ml of a 70% solution at -20°C). Cells were left to fix in ethanol for 30 min. on ice after which cells were pelleted by centrifugation (500 x g, 10 min., 4°C). The supernatant was removed carefully by aspiration and the pellet loosened by gentle vortexing. Fixed cells were resuspended in hydrochloric acid (HCl, 1 ml of a 2N solution containing 0.5% $^{v}/_{v}$ Triton-X-100) at room temperature for 30 min. to denature DNA to single stranded molecules prior to re-pelletting by centrifugation (500 x g, 10 min.). The supernatant was removed by aspiration and the cell pellet resuspended in sodium tetraborate (1 ml of a 0.1 M solution) to neutralise the acid. Aliquots of 1 x 10^6 cells were transferred into eppendorf tubes and cells were pelleted by centrifugation (500 x g, 5 min.). Cells were resuspended in PBS (50 µl containing 1% BSA and 0.5% Tween-20). Fluorescein- (FITC-) conjugated anti-BrdU antibody (20 µl, BD) was added to each cell sample and cells were incubated at room temperature. After a further 30 min., cells were pelleted by centrifugation (500 x g, 5 min.), the supernatant was removed by aspiration and cell pellets were resuspended in PBS (1 ml). PI (50 µl of a 100 µg/ml solution in PBS) was added to the cells 1 min. before analysis of the samples. Cells were analysed using a FACS Vantage cytometer (BD) with laser excitation (150 mW) set at 488 nm and standard FITC / PI emission filters (525 $\pm$ 30 nm and 575 $\pm$ 22 nm respectively). Data were analysed as dual parameter contour plots using Lysis II computer software (BD).

3.3 Results.

3.3.1 Growth Kinetics of Hepatoma Cell Lines.

The five cell lines studied exhibited marked differences in growth rate and behaviour at confluence (Fig. 3.1). RH1 and HTC cells grew rapidly to confluence with doubling times of 16 and 24 hours respectively (Fig. 3.1 a and b). At confluence these cells began to 'pile up' and then detached from the monolayer. $7800C_1$ and MH_1C_1 cells behaved quite differently with slower doubling times (36 hours; Fig. 3.1 c and d). These cells became density-inhibited at confluence, with little cell detachment apparent. Fao cells had a doubling time of approximately 24 hours but detached from the monolayer before reaching confluence (Fig. 3.1e). As a result, Fao cultures were considered to be confluent when cells covered 75-80% of the total area of the tissue culture flask.

3.3.2 Effect of Nafenopin on the Growth Kinetics of Hepatoma Cells in Complete Medium.

The presence of nafenopin (50 μM) in the complete medium of any of the five hepatoma cell lines tested had no significant effect on the growth rate or confluent density of the cells over an 8-12 day period (Fig. 3.1a-e). The growth rate of control cultures treated with DMF alone remained unchanged (Fig. 3.1 a-e).

3.3.3 Maintenance of Liver Specific Markers by Hepatoma Cells.

Western blot analysis, performed by Nicola French, revealed that Fao, MH_1C_1 and $7800C_1$ hepatoma cells express albumin and PPAR (Fig. 3.2a and 3.3a). Expression of both proteins was highest in the Fao cells (Table 3.1a and b). Albumin expression was not detected in HTC cells (Fig. 3.2a). HTC cells also expressed only low levels of PPAR (Fig. 3.3a). RH1 cells did not express albumin (Fig. 3.2a) or PPAR (data not shown). There was little or no increase in PPAR expression in any of the cell lines following a 48 hour exposure to nafenopin (Table 3.1b).

Expression of albumin and PPAR by Fao cells was confirmed by my own western blot analysis (Fig. 3.2b and 3.3b). A comparison of albumin expression in 1-day old primary rat hepatocytes and Fao cells revealed that endogenous expression of albumin was approximately 2-3-fold higher in the primary hepatocytes (Table 3.2a).

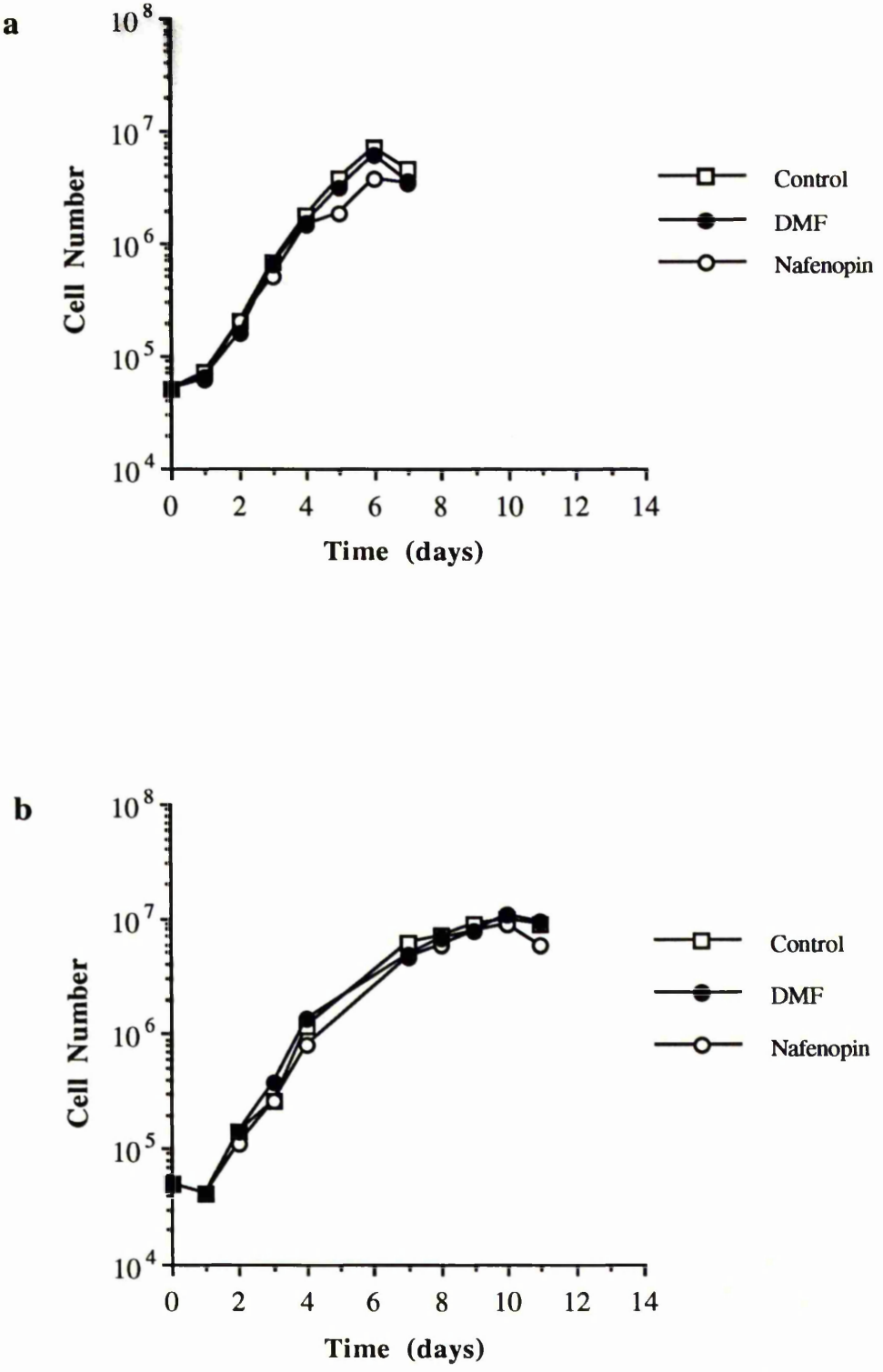
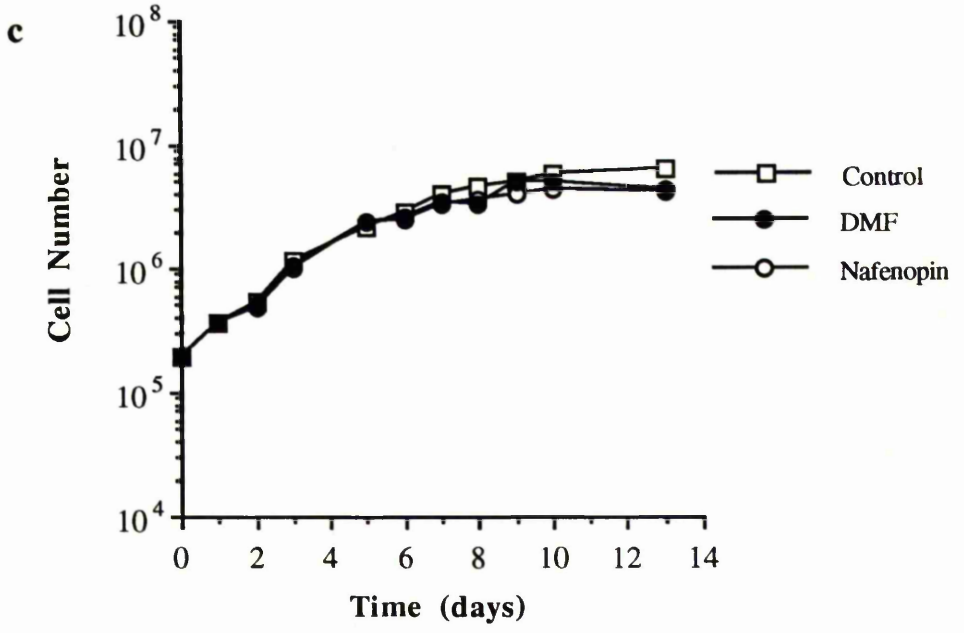


Figure 3.1. Growth kinetics of **(a)** RH1 and **(b)** HTC cells. Cells were seeded at 5 x 10^4 on day 0 and nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) was added at day 1. Data points represent the mean of duplicate counts from 2 independent experiments. In these and subsequent Figure 1 graphs, error bars have been omitted for clarity but were less than 10% of the mean.



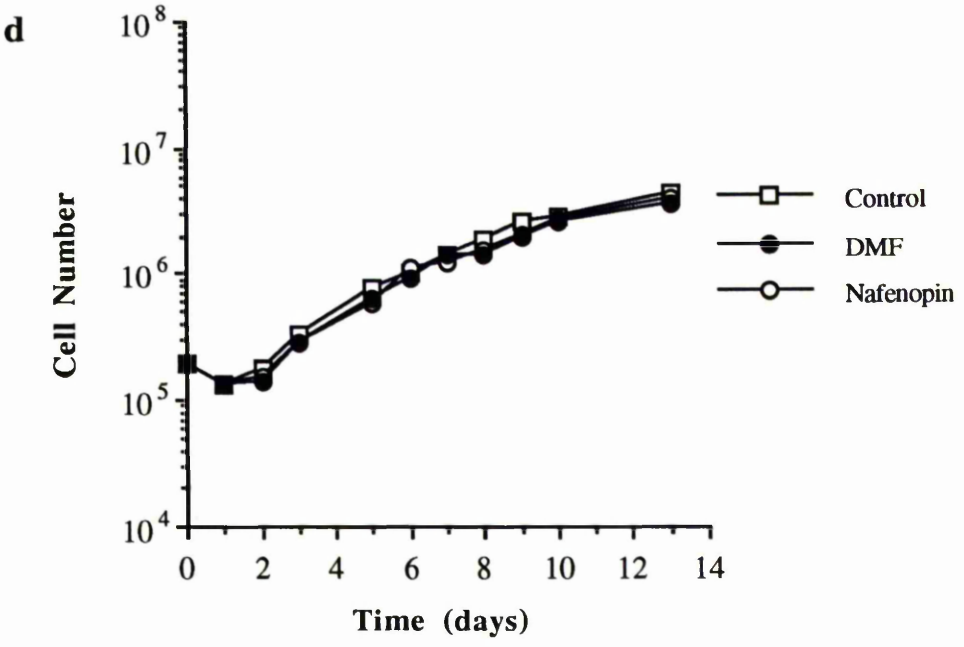


Figure 3.1. Growth kinetics of **(c)** $7800C_1$ and **(d)** MH_1C_1 cells. Cells were seeded at 2 x 10^5 on day 0 and nafenopin (50 μM) or DMF alone (0.25% $^v/_v$) was added at day 1. Data points represent the mean of duplicate counts from 2 independent experiments.

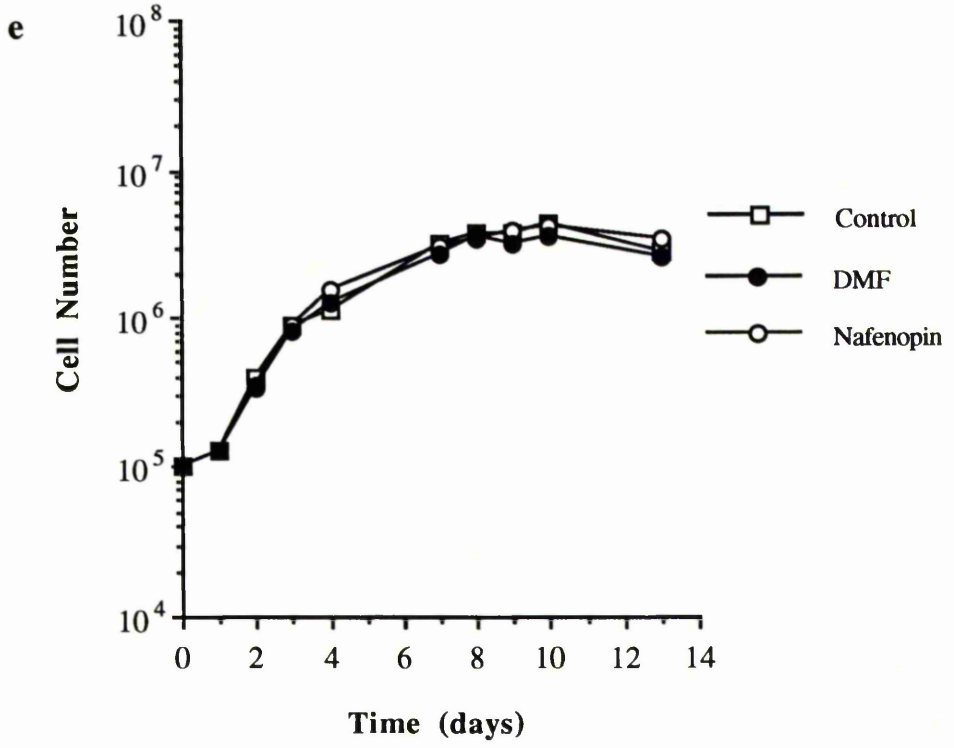


Figure 3.1. (e) Growth kinetics of Fao cells. Cells were seeded at 1 x 10^5 on day 0 and nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) was added at day 1. Data points represent the mean of duplicate counts from 3 independent experiments.

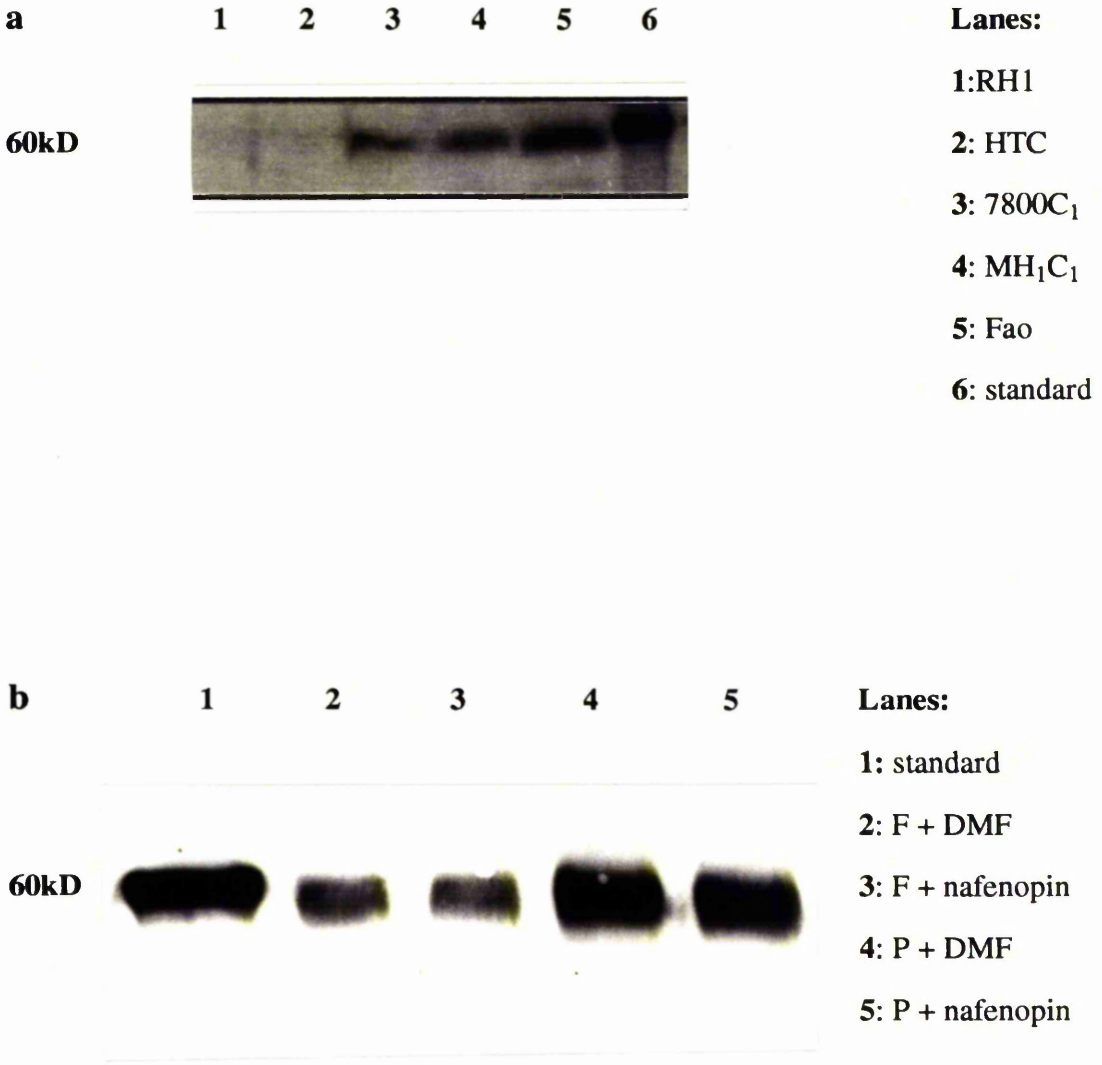


Figure 3.2. **(a)** Western blot analysis of hepatoma cells demonstrating constitutive albumin expression (performed by Nicola French, Zeneca CTL). In **a** and **b**, 30µg protein was loaded in each lane and the protein content of rat albumin standard was 0.5 µg.

(b) Western blot analysis of Fao hepatoma cells (F) and primary hepatocytes (P) demonstrating albumin expression in the presence of nafenopin (50 µM) or DMF alone (0.25% $^{v}/_{v}$). Western was performed by myself and is representative of 3 independent experiments.

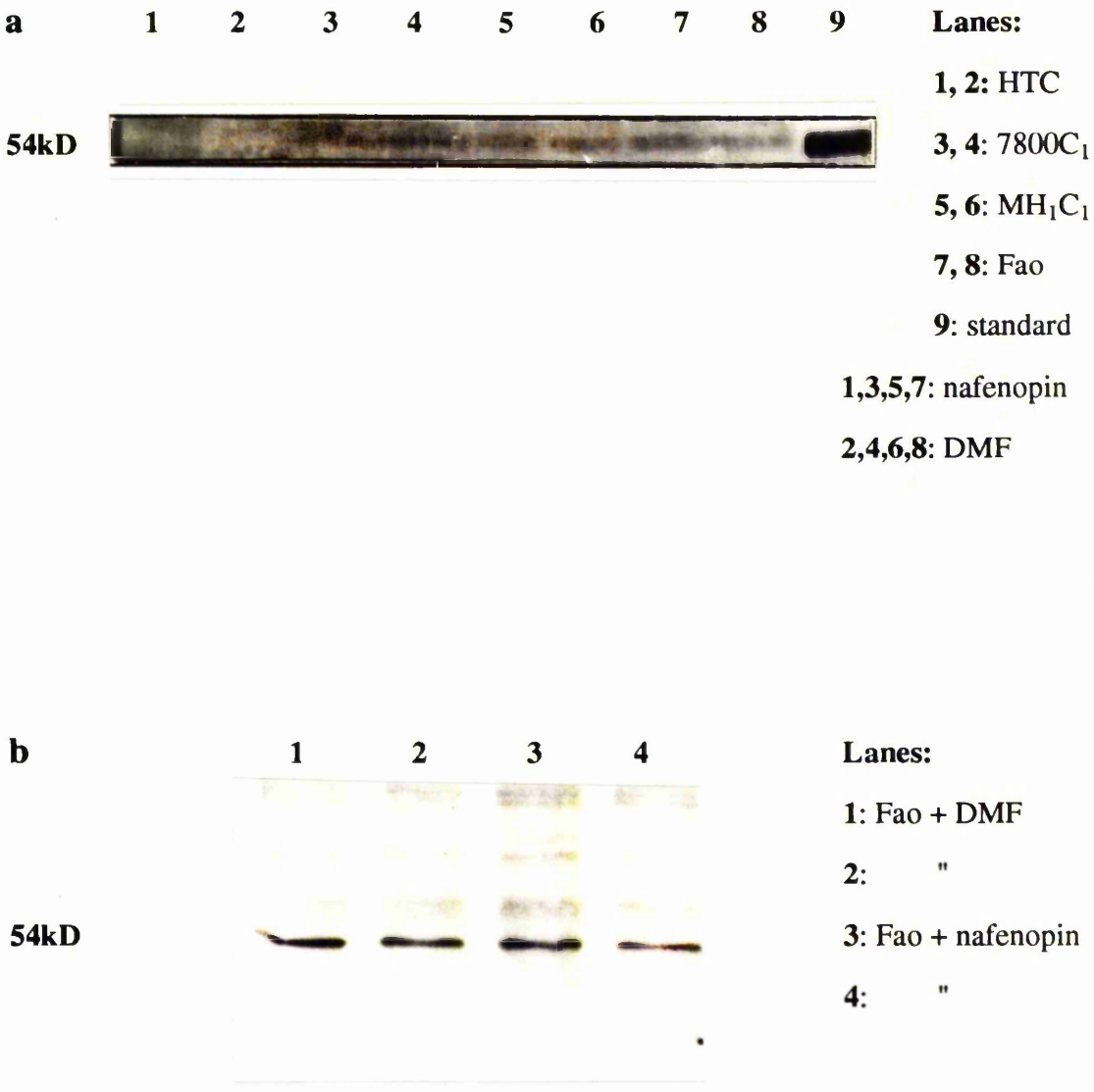


Figure 3.3. **(a)** Western blot analysis of hepatoma cells demonstrating PPAR expression in nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) treated cultures (performed by Nicola French, Zeneca CTL). Absolute protein content of PPAR standard unknown. In **a** and **b** 30 μg protein was loaded in each lane.

(b) Western blot analysis of Fao hepatoma cells demonstrating PPAR expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Western was performed by myself and is representative of 3 independent experiments.

a	DMF	
RH1	0.00	
HTC	0.00	
7800C1	0.20	
MH1C1	0.19	
Fao	0.44	
b	**DMF**	**Nafenopin (48 hours)**
HTC	0.02	0.05
7800C1	0.07	0.06
MH1C1	0.08	0.08
Fao	0.09	0.12
c	**DMF**	**Nafenopin (48 hours)**
RH1	0.04	0.04
HTC	0.05	0.06
7800C1	0.27	0.29
MH1C1	0.21	0.25
Fao	0.17	0.38

Table 3.1. Quantitation of **(a)** albumin, **(b)** PPAR and **(c)** cytochrome P4504A1 expression in nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) treated hepatoma cells by laser densitometry of western blots (conducted by Nicola French, Zeneca CTL). Values given represent the area under the peak (AU2). The albumin standard (0.5 μg), PPAR standard (absolute amount unknown) and cytochrome P4504A1 standard (1.5 pM) gave AU2 values of 1.36, 0.16 and 0.7 respectively.

a	DMF	Nafenopin (5 days)
Fao	1151.4	930.7
Hepatocytes	3142.6	2305.3
b	**DMF**	**Nafenopin (5 days)**
Fao	249.1	924.0
Hepatocytes (fresh)	187.2	-
Hepatocytes (1 day)	175.9	-
Hepatocytes (2 day)	107.5	441.3
Hepatocytes (4 day)	21.1	1104.0

Table 3.2. Quantitation of **(a)** albumin and **(b)** cytochrome P4504A1 expression in nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) treated Fao hepatoma cells and primary hepatocytes by laser densitometry of western blots (performed by myself). Values given represent the volume of the band. The albumin standard (0.5 μg) and cytochrome P4504A1 standard (1.5 pM) gave values of 4315 and 1131 respectively.

Once again, no induction of either PPAR or albumin was seen in Fao hepatoma cells in response to nafenopin (50 μM; Fig. 3.2b and 3.3b). Similarly, no induction of albumin was seen in primary hepatocytes in response to nafenopin (Fig. 3.2b).

3.3.4 Induction of Cytochrome P4504A1 in Hepatoma Cells by Nafenopin.

Western blot analysis performed by Nicola French revealed that of the five cell lines tested, Fao, MH_1C_1, and $7800C_1$ expressed the highest constitutive levels of cytochrome P4504A1 (Fig. 3.4a). A 2.2-fold increase in cytochrome P4504A1 expression was detected in Fao cells treated with nafenopin for 48 hours (Fig. 3.4a and Table 3.1c). A slight increase in expression was detected also in MH_1C_1 cells (Fig. 3.4a and Table 3.1c).

Constitutive expression of cytochrome P4504A1 by Fao cells and primary hepatocytes was confirmed by my own western blot analysis (Fig. 3.4b and c). Cytochrome P4504A1 expression was induced in both primary rat hepatocytes and Fao cells treated with nafenopin. Fao cells grown in the presence of nafenopin (50 μM) for 5 days expressed an approximately 3-4-fold higher level of cytochrome P4504A1 than control cells treated with DMF alone (0.25% $^{v}/_{v}$; Fig. 3.4b and Table 3.2b). An elevation in the level of cytochrome P4504A1 was detected in primary hepatocytes 24 and 72 hours after addition of nafenopin compared to DMF-treated controls (Fig. 3.4c). Furthermore, the level of cytochrome P4504A1 expression in hepatocyte cultures exposed to nafenopin for 72 hours was approximately 6-fold higher than the level of expression in freshly isolated hepatocytes (Fig 3.4c and Table 3.2b). Following western blot analyses, routine staining of the nitrocellulose filters with Ponceaus solution confirmed equal protein loading (data not shown).

3.3.5 Effect of Nafenopin on the Growth Kinetics of Fao Cells in Reduced Serum.

A reduction in the concentration of serum in the medium of Fao cultures to 2% did not significantly reduce the number of cells counted over the following 3 days (Fig. 3.5). A slight decrease in the growth rate of the cells occurred after this time. The number of Fao cells in cultures treated with medium containing 2% and 10% serum reached a plateau 6 and 8 days after seeding respectively. However, the density of cells

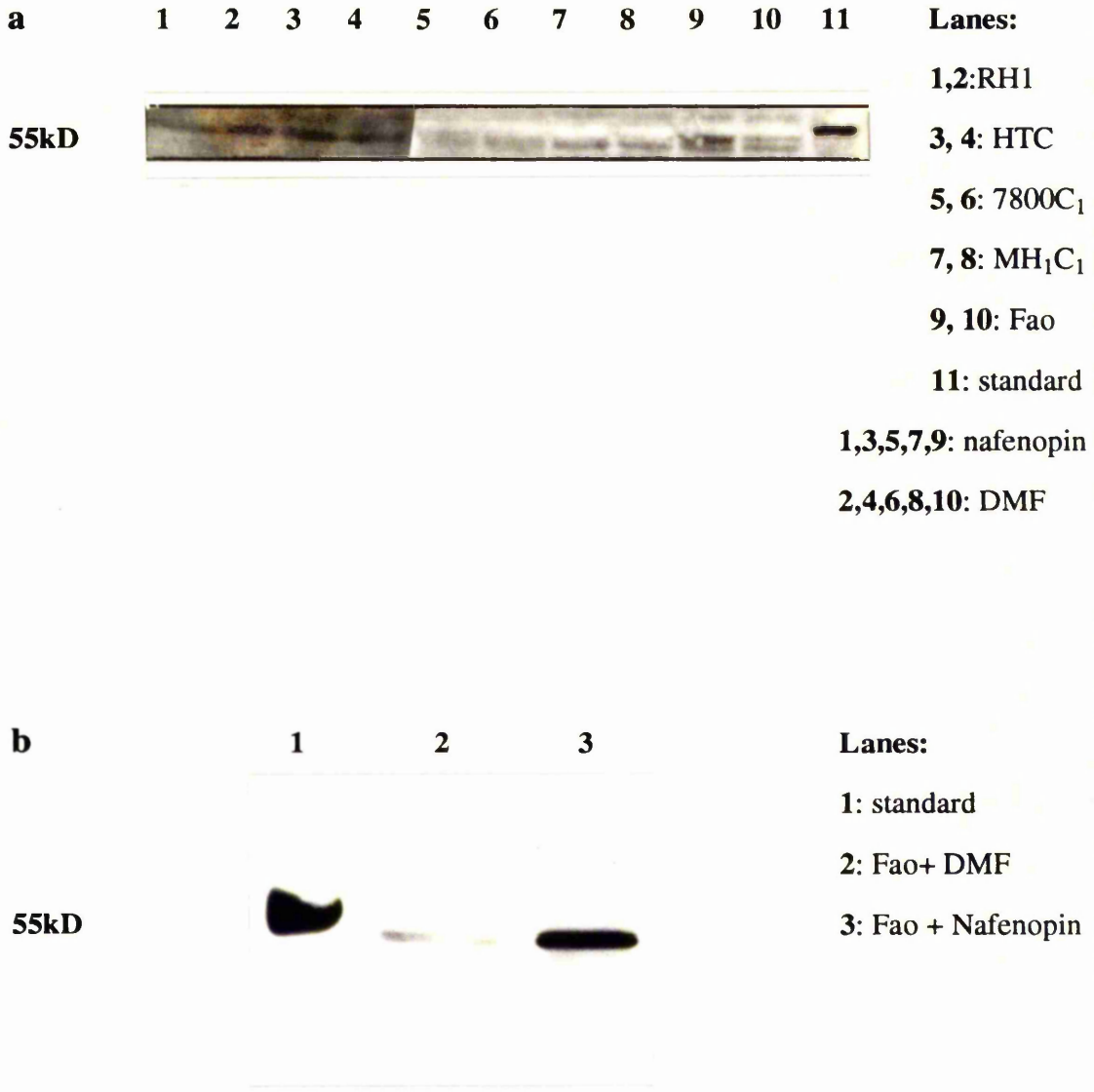


Figure 3.4. **(a)** Western blot analysis of hepatoma cells demonstrating cytochrome P4504A1 expression in nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) treated cultures (performed by Nicola French, Zeneca CTL). In **a**, **b** and **c**, 30 μg protein was loaded in each lane and protein concentration in P4504A1 standard was 1.5 pM.

(b) Western blot analysis of Fao hepatoma cells demonstrating cytochrome P4504A1 expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Western was performed by myself and is representative of 3 independent experiments.

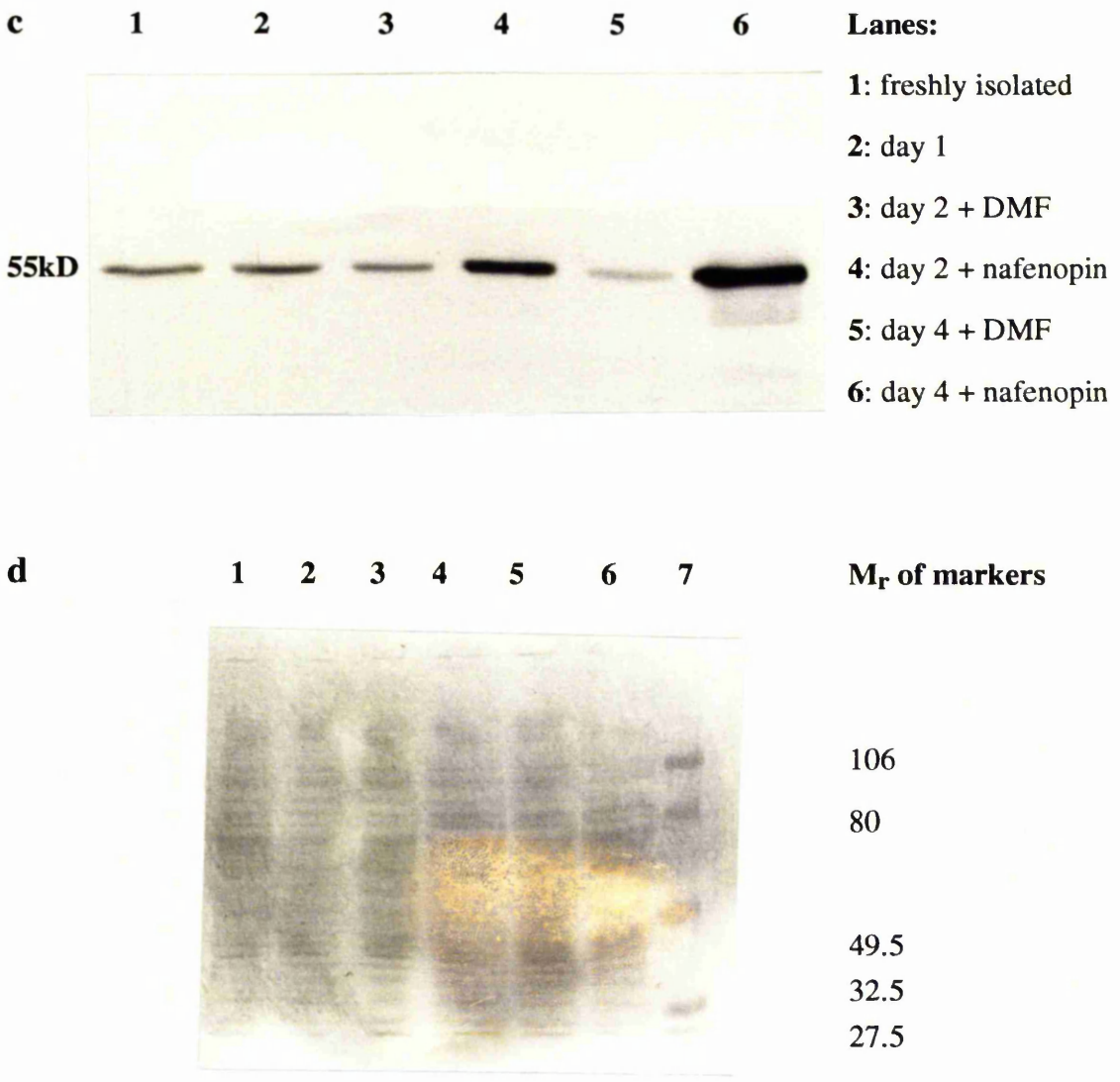


Figure 3.4. **(c)** Western blot analysis of primary hepatocytes demonstrating cytochrome P4504A1 expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Western was performed by myself and is representative of 3 independent experiments. **(d)** Ponceau s stained nitrocellulose filter demonstrating equal loading of protein. Lane 7 contains molecular weight markers. On a number of western blots in this thesis, a known protein standard was used to demonstrate the presence of the protein required. However, the $\log_{10}$ of the molecular weight of a protein could also be calculated by plotting the $\log_{10}$ of the molecular weight of known protein markers against their electrophoretic mobilities (Weber and Osborn, 1969). This provides a standard curve from which the molecular weight of the protein in question can be calculated.

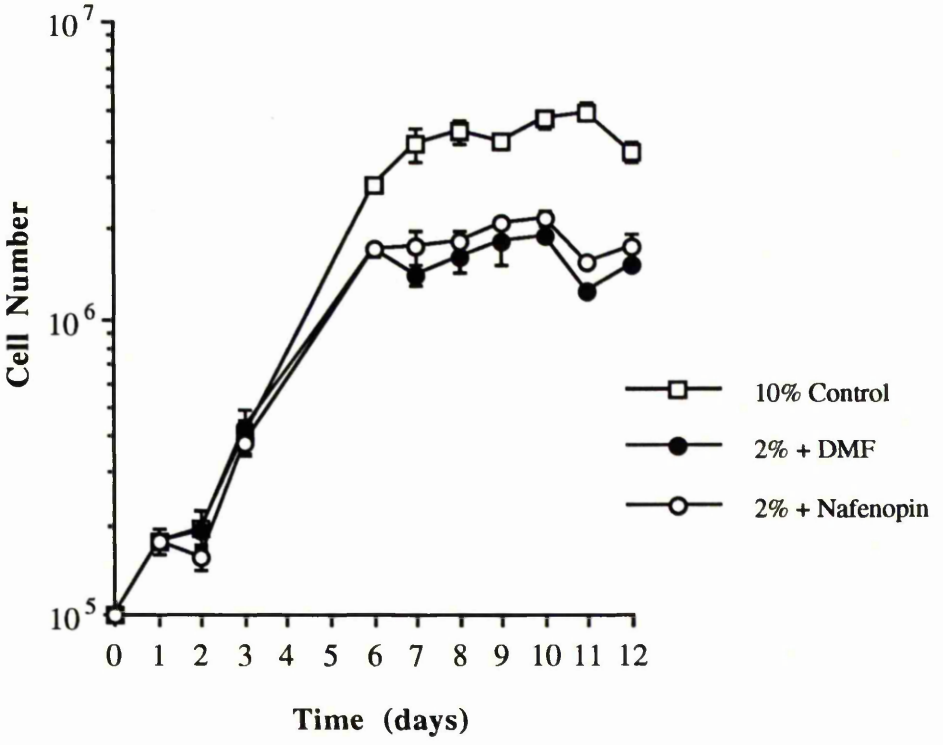


Figure 3.5. Growth kinetics of Fao cells in 2% serum in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Data points represent the mean and standard error of duplicate counts from 3 independent experiments.

in cultures grown in medium containing 2% serum was significantly lower than that of cells grown in medium containing 10% serum (n=3; $p \leq 0.01$; Fig. 3.5). Addition of nafenopin (50 μM) to Fao cultures grown in medium containing 2% serum had no significant effect on the number of cells counted over the 12 day experimental period (Fig. 3.5).

Complete withdrawal of serum from Fao cultures led to a reduction in the number of cells counted at each time point after serum withdrawal (Fig. 3.6). For example, 48 hours after serum withdrawal the number of cells present in serum-free cultures was 42% lower than in cultures treated with medium containing 10% serum (n=4; $p \leq 0.01$). Cell division, as assessed by an increase in total cell number, did not stop completely in serum-free cultures. Addition of nafenopin (50 μM) to serum-free Fao cultures led to a significant increase in the number of cells counted after serum withdrawal (Fig. 3.6). For example, 72 hours after serum withdrawal, the number of cells present in serum-free cultures treated with nafenopin was 79% higher than in untreated serum-free control cultures (n=4; $p \leq 0.05$). Treatment with DMF alone had no significant effect on the number of Fao cells counted in serum-free conditions (Fig. 3.6).

3.3.6 Effect of Nafenopin on the Cell Cycle Status of Fao Cells.

Single parameter flow cytometric analysis of the cell cycle distribution of Fao cells in 10% serum, stained with PI, revealed that 44 hours after seeding, 36.1% (n=3, se=0.9) of cells were in S, G_2 and M phases of the cell cycle (Fig. 3.7 and Table 3.3). In Fao cultures serum-starved for 20 hours and treated with DMF alone, the percentage of cells in these phases of the cell cycle decreased significantly to 27.5% (n=3, se=0.64; $p \leq 0.001$). The presence of nafenopin (50μM) in serum-free Fao cultures resulted in a small increase in the number of cells in S, G_2 and M to 33.1% (n=3, se=3.1; Fig. 3.7 and Table 3.3).

3.3.7 Effect of Nafenopin on S-Phase in Serum-Free Fao Cultures.

Dual parameter flow cytometric analysis of Fao cells pulsed with BrdU and stained with anti-BrdU antibody and PI revealed a typical horseshoe-shaped distribution where cells in S-phase could be distinguished easily from two separate

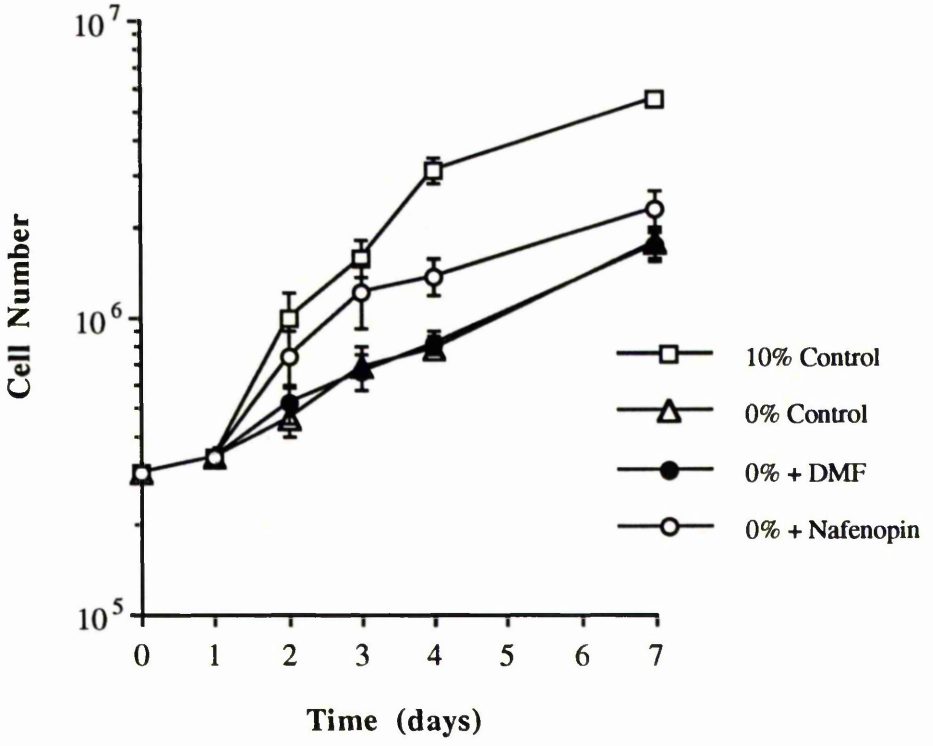


Figure 3.6. Growth kinetics of Fao cells in serum-free medium in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Data points represent the mean and standard error of duplicate counts from 4 independent experiments.

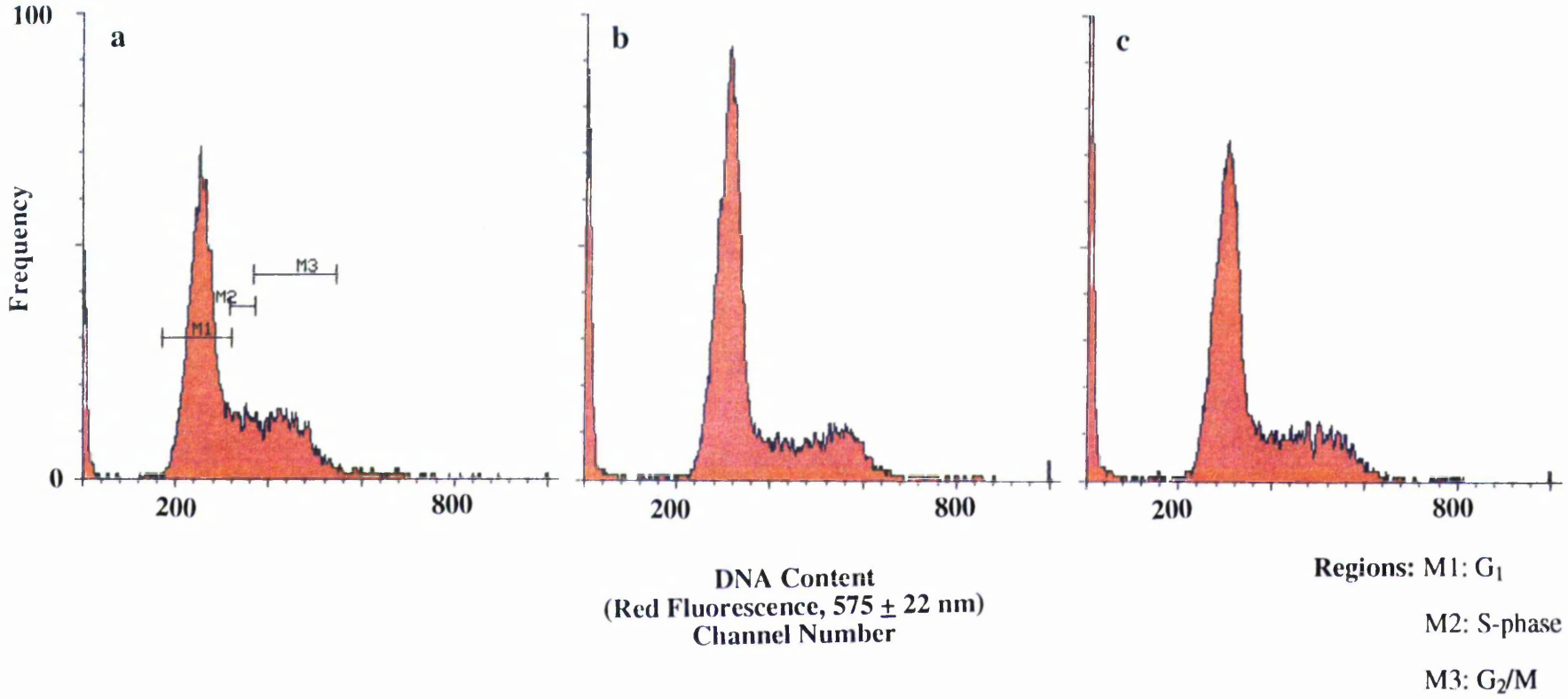


Figure 3.7. FCM analysis of Fao cells stained with PI, demonstrating the cell cycle distribution 48 hours after serum withdrawal. The distribution of Fao cells grown in medium containing either **(a)** 10% serum or 0% serum in the presence of **(b)** DMF alone (0.25% $^{v}/_{v}$) or **(c)** nafenopin (50 μM) is shown. Histograms are representative of 3 independent experiments.

	Treatment					
	10% Serum		**0% Serum + DMF**		**0% Serum + Nafenopin**	
Phase of cell cycle	**Mean**	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
G0 / G1	63.9	0.8	72.2	0.6	66.9	3.1
S	17.8	1.2	12.8	1.2	19.0	3.3
G2 / M	18.4	0.4	14.7	1.3	16.1	0.1
S + G2 / M	**36.1**	0.9	**27.5**	0.6	**33.1**	3.1

Table 3.3. Flow cytometric quantitation of the cell cycle distribution of Fao cells following serum withdrawal, calculated from single parameter DNA histograms (see Fig. 3.7). Data represents the mean and standard error of results obtained from 3 independent experiments.

populations of cells in G_0/G_1 and cells in G_2/M (Fig. 3.8). In Fao cultures grown in 10% serum, 28% (n=3, se=2.1) of cells were found in this S-phase population (Fig. 3.8 and Table 3.4). In contrast, serum starvation for 24 hours in the presence of DMF alone led to a significant reduction in the number of Fao cells in S-phase to 13.3% (n=3, se=0.7; $p \leq 0.01$). However, the presence of nafenopin (50 μM) in serum-free cultures increased the number of cells in S-phase significantly above the percentage seen in serum-free cultures treated with DMF alone to 16.8% (n=3, se=0.4; $p \leq 0.02$; Fig. 3.8 and Table 3.4).

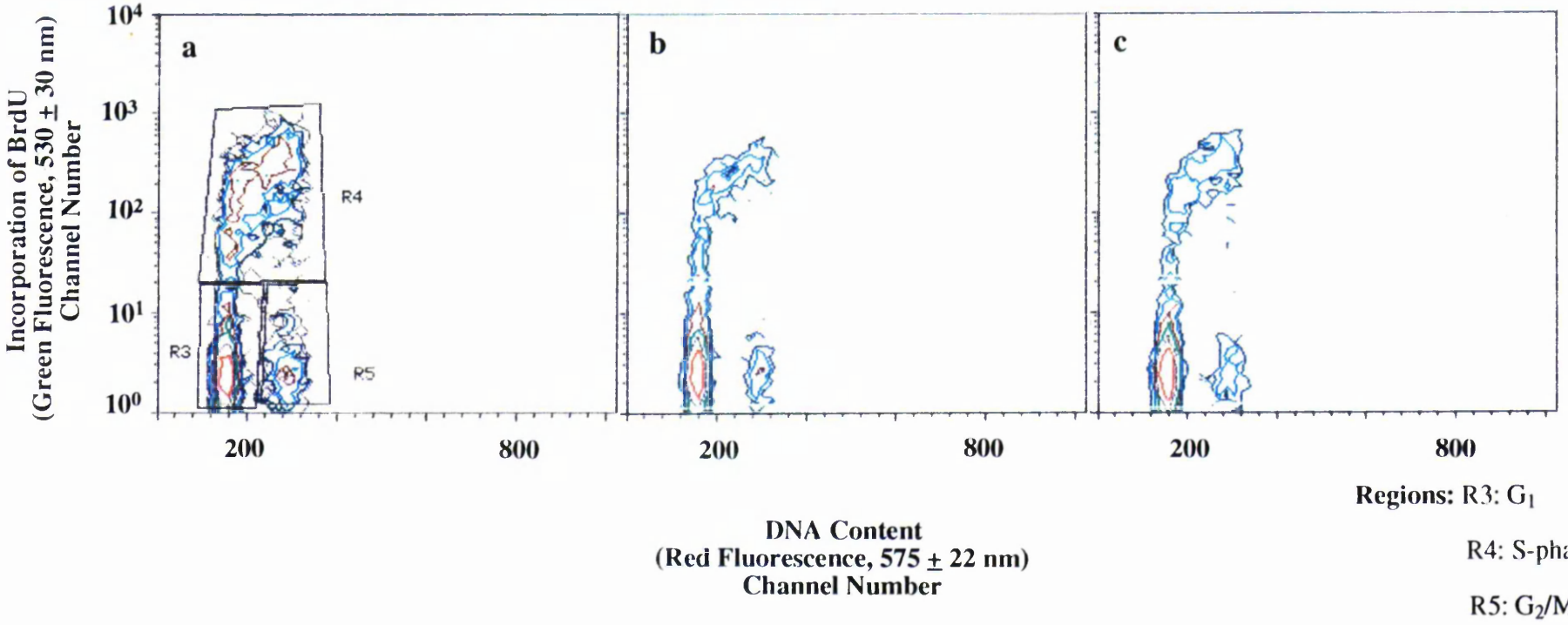


Figure 3.8. FCM analysis of Fao cells stained with FITC-labelled anti-BrdU antibody and PI, demonstrating the proportion of cells in S-phase 20 hours after serum withdrawal. The distribution of Fao cells grown in medium containing either **(a)** 10% serum or 0% serum in the presence of **(b)** DMF alone (0.25% $^{v}/_{v}$) or **(c)** nafenopin (50 µM) is shown. Contour plots are representative of 3 independent experiments.

	% Cells in S-Phase		
Treatment	**Mean**	**s.e.**	**Significance**
10% Serum	28.0	2.1	
			$p \leq 0.01$
0% Serum + DMF (0.25% v/v)	13.7	0.7	
			$p \leq 0.02$
0% Serum + Nafenopin (50μM)	16.8	0.4	

Table 3.4. Flow cytometric quantitation of the cell cycle distribution of Fao cells following serum withdrawal, calculated from 2-D contour plots (see Fig. 3.8). Data represents the mean and standard error of results from 3 independent experiments.

3.4 Discussion.

The molecular mechanisms of action of the peroxisome proliferator class of non-genotoxic carcinogens are unclear and progress in their elucidation is hindered by a lack of appropriate *in vitro* models that can be readily manipulated. To date little work has been carried out to evaluate the usefulness of cell lines for studies of peroxisome proliferator action. The initial aim of this thesis was to conduct a thorough characterisation of a range of hepatoma cell lines with a view to identifying a suitable *in vitro* model system for use in studying the molecular mechanisms of action of the peroxisome proliferators. An ideal cell model should express markers of differentiated liver function, maintain a response to peroxisome proliferators and be easy to manipulate in culture.

3.4.1 Characterisation of Hepatoma Cell Lines.

Characterisation of the five hepatoma cell lines, RH1, HTC, $7800C_1$, MH_1C_1 and Fao was carried out in collaboration with the Cell and Molecular Biology group at Zeneca CTL. Determination of the growth kinetics of the cell lines was conducted by myself, while expression of markers of differentiated liver function by the cells was examined by Nicola French. This collaboration ensured that a suitable hepatoma cell model was established early in the project.

The five hepatoma cell lines studied varied widely in growth rate and behaviour at confluence (Fig. 3.1, pages 100-102). It is important to note that the initial seeding density was not the same for each cell line. The optimum seeding density for each cell line was chosen on the basis of information gained from the suppliers and the results of preliminary experiments. These densities reflected the difference in growth kinetics between the different cell lines, with the more rapidly growing cell lines RH1, HTC and Fao seeded at a lower density than the more slow-growing MH_1C_1 and $7800C_1$ cell lines.

The disparate growth rates of the cell lines appeared to be accompanied by a variation in the differentiation status of the cells (Fig. 3.2, 3.3, 3.4, pages 103-109). The most rapidly growing cell line RH1 did not express albumin, PPAR or cytochrome P4504A1. Similarly, the HTC cell line did not express albumin or cytochrome P4504A1 and expressed only low levels of PPAR. In contrast the slow growing cell lines MH_1C_1

and $7800C_1$ expressed all three markers of differentiated liver function. A slow growing hepatoma cell line may seem to be an appropriate *in vitro* model for hepatocytes, which do not divide under normal conditions. However, the slow growth rates of the MH_1C_1 and $7800C_1$ cell lines led to problems with the culture of these cells, and would have hindered future experiments. The Fao cell line displayed the most desirable characteristics having an intermediate growth rate and high endogenous expression of albumin, PPAR and cytochrome P4504A1.

In addition to expressing markers of differentiated liver function, it was important that the chosen hepatoma cell line should maintain a response to peroxisome proliferators. A characteristic response of hepatocytes to peroxisome proliferators *in vivo* is the induction of a number of liver enzymes, including the microsomal enzyme cytochrome P4504A1 (Orton and Parker, 1982; Moody *et al.*, 1992). Therefore, the ability of each of the cell lines to mirror this response was investigated. Of the five cell lines studied, the Fao cell line exhibited the most marked induction of cytochrome P4504A1 in response to nafenopin (50 μM; Fig. 3.4a and b, page 108). Interestingly, Fao cells demonstrate a heterogeneous induction of cytochrome P4504A1 (Bayly *et al.*, 1993), with variations in expression occurring between neighbouring cells. Such intercellular heterogeneity of cytochrome P4504A1 induction is also seen in primary hepatocytes *in vitro* (James *et al.*, 1992) and in the liver *in vivo*, where levels of expression are higher in centrilobular hepatocytes than in periportal hepatocytes (Bars *et al.*, 1989).

These joint findings led to the choice of the Fao cell line as a suitable cell model for use in investigating the mechanisms of action of the peroxisome proliferators. The aim of this project was not to use the Fao cell line alone, but in complement with primary cultures of rat hepatocytes. Therefore in order to allow a correlation of the results of future experiments conducted with the Fao cell line and primary hepatocytes, some comparison of the differentiation status of the two cell types was required.

3.4.2 Comparison of Fao Hepatoma Cells and Primary Hepatocytes.

Studies of *in vitro* liver function should always be extrapolated to primary hepatocytes since these most closely resemble hepatocytes *in vivo*. Expression of

markers of differentiated liver function in Fao cells was compared therefore with the level of expression in freshly isolated primary rat hepatocytes. High endogenous levels of both albumin and cytochrome P4504A1 were detected in primary hepatocytes (Fig. 3.2b, 3.4c, pages 108-109). Not surprisingly, expression of these markers was higher in freshly isolated primary hepatocytes than in the Fao cells. As expected, cytochrome P4504A1 expression decreased markedly in hepatocytes over the first 4 days after isolation, with considerable loss of expression occurring over the first 24 hours. The decrease in cytochrome P4504A1 expression detected in primary hepatocytes following their isolation is likely to have occurred as a result of the failure of degenerating cultured hepatocytes to synthesise the enzyme. In an attempt to prolong differentiated hepatocyte function, hepatocyte growth medium was supplemented with insulin and hydrocortisone and cells were seeded onto rat tail collagen (James *et al.*, 1992). However, despite these additions, a differentiated phenotype was maintained for only a short time by hepatocytes in culture. As with the Fao cells, a marked induction of cytochrome P4504A1 was detected in primary hepatocytes from 24 hours after addition of nafenopin (50 μM; Fig. 3.4c, page 109). The extent of induction increased with time of exposure to nafenopin so that after a 72 hour exposure to nafenopin the level of cytochrome P4504A1 expression was approximately 6-fold higher than in the freshly isolated cells. This demonstration of a response of primary hepatocytes to nafenopin corroborates results reported elsewhere, in which peroxisome proliferation, enzyme induction and stimulation of replicative DNA synthesis have been detected in primary rat hepatocyte cultures, treated with a variety of peroxisome proliferators (Bieri *et al.*, 1984; Muakkassah-Kelly *et al.*, 1987; Thangada *et al.*, 1989). Taken together, these results indicate that the Fao cell line does express some of the same markers of differentiated liver function as primary hepatocytes, albeit at lower levels. Furthermore, the marked response of both cell types to the peroxisome proliferator nafenopin indicates their suitability for use in studies of peroxisome proliferator action.

3.4.3 Proliferative Response of Fao Cells to Nafenopin.

The hepatocarcinogenicity of the peroxisome proliferators is believed to stem, at least in part, from their ability to disrupt normal growth regulation. Under normal

circumstances, liver growth is controlled by a tightly regulated balance between cell gain in the form of proliferation and cell loss in the form of cell death (Tessitore *et al.*, 1989). Perturbed growth may occur via a disruption to either side of this equation. Thus, while the central theme of this thesis is to investigate the effects of peroxisome proliferators on apoptosis, some knowledge of the effects of peroxisome proliferators on mitosis is also required.

It has been established *in vivo* and *in vitro* that peroxisome proliferators stimulate cell division in hepatocytes (Bieri *et al.*, 1984; Marsman *et al.*, 1988). However, results presented in this chapter demonstrate no comparable increase in growth rate in the Fao cell line or in any of the other hepatoma cell lines studied in response to nafenopin (50 μM; Fig. 3.1, pages 100-102). This could suggest that the cells lack the ability to respond to nafenopin. However, since the Fao cell line and others have already been shown to respond to the same concentration of nafenopin by the up-regulation of cytochrome P4504A1, this explanation is unlikely. A fundamental difference between primary cells and hepatoma cells is that hepatoma cells proliferate, while hepatocytes are usually quiescent. The absence of a change in hepatoma cell growth rate in response to nafenopin may be explained by the inability of nafenopin to enhance the rate of division of cells that are already growing at their maximal rate in culture. Thus, in order to mirror the effect of the peroxisome proliferator nafenopin on proliferation seen in hepatocytes, it was necessary to reduce the growth rate of the hepatoma cells.

It has been demonstrated that the rate at which cells proliferate in culture can be decreased by reducing the concentration of serum and thus the concentration of mitogenic factors in the culture medium (Panet *et al.*, 1983; Vichi and Tritton, 1989). A reduction in the concentration of serum present in Fao cultures from 10% to 2% did not immediately affect the number of viable cells attached to the monolayer (Fig. 3.5, page 110). The difference between the two serum concentrations was only reflected by the fact that cells growing in 2% serum reached a plateau at a lower density than cells in 10% serum. Since the rate of proliferation of Fao cells was not significantly affected by this reduction in serum concentration, it is perhaps not surprising that nafenopin (50 μM) did not alter the number of Fao cells counted over the course of the experiment.

Complete withdrawal of serum from the medium of Fao cultures significantly reduced the number of cells counted within 48 hours ($p \leq 0.01$; Fig. 3.6, page 112). In these serum-free Fao cultures, the presence of nafenopin did result in a significant increase in the number of cells in the monolayer ($p \leq 0.05$). These results indicate that the hepatomitogenic effect of peroxisome proliferators can be reproduced in Fao hepatoma cells but only if the cells are proliferating at a sub-maximal rate. In order to confirm that the changes in cell number detected in serum-free Fao cultures were due to differences in the rate of cell proliferation, the cells were analysed by FCM.

Single parameter FCM of cells stained with PI provides a method for determining their cell cycle phase distribution. Intercalation of PI with DNA results in red fluorescence when excited at 488 nm. This method of staining allows cells to be examined with respect to their DNA content, giving a measure of the percentage of cells in G_1, S and G_2/M phases of the cell cycle. FCM of Fao cells stained with PI demonstrated that the percentage of cells present in S, G_2 and M phases of the cell cycle was significantly reduced 24 hours after withdrawal of serum compared to control cultures growing in 10% serum ($p \leq 0.001$; Fig. 3.7, page 113). Moreover, the presence of nafenopin (50 μM) in these serum-free cultures resulted in an increase in the percentage of cells present in S, G_2 and M, returning the levels almost back to those seen in serum-treated controls. These results confirmed that the changes in cell number detected in serum-free and nafenopin-treated Fao cultures were the result of changes in the rate of proliferation of the cells.

While analysis of cells by FCM using a single parameter such as PI staining is useful for obtaining a general picture of the cell cycle distribution of a cell population, the boundaries between the different phases of the cell cycle are often indistinct. This is particularly true for the S-phase population, and can lead to inaccurate estimations of the population size. This problem can be overcome by the use of dual staining parameters. An example of one such method involves labelling cells with BrdU, a uridine analogue which is incorporated into the DNA of cells undergoing DNA synthesis in place of thymidine. A short exposure of cells to BrdU ensures that only those cells that are synthesising DNA at the time, incorporate BrdU (McNally and Wilson, 1986). The subsequent use of an antibody to BrdU, conjugated to a fluorescent tag such as FITC,

facilitates the detection of those cells that have incorporated BrdU into their DNA. FCM of cells stained with anti-BrdU antibody and PI provides a comparison of DNA content (measured as red fluorescence) and DNA synthesis (measured as green fluorescence). As cells progress through S-phase their DNA content increases (increased red) as a result of increased DNA synthesis (increased green). Thus on a linear plot of green fluorescence against red fluorescence, the population of cells in S-phase appears as the top of a horseshoe-shaped cell population.

FCM analysis of Fao cells pulsed for 30 min. with BrdU and stained with anti-BrdU antibody and PI revealed that serum starvation in the presence of DMF alone caused the number of cells in S-phase to decrease significantly by 14% ($n=3$; $p \leq 0.01$; Fig. 3.8, page 116). The presence of nafenopin (50 μM) in these serum-free cultures led to a significant 3-4% increase in the number of cells in S-phase compared to serum-free, DMF solvent control cultures ($p \leq 0.02$). This increase in the number of cells in S-phase was not as large as expected. It is possible that the 30 min. incubation with BrdU used in this experiment was not sufficient to detect the difference in the proliferative rate of Fao cells in the presence of nafenopin. Further experiments using increased incubation times with BrdU would be required to validate such a hypothesis. However, as little as a 4% increase in the number of cells in S-phase could be important for tumour promotion, especially if it is combined with a reduction in the rate of cell death (see Chapters 4 and 5). The results presented also provide further evidence that a mitogenic response to the peroxisome proliferator nafenopin is retained to some degree by the Fao cell line.

3.4.4 Summary.

A large number of hepatoma cell lines are currently employed for *in vitro* studies of liver function. While these cell lines do not obviate the need for *in vivo* studies or the use of primary hepatocytes, they provide useful complementary mechanistic information. Following a systematic study of a range of hepatoma cell lines, the Reuber hepatoma cell line Fao has been identified as a suitable model for use in assessing the mechanism(s) of action of the peroxisome proliferators: this cell line is well differentiated, expressing albumin, PPAR and cytochrome P4504A1, is easy to

manipulate in culture and has retained a response to peroxisome proliferators, measured here by up-regulation of cytochrome P4504A1.

A tightly regulated balance exists between mitosis and apoptosis in the liver. Therefore, in order to interpret the results of investigations into the effect of peroxisome proliferators on apoptosis, some knowledge of their effects on mitosis is required. Results presented in this chapter demonstrate that nafenopin has a slight, but significant, mitogenic effect on Fao hepatoma cells. Interestingly, this effect was only exerted when the cells were proliferating at a sub-maximal rate, reflecting the response of quiescent hepatocytes to the peroxisome proliferators.

A comparison of Fao cells and primary hepatocytes demonstrated that expression of markers of differentiated function was higher in freshly isolated hepatocytes. However, differentiated function was lost in primary hepatocyte monolayers within 3-4 days of their establishment. Thus, used in isolation, both hepatocytes and hepatoma cells have drawbacks. In this thesis, complementary use will be made of both primary hepatocytes and the Fao hepatoma cell line to investigate the ability of the peroxisome proliferators to suppress apoptosis. The following chapter describes the kinetics of spontaneous and chemically-induced apoptosis in both cell systems and the effect of peroxisome proliferators on this apoptosis.

Chapter 4.

Effect of Peroxisome Proliferators on Spontaneous and Drug-Induced Hepatocyte and Hepatoma Cell Death.

4.1 Introduction.

4.1.1 Hepatocyte Apoptosis *In Vivo*.

Hepatocyte apoptosis is reported to occur *in vivo* in response to a number of stimuli. The mechanism of induction of apoptosis in the liver can be divided broadly into two categories. In the normal liver, apoptosis appears to have a role in the maintenance of homeostasis in the liver but apoptosis can also be induced by exposure to a number of exogenous chemicals.

4.1.1.1 Physiological Hepatocyte Cell Death and Maintenance of Liver Homeostasis.

Parenchymal hepatocytes are the functional cells of the liver and constitute the majority (80-90%) of liver mass (Michalopoulos, 1990). Although under normal circumstances adult hepatocytes rarely divide, high levels of proliferation occur during the compensatory liver growth that occurs in response to a loss of liver tissue induced by chemical or physical injury. Removal of up to two thirds of the liver by partial hepatectomy, or by treatment with a toxin such as carbon tetrachloride (CCl_4), has been shown to induce a wave of regenerative hypertrophy and hyperplasia in adult rats, leading to restoration of original organ mass (Thompson *et al.*, 1986; Michalopoulos, 1990). Liver regeneration involves almost all (95%) of the remaining hepatocytes and occurs extremely rapidly. Following a two-thirds partial hepatectomy, original liver mass is usually restored in experimental animals within 6-8 days (Michalopoulos, 1990).

Liver hyperplasia can also be induced in experimental animals by treatment with liver mitogens such as lead nitrate (Columbano *et al.*, 1984), phenobarbital (Argyris and Magnus, 1968), cyproterone acetate (Bursch *et al.*, 1986) and nafenopin (Moody *et al.*, 1977) and has been detected transiently in rats transplanted with a highly deviated ascites hepatoma (Tessitore *et al.*, 1987). In contrast to the regenerative hyperplasia that follows tissue damage, the response of hepatocytes to liver mitogens is an additive response, involving increases in DNA synthesis, DNA content, nuclear ploidy and mitosis (Bursch *et al.*, 1986). Withdrawal of liver mitogens from experimental animals is followed by liver involution, the rate of which is determined by the rate of removal of the mitogen from the liver (Bursch *et al.*, 1986). This regression of hyperplasia is associated with elevated rates of cell death by apoptosis, but not necrosis (Columbano *et*

al., 1984; Bursch *et al.*, 1986; Bursch *et al.*, 1992). Increased numbers of apoptotic cells have also been detected during the involution of liver hyperplasia in ascites tumour-bearing rats (Tessitore *et al.*, 1989) and during the liver involution that accompanies starvation (Bursch *et al.*, 1992). The apoptosis that follows mitogen withdrawal in the liver can be prevented by re-administration of the appropriate mitogen (Bursch *et al.*, 1984; Bursch *et al.*, 1986; Bursch *et al.*, 1990). Thus it appears that the balance between mitosis and apoptosis is tightly regulated in the maintenance of liver homeostasis.

4.1.1.2 Chemically-Induced Hepatocyte Cell Death.

A variety of chemicals, most notably CCl_4 and dimethylnitrosamine (DMN), induce centrilobular necrosis in the liver (Pritchard *et al.*, 1987). However, a number of chemicals that are toxic to the liver exert their effects via induction of apoptosis instead of, or prior to necrosis. For example, DMN induces apoptosis in rat liver prior to the onset of centrilobular necrosis (Pritchard and Butler, 1989). Initially, endothelial cells of the liver die by apoptosis, exposing underlying hepatocytes to the chemical and resulting in hepatocyte apoptosis. Tissue necrosis follows with increasing time of exposure to DMN, as a result of extensive damage to the vasculature system (Pritchard and Butler, 1989).

Other chemicals reported to induce apoptosis in rat liver include 1,1,-dichloroethylene (Reynolds *et al.*, 1984), cycloheximide (Ledda-Columbano *et al.*, 1992), nitrogen mustard (Bursch *et al.*, 1992), and ethanol (Benedetti *et al.*, 1988a). Apoptotic bodies in the livers of ethanol-treated rats have been shown to be distributed preferentially around the terminal hepatic vein. This apoptosis has been attributed in part to a low oxygen tension surrounding the terminal hepatic vein (Benedetti *et al.*, 1988b). In addition it has been proposed that this region of the liver contains older hepatocytes (Benedetti *et al.*, 1988b). Thus, the presence of more apoptotic bodies in the area around the terminal hepatic vein of the liver suggests that older hepatocytes may be more susceptible to the induction of apoptosis (Benedetti *et al.*, 1988b).

4.1.2 Hepatocyte Apoptosis *In Vitro*.

Apoptosis can also be induced chemically in hepatocytes *in vitro*. Apoptosis has been detected in primary hepatocyte cultures treated with menadione (McConkey *et al.*, 1988b), TNF α (Shinagawa *et al.*, 1991), the PKC inhibitors staurosporin or polymixin B (Sanchez *et al.*, 1992), cycloheximide (Ledda-Columbano *et al.*, 1992), TGFβ_1 (Oberhammer *et al.*, 1991), activin (Schwall *et al.*, 1993) and the phosphatase inhibitor okadaic acid (Boe *et al.*, 1991).

4.1.3 Biochemistry of Hepatocyte Apoptosis.

The majority of studies of hepatocyte apoptosis to date have focused on the morphological changes that occur in the cell, detected primarily by electron and light microscopy (Bursch *et al.*, 1986; Tessitore *et al.*, 1989; Boe *et al.*, 1991) and fluorescence microscopy of cells stained with DNA intercalating dyes (Oberhammer *et al.*, 1991). Hepatocyte apoptosis has been characterised further by tissue transglutaminase activation and by the involvement of Ca^{2+} and PKC (McConkey *et al.*, 1988a; Fesus *et al.*, 1989). These features of apoptosis are all described in detail in the general introduction (pages 43-59). In addition, it has been proposed that the negative growth regulator TGFβ_1 is involved in the induction of apoptosis in the liver (Bursch *et al.*, 1993). The role of TGFβ_1 in hepatocyte apoptosis is discussed in detail in Chapter 5.

The importance of internucleosomal DNA fragmentation in apoptosis is currently a matter of some controversy (see pages 43-47). The issues surrounding this debate are exemplified by the hepatocyte system. DNA fragmentation is reported to occur during hepatocyte apoptosis induced by TNFα (Shinagawa *et al.*, 1991) and the PKC inhibitors polymixin B and staurosporin (Sanchez *et al.*, 1992). In addition to these hepatocyte studies, DNA fragmentation has also been detected during apoptosis in a number of hepatoma cell lines (Lin and Chou 1992; Evans and Dive, 1993). However, exposure of primary hepatocytes to okadaic acid and TGFβ_1 is reported to result in the morphological features of apoptosis without any associated internucleosomal DNA fragmentation (Boe *et al.*, 1991; Oberhammer *et al.*, 1993a). The finding that necrotic hepatocytes also yield a DNA 'ladder' (Fukuda *et al.*, 1993) has cast further doubt on the significance of internucleosomal DNA fragmentation during hepatocyte apoptosis. This

latter finding could be explained by a recent report demonstrating that if apoptotic cells are not phagocytosed rapidly enough following extensive apoptosis in the liver they undergo a process of secondary necrosis (Nagata, 1994). Furthermore, close examination of the data presented by Fukuda *et al.* (1993) shows that the cells used in DNA analysis were a combination of viable, apoptotic and necrotic hepatocytes. It has been proposed that a more universal form of DNA fragmentation in apoptosis is the cleavage of DNA into high molecular weight (>50 kb) fragments (Oberhammer *et al.*, 1993b; Walker *et al.*, 1993; see page 45). This phenomenon has been demonstrated in nuclei from liver cells (Walker *et al.*, 1994).

4.1.4 Apoptosis and Hepatocarcinogenesis.

The liver has been suggested to provide an ideal system for studies of initiation and promotion during carcinogenesis because hepatocytes in the intermediate stages of carcinogenic transformation can be detected as phenotypically altered preneoplastic foci (Schulte-Hermann *et al.*, 1990). Hepatocytes in such foci exhibit an approximately ten-fold higher incidence of DNA synthesis and mitosis than occurs in normal liver (Schulte-Hermann *et al.*, 1990). Despite this increase in mitosis, no net liver growth occurs because the increase in mitosis is counterbalanced by a similar increase in the incidence of cell death by apoptosis. It has been proposed that apoptosis occurs in these foci in an attempt by the liver at autoregulation (Schulte-Hermann *et al.*, 1990). Following exposure to a tumour promoter, such as phenobarbital, the growth of preneoplastic foci is selectively accelerated. The clonal expansion of preneoplastic foci in response to tumour promoters may be the result of enhanced sensitivity of viable cells surrounding preneoplastic foci to the effects of growth inhibitors (Jirtle and Meyer, 1991). This hypothesis is supported by the finding that chronic exposure of rats to the tumour promoter, phenobarbital reduces the ability of normal hepatocytes to respond to the mitogenic effects of EGF (Jirtle and Meyer, 1991). In addition it has been proposed that the clonal expansion of such preneoplastic foci in response to tumour promoters may also occur as a result of suppression of apoptosis within the foci (Bursch *et al.*, 1984; Schulte-Hermann *et al.*, 1990). The importance of suppression of apoptosis by tumour promoters in the growth of preneoplastic foci is highlighted by the fact that withdrawal

of phenobarbital leads to the regression of foci, associated with an elevation in levels of apoptosis (Bursch *et al.*, 1990). The anomalous survival of such preneoplastic cells long enough to allow the development of further mutations could contribute to the further development of neoplasia.

4.1.5 Apoptosis and Peroxisome Proliferator-Induced Hepatocarcinogenesis.

Peroxisome proliferators have been shown to promote the development of phenotypically different foci to phenobarbital (see page 33). However, a contributory factor in peroxisome proliferator-induced hepatocarcinogenesis may also be the suppression of apoptosis (see page 37). In support of this hypothesis is the finding that the peroxisome proliferator nafenopin can suppress the hepatocyte apoptosis that occurs during the involution of cyproterone acetate-induced liver hyperplasia (Bursch *et al.*, 1986). In addition, the peroxisome proliferators clofibrate, Wy-14,643 and ETYA have been shown to protect preadipocytes from apoptosis induced by retinoic acid treatment (Chawla and Lazar, 1994). In another study, protection against paracetomol-induced hepatotoxicity in mice was given by clofibrate and DEHP (Nicholls-Grzemski *et al.*, 1992). While these results indicate that peroxisome proliferators may suppress apoptosis in the liver there is as yet no conclusive evidence that this suppression plays a role in the hepatocarcinogenicity of the peroxisome proliferators.

4.1.6 Aims of Chapter.

The work described in this chapter was designed to investigate and characterise the kinetics of apoptosis in spontaneously degenerating cultures of primary rat hepatocytes and Fao hepatoma cells. The ability of peroxisome proliferators to suppress such spontaneous hepatocyte and hepatoma cell death has been examined and compared. In order to determine whether the putative protective effect of peroxisome proliferators occurs regardless of the stimulus used to induce apoptosis, the ability of peroxisome proliferators to protect hepatoma cells from chemically-induced cell death has also been examined. The majority of the work described was conducted using the peroxisome proliferator and hypolipidaemic agent nafenopin. However, results were corroborated using another peroxisome proliferator, Wy-14, 643.

The *bcl-2* proto-oncogene and its protein product have been implicated in the suppression of apoptosis in a number of cell systems (Vaux *et al.*, 1988; Mah *et al.*, 1993; Deng and Podack, 1993; Naumovski and Cleary, 1994; see page 70). Therefore a possible relationship between the ability of peroxisome proliferators to suppress apoptosis and expression of the protein product of *bcl-2* has been examined.

4.2 Methods.

Throughout this chapter, culture of primary hepatocytes and Fao hepatoma cells, treatment of cells with nafenopin and Wy-14,643, cell counts and viability measurements were carried out as described in the general methods section (pages 77-87).

4.2.1 Detection of Apoptosis in Primary Hepatocyte and Fao Hepatoma Cell Cultures.

4.2.1.1 Analysis of Primary Hepatocytes in Culture.

The morphology of hepatocyte monolayers was studied over a 2 week period following seeding. Changes in cell morphology were monitored by phase contrast microscopy using a Leitz Labovert FS microscope. Photographs were taken using a Leitz Orthomat E camera.

4.2.1.2 Detection of Apoptosis in Spontaneously Degenerating Hepatocyte Monolayers.

Cells detaching from hepatocyte monolayers as they approached confluence were collected as follows: medium was removed by aspiration and fresh medium (4 ml) added to the flask. After 2 hours, medium containing cells that had detached over that 2 hour period was transferred to a sterile universal tube and the cells were pelletted by centrifugation (300 x g, 5 min.). Fresh medium (4 ml) was added to the flask, which was returned to the incubator at 37°C. The pellet of detached cells was resuspended in fresh medium (20-50 µl) and aliquots were examined by TB exclusion and AO staining. The remaining cells were pelletted in an eppendorf (300 x g, 5 min.). The supernatant was removed by aspiration and the cell pellet was frozen at -70°C. This procedure was repeated at 2 hourly intervals until approximately 1 x 10^6 cells had been collected. Frozen cell pellets were pooled at the end of the experiment and used as samples for CAGE, using the protocol described in the general methods section (page 83).

As a result of difficulties encountered in the collection of detaching hepatocytes, spontaneously degenerating hepatocyte monolayers were also fixed and stained with Ho258. Samples for CAGE were subsequently produced by lysing cells directly from the

monolayer as follows: medium from degenerating and viable hepatocyte monolayers was removed by aspiration and lysis buffer (200 µl) containing proteinase K (see page 83) was added to each flask. Cells were incubated in lysis buffer for 10 min. at 37°C. Lysis buffer containing the hepatocytes was removed from the flask by gentle pipetting and transferred to an eppendorf tube. Samples were incubated at 50°C for a further 30 min. prior to CAGE as described in the general methods section (page 83). In addition to CAGE, the DNA from viable and degenerating monolayer cells was examined by FIGE (page 83).

4.2.1.3 Detection of Apoptosis in Confluent Fao Hepatoma Cell Cultures.

Fao hepatoma cells were seeded at 1 x 10^6 / 75cm^3 flasks in complete Ham's F12 medium (10 ml) and were grown to confluence. Cells detaching from Fao monolayers as they approached confluence were collected at 2-hourly intervals and examined using the method described in section 4.2.1.2 (page 132). Cells remaining attached to the monolayer at the end of the experiment were removed by trypsinisation and analysed by TB exclusion, by AO staining and by CAGE .

4.2.2 Analysis of the Effect of Peroxisome Proliferators on Spontaneous Primary Hepatocyte and Fao Hepatoma Cell Apoptosis.

4.2.2.1 Analysis of the Effect of Peroxisome Proliferators on Primary Hepatocyte Viability.

Freshly isolated primary hepatocytes were seeded as described (see page 78). After 24 hours, medium was removed from the cultures by aspiration and replaced with that containing nafenopin (50 µM), Wy-14,643 (1-50 µM) or DMF alone (0.25% $^{v}/_{v}$). The morphology of nafenopin-, Wy-14,643- and DMF-treated hepatocytes was analysed over the following 2 weeks by phase contrast microscopy and Ho258 staining.

4.2.2.2 Analysis of the Effect of Withdrawal of Nafenopin from Primary Hepatocyte Cultures.

Freshly isolated primary rat hepatocytes were seeded and treated with nafenopin (50µM) or DMF alone (0.25% $^{v}/_{v}$) as described in section 4.2.1.2. Hepatocyte

monolayers were maintained in nafenopin for 6 weeks at 37°C in a humidified atmosphere of 5% CO_2. Medium containing fresh nafenopin or DMF was replaced every 48 hours. After 6 weeks, medium was removed from the cultures by aspiration and monolayers were washed twice with nafenopin-free medium. Medium was then replaced with that containing nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Hepatocyte morphology was analysed for the following 8 days by phase contrast microscopy and Ho258 staining.

4.2.2.3 Analysis of the Effect of Nafenopin on Spontaneous Fao Apoptosis.

Fao hepatoma cells were seeded at 1 x 10^6 / 75cm^3 flask in complete Ham's F12 medium (10ml). After 4 days, medium was removed by aspiration and replaced with that containing nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Over the following 6 days, cells that detached from the monolayer were collected and apoptosis was assessed as described in section 4.2.1.2 (page 132).

4.2.3 Chemically-Induced Fao Apoptosis.

4.2.3.1 Treatment of Fao Cells with Etoposide and Hydroxyurea.

Fao cells were seeded at 1 x 10^6 cells / 75 cm^3 flask in complete Ham's F12 medium (10 ml). After 48 hours, medium was removed by aspiration and replaced with that containing the topoisomerase II inhibitor etoposide (1-100 μM from a freshly prepared 4 mM stock in DMF) or the ribonucleotide reductase inhibitor hydroxyurea (1-20 mM from a freshly prepared 100 mM stock in Ham's F12 medium). The viability of attached and detached cells was assessed over 48 hours following drug addition by TB exclusion, AO staining and CAGE of DNA.

4.2.3.2 Analysis of the Effect of Nafenopin on Apoptosis Induced by DNA-Damaging Agents.

Fao cells were seeded at 1 x 10^5 cells / 25 cm^3 flask in complete Ham's F12 medium (4 ml). After 24 hours, medium was removed by aspiration and replaced with that containing nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). After a further 24 hours, etoposide (10 μM) or hydroxyurea (1 mM) were added as described above in section

4.2.3.1. Cell number and viability were assessed over the following 8 days by TB exclusion.

4.2.4 Analysis of Bcl-2 Expression in Primary Hepatocytes and Fao Hepatoma Cells.

Protein samples were made from Fao hepatoma cells and primary hepatocytes treated with nafenopin or DMF alone as described in section 3.2.2 (page 95). SDS-PAGE and western blotting were carried out as described in the general methods section (page 85). Bcl-2 expression in Fao hepatoma cells and primary hepatocytes was detected by incubation of nitrocellulose filters with a monoclonal rabbit anti-Bcl-2 primary antibody (gift from Gerard Evan, ICRF), diluted to 1:10 000, followed by antibody localisation using a horseradish peroxidase-linked donkey anti-rabbit secondary antibody (Amersham), diluted to 1:8000. Nitrocellulose filters were stained with Ponceaus solution to ensure even protein loading in each lane.

4.3 Results.

4.3.1 Mode of Cell Death in Spontaneously Degenerating Primary Hepatocyte and Fao Hepatoma Cell Cultures.

4.3.1.1. Spontaneous Degeneration of Primary Rat Hepatocytes in Culture.

Freshly isolated hepatocytes seeded at 1.8 x 10^6 / flask attached to the culture flask within 4 hours of seeding and formed a confluent monolayer within 24 hours. 24-48 hours after seeding, viable hepatocytes typically contained 1 or 2 nuclei and exhibited a regular, polygonal shape with phase bright cell borders, resembling bile canaliculi *in vivo* (Fig. 4.1a). However within 4-6 days of seeding, hepatocytes began to lose the phase bright nature of their cell borders, accumulated multiple nuclei and became irregular in shape (Fig. 4.1b). Degenerating hepatocytes developed stress fibres and detached from the monolayer. Proliferation of fibroblasts, characterised by positive staining with anti-vimentin antibodies (Nicola French, CTL, personal communication), occurred in those areas where hepatocytes had detached from the monolayer and eventually took over the cultures (Fig. 4.1c).

4.3.1.2 Mode of Cell Death in Spontaneously Degenerating Hepatocyte Monolayers.

Examination of a sample of primary hepatocytes that detached from a 6-day old monolayer over a 2 hour period revealed that 79.4% of the cells had plasma membranes permeable to TB (n=3, se=9.2). Fluorescence microscopy of these detached hepatocytes, stained with AO, revealed few cells (<1%) with chromatin condensation patterns typical of apoptosis. Instead, diffuse staining of the whole cell was detected and no recognisable nuclear morphology was apparent (data not shown). In contrast, following fluorescence microscopy of fixed primary hepatocyte monolayers stained with Ho258, cells with brightly stained condensed and fragmented nuclei typical of apoptosis were evident (Fig. 4.2). Apoptotic cells were visible in hepatocyte monolayers as early as 24 hours after seeding and were detected at levels of 0.5-1% on each of the following 8 days (n=6; Fig. 4.7) The majority of cells remaining exhibited diffuse nuclear staining although the occasional mitotic figure was apparent (data not shown).

Analysis of DNA from detached hepatocytes by CAGE resulted in the production of a smear indicative of non-specific DNA degradation (data not shown). When samples

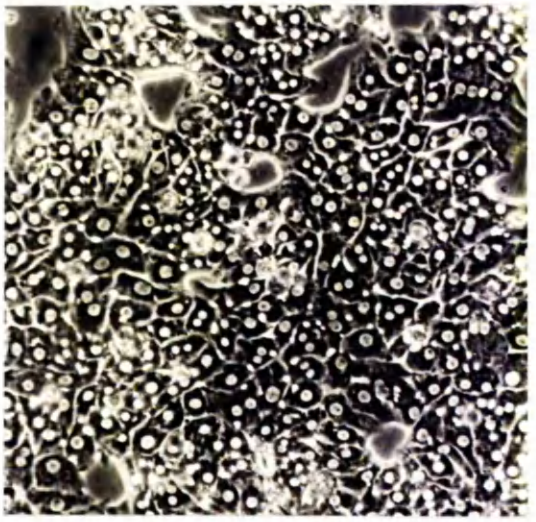
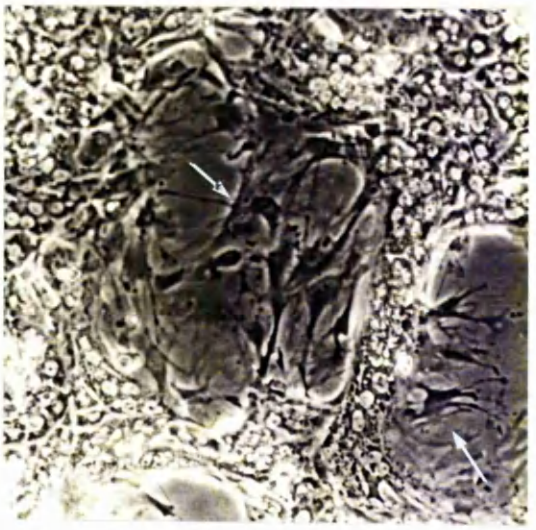
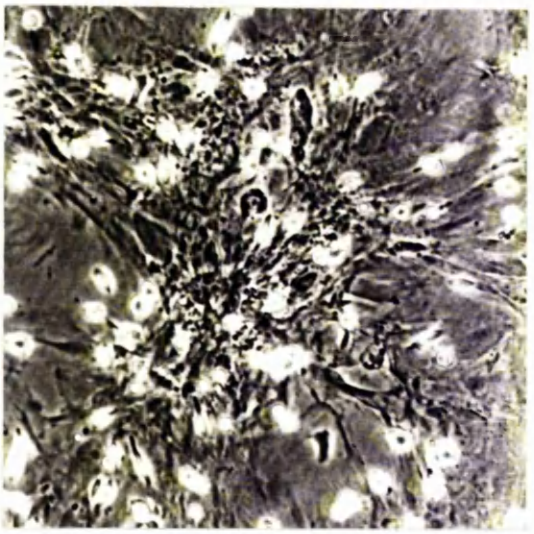


Figure 4.1. (a) Phase contrast photograph of a viable primary hepatocyte monolayer 48 hours after seeding. Hepatocytes are regular in shape, have phase bright borders and only 1-2 nuclei. Bar=40 μM.



(b) Phase contrast photograph of a degenerating hepatocyte monolayer 6 days after seeding. Hepatocytes are irregular in shape, and have accumulated multiple nuclei. Areas of fibroblast proliferation (arrows) are apparent. Bar=40 μM.



(c) Phase contrast photograph of a degenerating hepatocyte monolayer 8 days after seeding. Hepatocytes have detached from the monolayer and fibroblasts are predominant. Bar=40 μM. Photographs are representative of many independent experiments.

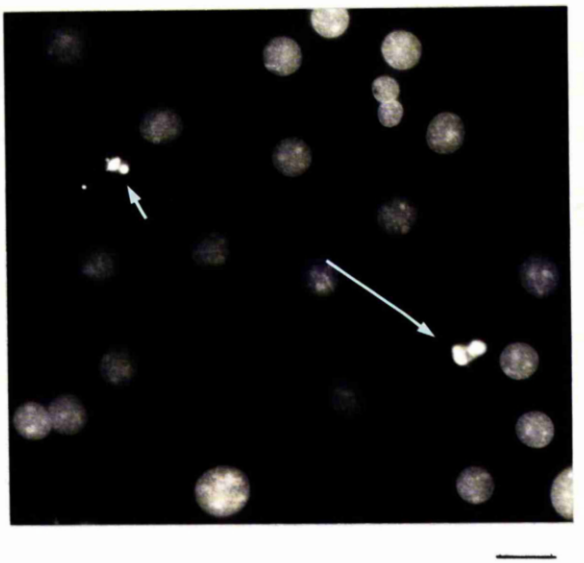


Figure 4.2. Fluorescent micrograph of a 6-day old degenerating hepatocyte monolayer stained with Ho258. The brightly stained condensed and fragmented nuclei of apoptotic hepatocytes (arrows) can be distinguished from diffusely stained nuclei of viable cells. Bar=10 μM. Photograph is representative of many independent experiments.

were prepared from degenerating hepatocyte monolayers by lysing the cells directly on the plate, only high molecular weight DNA was detected (data not shown). Analysis of DNA from both viable and degenerating hepatocyte monolayers by FIGE resulted in some non-specific DNA smearing (data not shown).

4.3.1.3 Mode of Cell Death in Fao Cultures Approaching Confluence.

In Fao monolayers, the percentage of cells with membranes permeable to TB was always less than 10% (x=4.5, se=0.76, n=20). Fluorescence microscopy of monolayer cells stained with AO revealed diffuse nuclear staining in >90% of cells examined (Fig. 4.3a). Some cells with mitotic figures were also apparent. As Fao cultures approached confluence, cells were seen to detach from the monolayer. Fluorescence microscopy of these detached cells stained with AO revealed cells with brightly stained condensed and fragmented nuclei typical of apoptosis (Fig. 4.3b). For example, of the Fao cells that detached from a confluent monolayer over a 2 hour period, 51% of cells (n=4, se=7.2) exhibited a condensed and fragmented nuclear morphology. The remainder of detached cells were weakly staining cell ghosts where the mode of cell death could not be established or exhibited mitotic figures, which were clearly distinguishable from the condensed nuclei of apoptotic cells (data not shown). In the same sample of detached cells, 20% of cells (n=4, se=5.9) had cell membranes permeable to TB. If the time period over which detached cells were collected was increased, the percentage of cells exhibiting an apoptotic morphology when stained with AO decreased and the number of cell ghosts increased. In accordance with this finding, an increase in the number of cells allowing uptake of TB was also detected (data not shown).

CAGE of DNA from detached Fao cells revealed a 180-200 base pair DNA banding pattern or 'ladder' indicative of internucleosomal DNA cleavage reported for apoptosis in many cell types (Fig. 4.4). In contrast, DNA from viable monolayer Fao cells was consistently of high molecular weight (Fig. 4.4). DNA from detached Fao cells collected over a time interval longer than 3 hours exhibited a smear typical of non-specific degradation of DNA (data not shown).

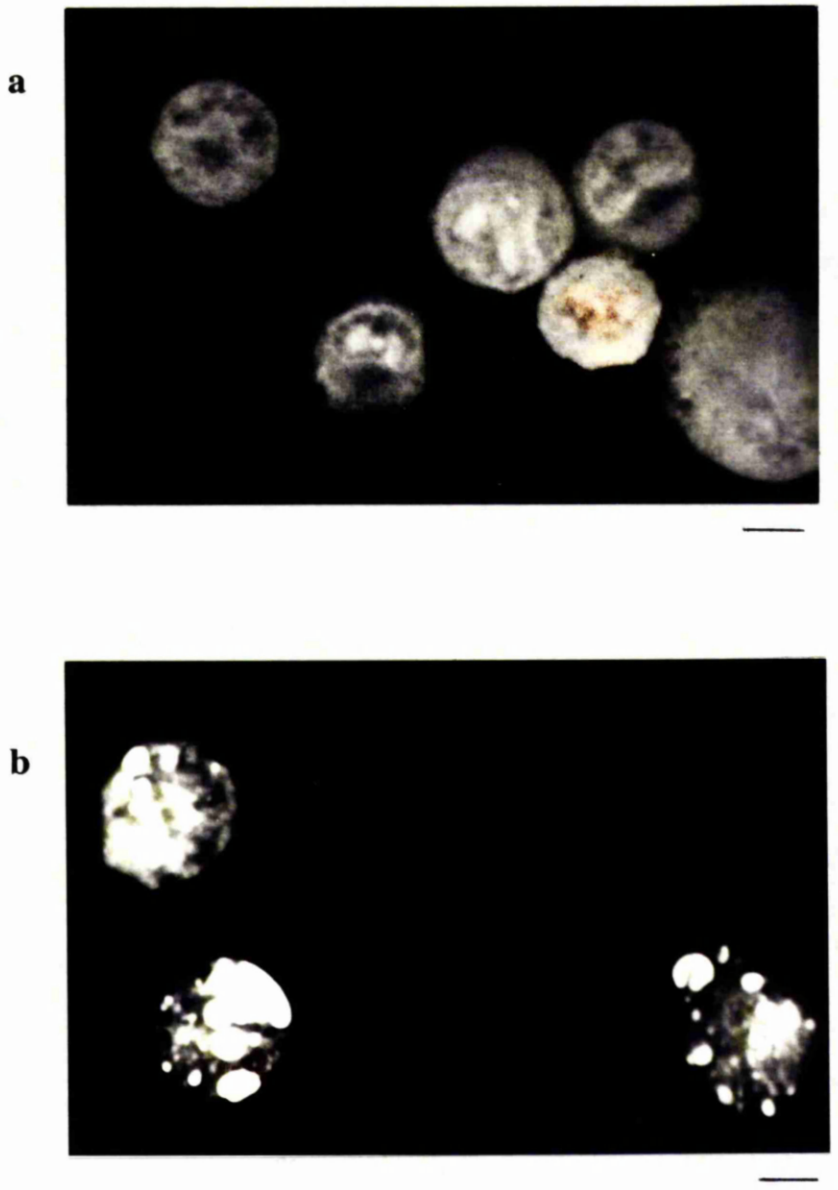
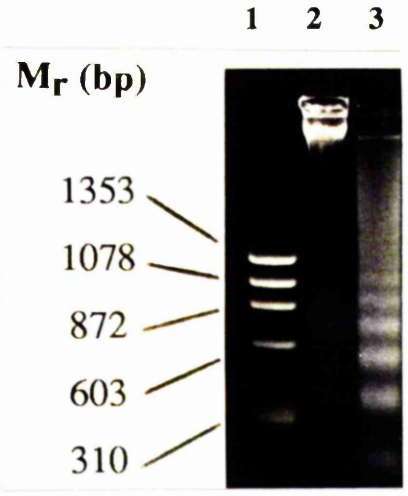


Figure 4.3. Confocal photograph of **(a)** viable monolayer and **(b)** apoptotic detached Fao cells stained with AO. The diffuse nuclear staining of the viable cells can be contrasted with the brightly stained compact chromatin masses in the detached cells. Bar= 5μM. Photograph is representative of many independent experiments.



Lanes:

1: Hae III size marker

2: Viable attached cells

3: Apoptotic detached cells

Figure 4.4. CAGE of DNA from viable attached and apoptotic detached Fao cells. The high molecular weight DNA in the monolayer cells illustrates their viability, whereas DNA fragmentation patterns typical of apoptosis can be seen in the detached cells. Gel is representative of 3 independent experiments.

4.3.2 Effect of Peroxisome Proliferators on Spontaneous Hepatocyte and Fao Hepatoma Cell Death.

4.3.2.1 Maintenance of Hepatocyte Viability by Peroxisome Proliferators.

4.3.2.1.1 Nafenopin

The viability of primary hepatocytes was maintained by the addition of nafenopin (50 μM) to the culture medium 24 hours after establishment of the monolayer (Fig. 4.5a). Hepatocytes grown in the continual presence of nafenopin retained their polygonal shape and phase bright cell borders and contained only 1 or 2 nuclei. An accumulation of lipid droplets was also apparent in nafenopin-treated hepatocytes (determined by Texas Red staining, Ruth Roberts, Zeneca CTL, personal communication). Treatment with DMF alone had no protective effect on the viability of primary hepatocyte cultures (Fig. 4.5b).

Some hepatocyte degeneration was seen in cultures treated with nafenopin, but this occurred in regions of the monolayer which were not fully confluent on addition of nafenopin. Furthermore, the viability of hepatocytes seeded at a density lower than 1.8 x 10^6 / flask was not maintained to the same extent as that of confluent monolayers (data not shown). Since the most confluent areas of the monolayer were generally in the centre of the flask, the viability of hepatocytes was maintained most significantly in this area. The viability of confluent hepatocyte monolayers was maintained by nafenopin for at least 6 weeks. However, withdrawal of nafenopin from the cultures led once again to the onset of degeneration and cell death of hepatocyte cultures within approximately 8 days (Fig. 4.5c).

4.3.2.1.2 Wy-14, 643.

Hepatocytes treated with Wy-14,643 (1, 10 and 50 μM) also retained a viable morphology for a longer period of time than controls treated with DMF alone (Fig. 4.6). The extent of hepatocyte viability maintained by 10μM Wy-14,643 was equivalent to that achieved using 50μM nafenopin. As was seen with the nafenopin-treated cultures some hepatocyte degeneration occurred in semi-confluent areas of the monolayer.

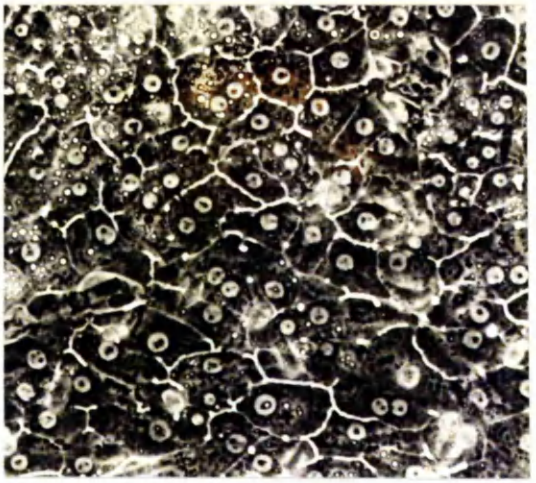
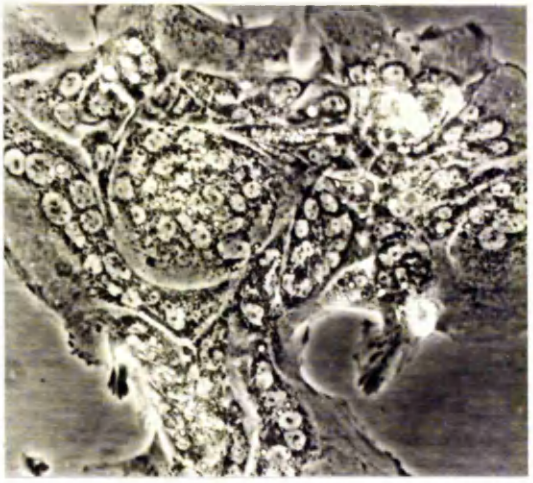
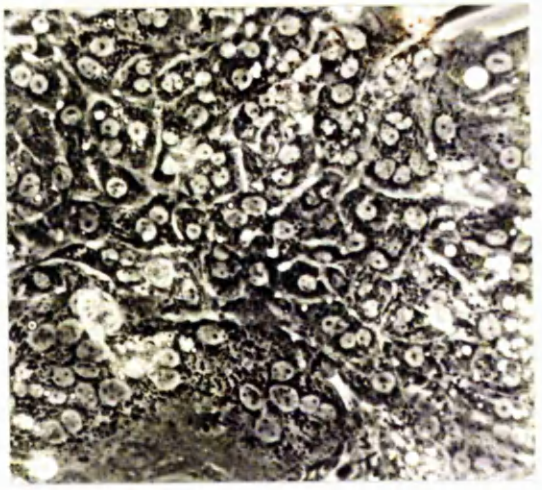


Figure 4.5. (a) Phase contrast photograph of a viable primary rat hepatocyte monolayer maintained in the presence of nafenopin (50 μM) for 2 weeks. Cells have retained a regular shape and phase bright cell borders, have only 1-2 nuclei and have accumulated lipid droplets. Bar=20 μM.



(b) Phase contrast photograph of a degenerating primary rat hepatocyte monolayer treated with DMF alone (0.25% $^{v}/_{v}$), 6 days after seeding. Bar=20 μM.



(c) Phase contrast photograph of a degenerating primary rat hepatocyte monolayer 8 days after withdrawal of nafenopin (50 μM) from the culture medium. Bar=20 μM. Photographs are representative of 6 independent experiments.

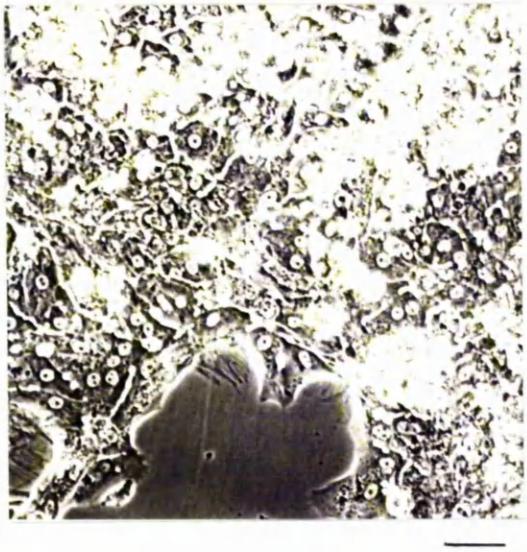


Figure 4.6. (a) Phase contrast photograph of a DMF-treated control primary hepatocyte monolayer 6 days after seeding. Bar=40 μM.

(b) Phase contrast photograph of a primary hepatocyte monolayer maintained in Wy-14,643 (10 μM) for 6 days. Bar=40 μM.

4.3.2.2 Effect of Peroxisome Proliferators on Spontaneous Hepatocyte Apoptosis.

4.3.2.2.1 Nafenopin

Fluorescence microscopy of fixed primary hepatocyte monolayers stained with Ho258 was used to quantify the number of apoptotic hepatocytes present in nafenopin- and DMF-treated monolayers over the first 8 days following seeding. Cells with condensed and fragmented nuclei typical of apoptotic cells were detected in DMF-treated control cultures as early as 24 hours after establishment of the monolayer and were present at levels of 0.5-1% over the following 8 days (n=6; Fig. 4.7). The majority of remaining cells exhibited diffuse nuclear staining patterns, although some mitotic figures were also apparent. Cells with an apoptotic morphology were also detected in hepatocyte monolayers treated with nafenopin. However, results from 6 independent experiments indicated that the number of apoptotic hepatocytes present in these nafenopin-treated monolayers was always significantly lower ($p < 0.05$) than the number seen in solvent-treated controls over the 8-day experimental period (Fig. 4.7).

Fluorescence microscopy of Ho258-stained cells was also used to examine the occurrence of apoptosis in primary hepatocyte monolayers that had been maintained in nafenopin (50 μM) for 6 weeks and from which nafenopin had subsequently been withdrawn. Cells with an apoptotic morphology were detected at low levels (0.2-0.5%) in these monolayers over the 8 days following nafenopin withdrawal. In 3 independent experiments, the number of apoptotic cells detected in these monolayers showed a higher trend than in hepatocyte cultures that remained in nafenopin, where very few apoptotic cells were detected (Fig. 4.8). However, the difference in apoptotic cell number in nafenopin-treated and nafenopin-withdrawn hepatocyte cultures was only found to be statistically significant at one of the time points measured (day 6 after nafenopin withdrawal; $p < 0.05$).

4.3.2.2.2 Wy-14, 643.

A preliminary experiment carried out to examine the effect of Wy-14,643 on spontaneous hepatocyte apoptosis demonstrated a reduction in the number of apoptotic hepatocytes present in cultures treated with Wy-14,643 (10 and 50 μM) compared to solvent-treated controls over the first 8 days after seeding (Fig. 4.9). Since the

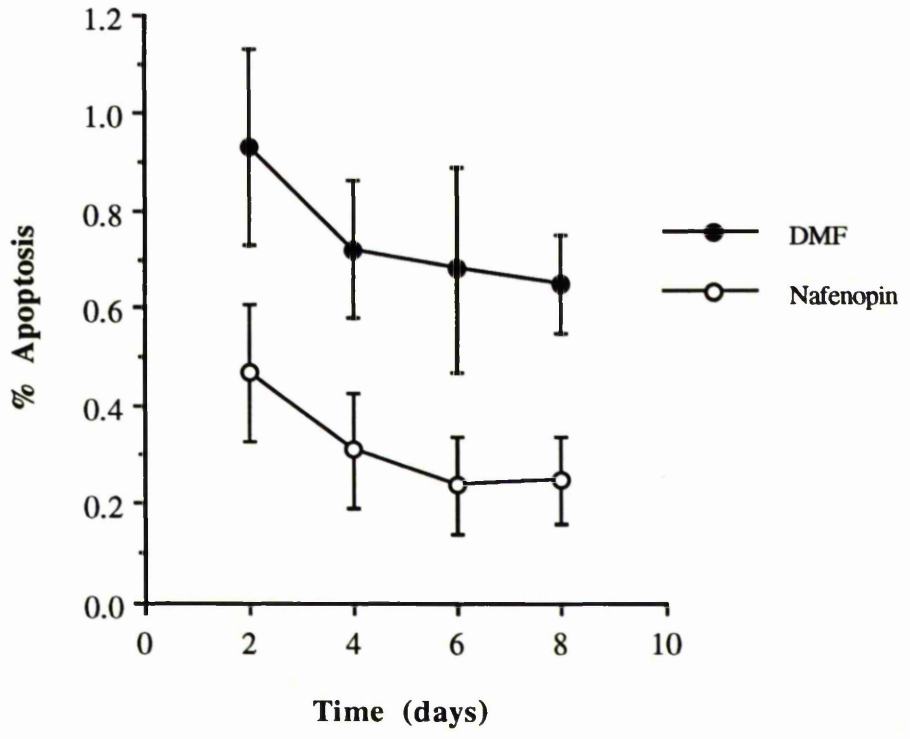


Figure 4.7. Percentage of cells with an apoptotic morphology detected by Ho258 staining of primary rat hepatocyte monolayers, cultured in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) for 8 days following establishment. Data points represent the mean and standard error of 6 independent experiments in which at least 800 cells were counted every 2 days.

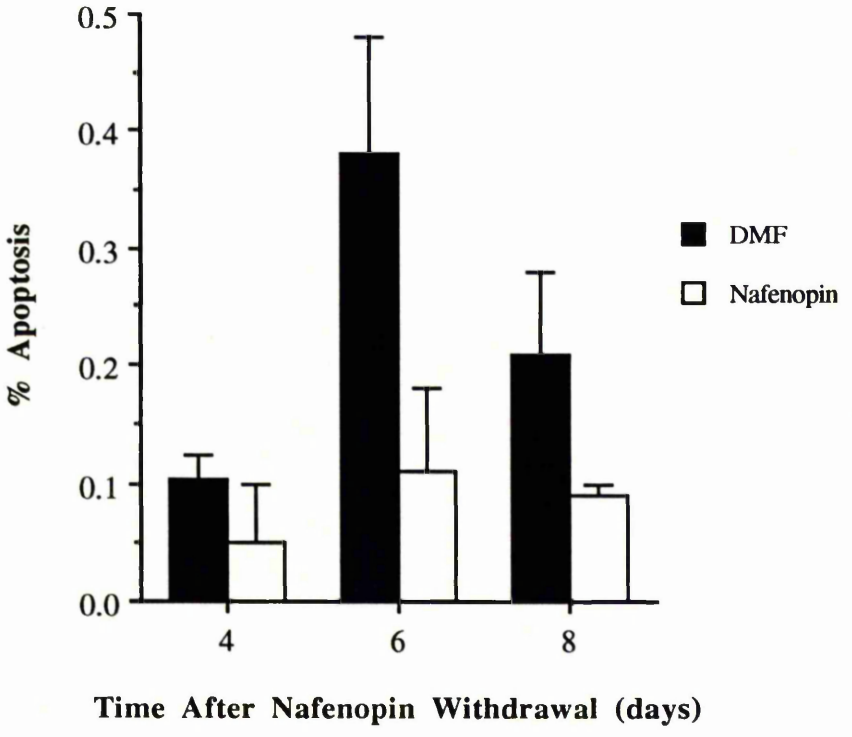


Figure 4.8. Percentage of cells with an apoptotic morphology detected by Ho258 staining of 6-week old primary hepatocyte monolayers maintained in nafenopin (50 μM). Nafenopin was withdrawn from hepatocyte cultures after 6 weeks and replaced with nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Data points represent the mean and standard error of 3 independent experiments in which at least 800 cells were counted every 2 days after nafenopin withdrawal.

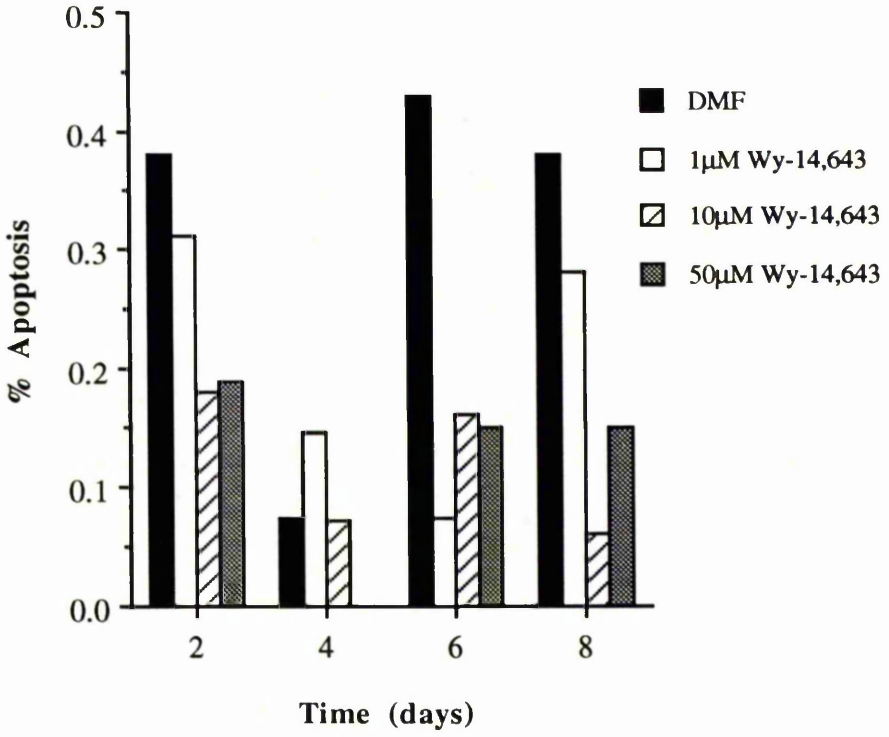


Figure 4.9. Percentage of cells with an apoptotic morphology detected by Ho258 staining of primary rat hepatocyte monolayers, cultured in the presence of Wy-14,643 (1-50 μM) or DMF alone (0.25% $^{v}/_{v}$) for 8 days following establishment. Data points represent preliminary results from 1 experiment in which at least 800 cells were counted every 2 days.

experiment was only carried out once, no statistical analysis of the results could be performed. A slight reduction in apoptotic hepatocyte number was also detected in monolayers treated with Wy-14,643 (1 μM), although the reduction was not as marked as that seen with higher concentrations of Wy-14,643 (Fig. 4.9). Further experiments using Wy-14,643 are described in Chapter 5.

4.3.2.3 Effect of Peroxisome Proliferators on Spontaneous Fao Apoptosis.

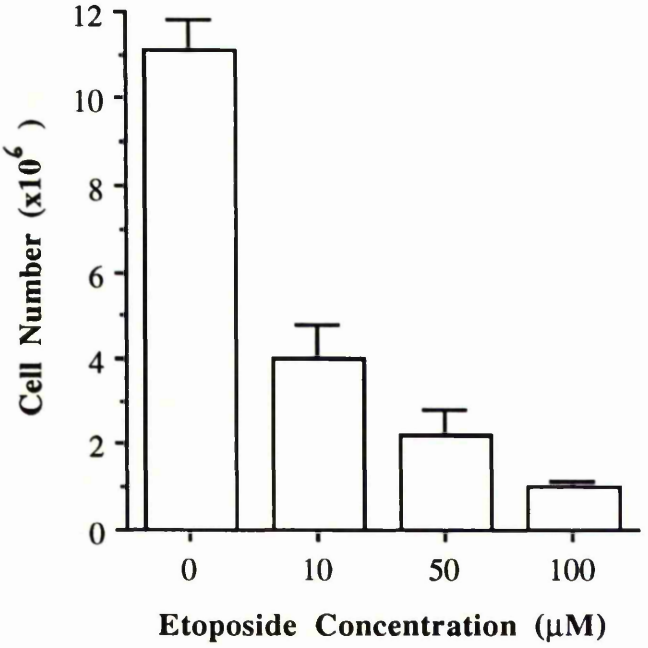
The presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) in the medium of Fao hepatoma cultures as they approached confluence did not affect the number of cells detaching from the monolayer (data not shown). Furthermore, the confluent density of the cells was not affected by either of these additions (Fig. 3.1e, page 102).

4.3.3 Effect of Peroxisome Proliferators on Chemically-Induced Fao Hepatoma Cell Death.

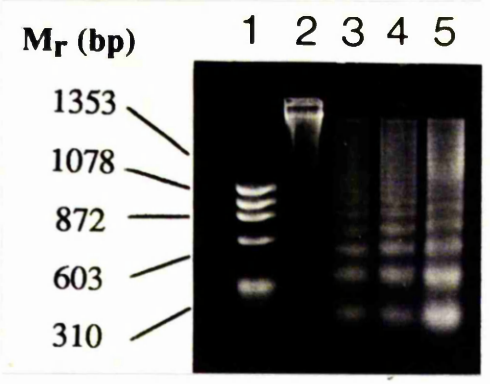
4.3.3.1 Induction of Apoptosis in Fao Hepatoma Cells by DNA-Damaging Agents.

Treatment of Fao cells with etoposide (10-100 μM) led to rapid detachment of Fao cells from the monolayer. Treatment with etoposide (10 μM) for 48 hours, resulted in the loss of 63% of Fao cells from the monolayer compared to untreated controls (n=3, se=4.3; Fig. 4.10a). Similarly, treatment of Fao cells with hydroxyurea (1-20 mM) resulted in the rapid onset of cell death with 83% of cells detaching from the monolayer after treatment with hydroxyurea (1 mM) for 48 hours (n=3, se=2.8; Fig. 4.11a). Following a 24 hour exposure to etoposide (10 μM), 45% of cells that detached from the monolayer over a 2 hour period exhibited chromatin condensation patterns typical of apoptosis (n=4, se=7.8) whereas only 10% had membranes permeable to TB (n=4, se=5.1). The corresponding values for cells detaching after treatment with hydroxyurea (1 mM) were 41% (n=4, se=10) and 6% (n=4, se=3) respectively. Furthermore, the DNA from cells that detached from Fao monolayers after administration of etoposide or hydroxyurea exhibited a DNA ladder indicative of the internucleosomal DNA fragmentation typical of apoptosis in many cell systems (Fig. 4.10b and 4.11b). On the basis of these results, the concentrations of etoposide and hydroxyurea chosen for further experiments were 10 μM and 1 mM respectively.

a



b



Lanes:

1: Hae III size marker

2: Viable attached cells

3-5:Apoptotic detached cells

3: 10 μM etoposide

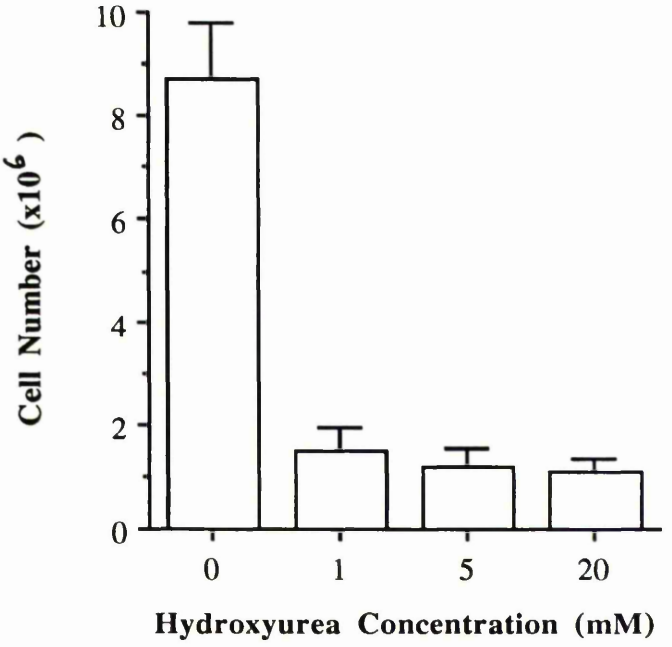
4: 50 μM etoposide

5: 100 μM etoposide

Figure 4.10. **(a)** Effect of etoposide (1-100 μM) on the viability of Fao Cells. Data points represent the mean and standard error of 3 independent experiments in which the number of viable cells remaining attached to the monolayer was counted 48 hours after etoposide treatment.

(b) CAGE of DNA from attached and detached Fao cells 24 hours after addition of etoposide (1-50 μM). Gel is representative of 3 independent experiments.

a



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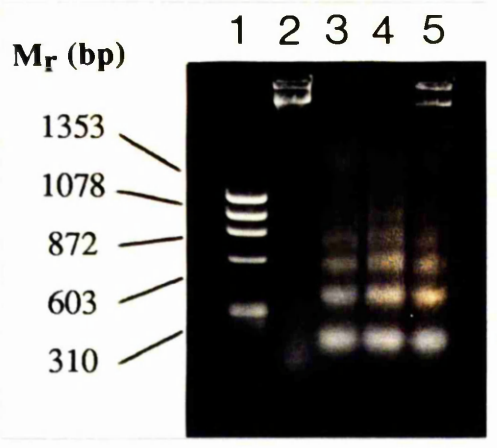


Lanes:

1: Hae III size marker

2: Viable attached cells

3-5:Apoptotic detached cells

3: 1mM hydroxyurea

4: 5 mM hydroxyurea

5: 20 mM hydroxyurea

Figure 4.11. (a) Effect of hydroxyurea (1-20 mM) on the viability of Fao Cells. Data points represent the mean and standard error of 3 independent experiments in which the number of viable cells remaining attached to the monolayer was counted 48 hours after hydroxyurea treatment.

(b) CAGE of DNA from attached and detached Fao cells 24 hours after addition of hydroxyurea (1-20 mM). Gel is representative of 3 independent experiments.

4.3.3.2 Effect of Nafenopin on Fao Apoptosis Induced by DNA-Damaging Agents.

Co-addition of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$) to Fao cultures treated with etoposide (10 μM) or hydroxyurea (1 mM) had no significant effect on the number of viable cells remaining attached to the monolayer (Fig. 4.12a and b).

4.3.4 Expression of Bcl-2 in Primary Hepatocytes and Fao Hepatoma Cells.

Western blot analysis revealed that Fao hepatoma cells express detectable levels of Bcl-2 (Fig. 4.13a). In contrast, no Bcl-2 expression was detected in freshly isolated or 2-day old primary hepatocytes (Fig. 4.13b). Interestingly, some Bcl-2 expression was detected in primary hepatocyte monolayers 4 days after seeding (Fig. 4.13b). However, it was not apparent whether the protein was expressed by hepatocytes themselves or by non-parenchymal cells present in the cultures. Treatment of Fao and primary hepatocyte cultures with nafenopin (50 μM) did not affect the level of expression of Bcl-2 (Fig. 4.13 a and b).

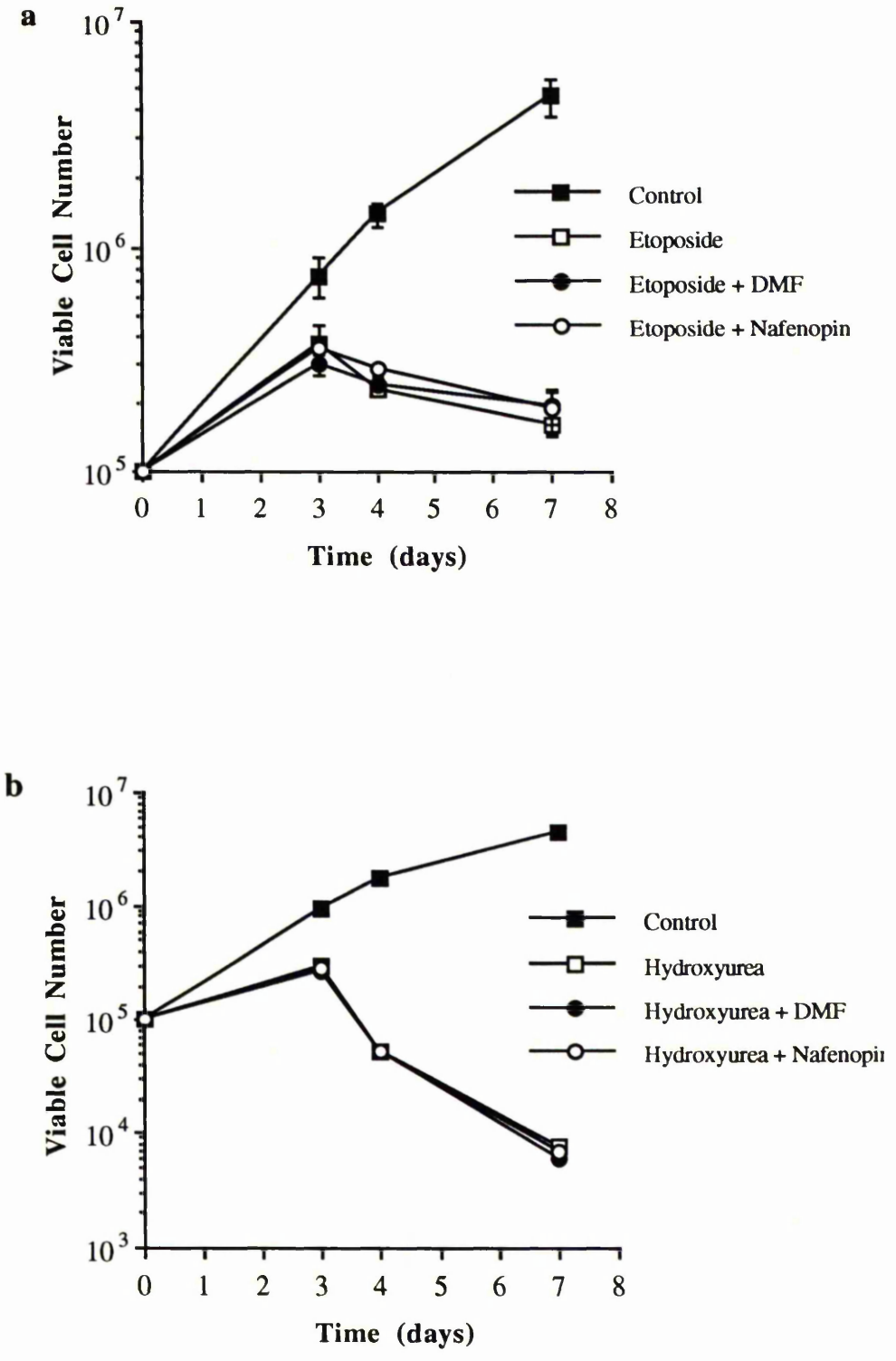


Figure 4.12. Effect of nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) on Fao cell viability in the presence of **(a)** etoposide (10 μM) and **(b)** hydroxyurea (1 mM). Data points represent the mean and standard error of 3 independent experiments in which the number of viable cells remaining attached to the monolayer after drug addition was counted at 1 time point per day.

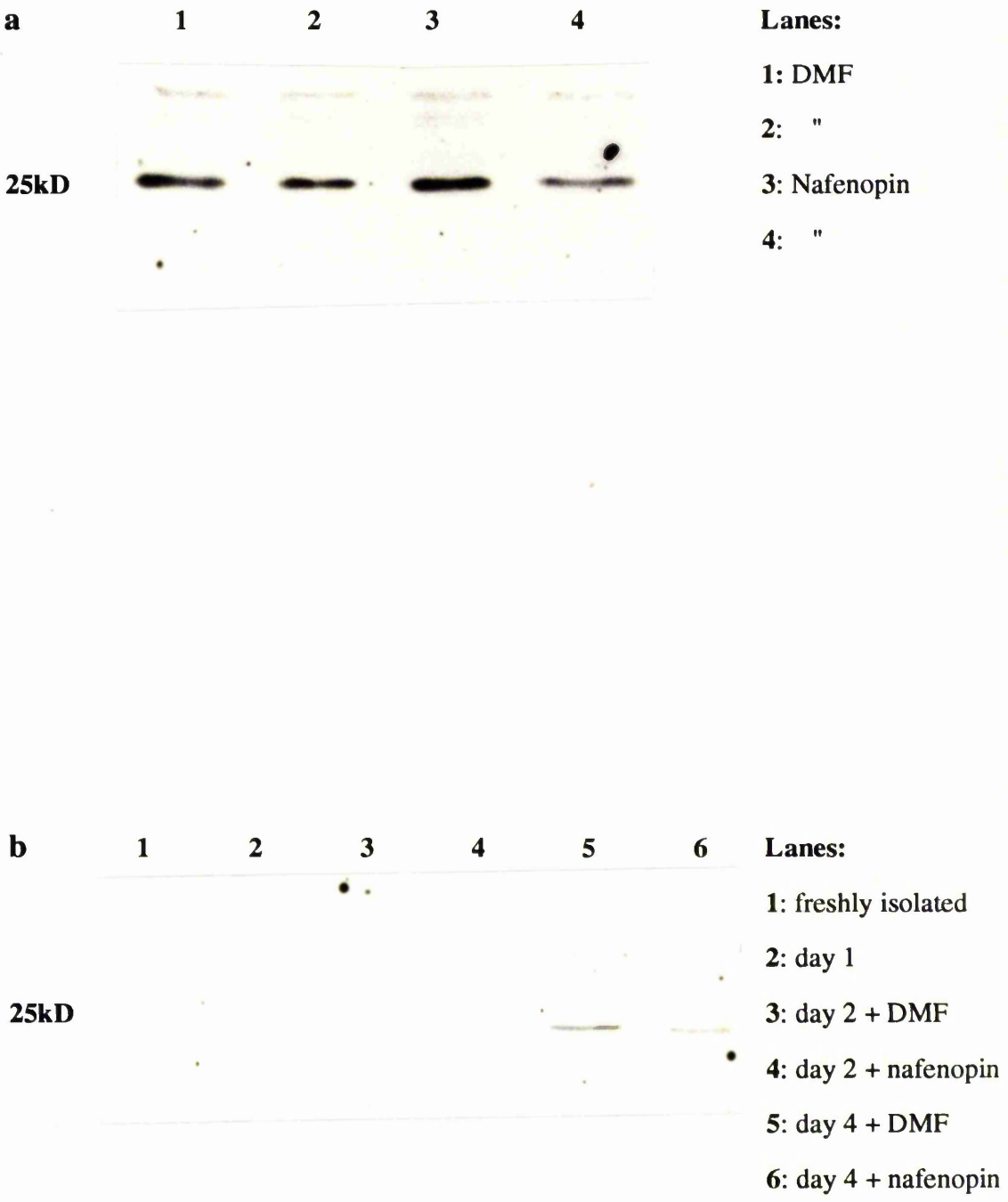


Figure 4.13. Western blot analysis of **(a)** Fao hepatoma cells and **(b)** primary hepatocytes demonstrating Bcl-2 expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). 30 μg protein was loaded in each lane. Westerns are representative of 3 independent experiments.

4.4 Discussion.

It has been proposed that the promotion of tumourigenic lesions may occur not only as a result of enhanced mitogenesis within initiated foci but also by suppression of apoptosis (Bursch *et al.*, 1992). While the role of sustained hyperplasia in peroxisome proliferator-induced hepatocarcinogenesis has been investigated quite extensively, few detailed studies have been conducted to investigate the importance of suppression of apoptosis in this process. Chapter 3 described the validation of the Fao rat hepatoma cell line as a suitable cell model for use with primary rat hepatocytes in *in vitro* investigations of the mechanism of action of the peroxisome proliferators. It is fundamental that any cell model used in this project should retain the ability to undergo apoptosis and more importantly, that cells undergoing apoptosis should be readily detectable. Therefore an assessment of apoptosis in the two cell systems was required.

4.4.1 Apoptosis in Spontaneously Degenerating Primary Hepatocyte Cultures.

Primary hepatocyte monolayers were seen to degenerate rapidly following their establishment: after only 6 days in culture, hepatocytes had sustained considerable changes in morphology and had started to detach from the monolayer (Fig. 4.1, page 137). At any one time, only 15-20% of these detached cells had intact plasma membranes, as demonstrated by their ability to exclude TB. Furthermore, little evidence of chromatin condensation or nuclear fragmentation was apparent in detached cells stained with AO. Indeed, the majority of detached cells were only diffusely stained and no nuclear morphology was apparent. The absence of cells with an apoptotic morphology is unlikely to be the result of secondary degeneration occurring after hepatocyte detachment. This is supported by the finding that cells were equally degraded if the time interval over which detaching hepatocytes were collected was reduced. Thus, extensive chromatin degradation and loss of plasma membrane integrity must have occurred prior to or concomitant with cell detachment. An additional problem encountered during the analysis of detached cells was the heterogeneity of the cell population in degenerating hepatocyte monolayers. During liver perfusions, 5% of the cells isolated are fibroblasts and other non-parenchymal cells. Since these cell types were often seen to multiply in degenerating hepatocyte cultures, it was difficult to

establish whether all the cells detaching from a hepatocyte monolayer were actually hepatocytes.

More success was achieved in detecting apoptotic hepatocytes using the fluorescent stain Ho258. This dye is much less 'promiscuous' than AO. While AO is used routinely for staining detached cells at low density in suspension, it is less suitable for staining confluent monolayers. AO binds to all nucleic acids and is highly sensitive to pH changes giving a high level of background staining which interferes with the detection of apoptotic cells in hepatocyte monolayers. In contrast, Ho258 binds only to double stranded DNA and is less pH dependent, so that the nuclei of apoptotic cells in a confluent monolayer are apparent. Using Ho258, cells with condensed and fragmented nuclei, typical of apoptosis, were apparent in primary hepatocyte monolayers as early as 24 hours after seeding (Fig. 4.2, page 138). Furthermore, the problem of cell heterogeneity could be overcome in these monolayers by changing the microscope from fluorescence to phase mode and analysing the morphology of Ho258-positive cells. Only a very small percentage of apoptotic cells were identified in the monolayer at any one time point (<1%), reflecting the short half life of the apoptotic cells. It is not unreasonable to assume that the small amounts of apoptosis detected here are likely to account for a substantial proportion of the cell death seen in spontaneously degenerating primary hepatocyte cultures. It has been calculated from results obtained *in vivo*, that the histologically visible phase of apoptosis in normal rat liver lasts for approximately 3 hours (Bursch *et al.*, 1990). On the basis of this calculation it has been estimated that as few as 3% of cells exhibiting a morphology typical of apoptosis at any one time could account for an overall loss of 25% of liver in only a few days. Indeed, even during the rapid liver regression that follows withdrawal of tumour promoters *in vivo*, only 1-2% of hepatocytes exhibit chromatin condensation patterns typical of apoptosis at any one time (Bursch *et al.*, 1984; Bursch *et al.*, 1986).

Unfortunately the presence of apoptotic hepatocytes in degenerating monolayers, as defined by nuclear morphology, could not be confirmed by DNA analysis. CAGE of DNA from cells that detached from degenerating hepatocyte monolayers yielded a smear indicative of non-specific DNA degradation. This is not surprising considering the degraded appearance of the cells. When the DNA from hepatocyte monolayers known to

contain apoptotic cells was analysed, only high molecular weight DNA was detected. The apparent absence of internucleosomal DNA fragmentation in primary hepatocytes may be the result of the small percentage of apoptotic cells present. Alternatively it is possible that primary hepatocytes do not possess the endonuclease responsible for internucleosomal DNA fragmentation (Oberhammer *et al.*, 1993b). It has been proposed that a more universal form of DNA fragmentation during apoptosis is the cleavage of DNA into high molecular weight (50-300 kb) DNA fragments (Walker *et al.*, 1991; Oberhammer *et al.*, 1993b). In this chapter, FIGE was used in order to examine the integrity of primary hepatocyte DNA. However, the results obtained were not conclusive. DNA from both viable and degenerating hepatocyte monolayers yielded only a smear indicative of non-specific DNA degradation. Since viable hepatocytes also yielded a smear, it is likely that the method used to prepare the DNA samples for FIGE was responsible for this non-specific fragmentation. A less abrasive method of sample preparation would be necessary in order to fully establish the nature of DNA fragmentation in apoptotic primary hepatocytes.

4.4.2 Spontaneous Apoptosis in Fao Hepatoma Cultures Approaching Confluence.

In addition to expressing markers of differentiated liver function and maintaining a response to peroxisome proliferators, the Fao cell line has also retained the ability to undergo the active process of apoptosis. Results presented in this chapter demonstrate that as Fao cells approach confluence, cell death occurs by a process with the morphological and biochemical characteristics of apoptosis (Fig. 4.3, page 140). It is interesting to note that in contrast to the primary hepatocytes, cells with chromatin condensation and internucleosomal DNA fragmentation patterns typical of apoptosis were only seen following their detachment from the monolayer (Fig. 4.4, page 141). This phenomenon was first reported in JB1 hepatoma cells (Evans *et al.*, 1991) and has since been demonstrated in lung, colon, prostate and mammary carcinoma cells (Oberhammer *et al.*, 1993b). The detachment of apoptotic cells from the monolayer *in vitro* mirrors the loss of contact with neighbouring viable cells reported for epithelial cells undergoing apoptosis *in vivo* (Wyllie *et al.*, 1980). However, in primary hepatocyte cultures, cells with a morphology typical of apoptosis did not detach, but remained in the monolayer.

Interestingly apoptotic hepatocytes were often seen on a higher plane than viable cells, indicating that loss of cell-cell contacts may have occurred but that cells were retained in the monolayer by cell-matrix contacts. This suggests that primary hepatocytes lay down a more extensive extracellular matrix than hepatoma cells. The major basement membrane proteins of normal rat liver are type IV collagen, fibronectin and laminin (Bissell *et al.*, 1986). The extent of hepatocyte-matrix interactions is demonstrated by the finding that hepatocytes cannot be removed intact from a monolayer using conventional enzymatic methods such as trypsin, collagenase, dispase or pronase (Neil James, Zeneca CTL, personal communication). It is interesting to speculate that Fao cells cultured on matrix proteins similar to those deposited by primary hepatocytes may not detach so readily from the monolayer as they do from plastic. Alternatively, the weaker cell-matrix contacts may reflect the transformed nature of the hepatoma cells.

In vivo, apoptotic cells are rapidly recognised and phagocytosed by macrophages or neighbouring cells (Wyllie *et al.*, 1980). However, *in vitro,* in the absence of phagocytic cells, apoptotic cells undergo secondary degeneration processes, resulting ultimately in loss of plasma membrane integrity. Thus detached Fao cells, collected over a time period exceeding 2 hours no longer exhibited chromatin condensation or DNA fragmentation patterns typical of apoptosis. Indeed, samples of Fao cells collected over longer time intervals contained a higher proportion of cell ghosts and the DNA from these cells exhibited a smear, indicative of non-specific degradation, on agarose gels.

4.4.3 Effect of Peroxisome Proliferators on Spontaneous Hepatocyte and Hepatoma Cell Death.

In this chapter, the effect of two peroxisome proliferators, nafenopin and Wy-14,643, on hepatocyte and Fao apoptosis was investigated. Nafenopin and Wy-14,643 are hypolipidaemic agents and both are potent rodent liver carcinogens. The use of these two agents gives a broader picture of the effects of peroxisome proliferators on apoptosis.

4.4.3.1 Suppression of Spontaneous Hepatocyte Apoptosis by Peroxisome Proliferators.

Results presented in this chapter demonstrate that primary rat hepatocyte viability can be maintained reversibly by the continual presence in the growth medium of either nafenopin (Fig. 4.5, page 143) or Wy-14,643 (Fig. 4.6, page 144). Hepatocyte viability was maintained by nafenopin for at least 6 weeks and by Wy-14,643 for at least 2 weeks, a difference that reflects merely the different experimental time course used for the two peroxisome proliferators. The morphology of hepatocytes maintained in nafenopin and Wy-14,643 was the same as that of freshly seeded hepatocytes, with one exception: hepatocytes maintained in medium containing either peroxisome proliferator accumulated lipid droplets, demonstrated using Texas Red staining (Ruth Roberts, personal communication). This lipid accumulation is likely to reflect the major effects of peroxisome proliferators on lipid metabolism. However, the relevance, if any, of lipid accumulation to maintenance of hepatocyte viability by peroxisome proliferators is unknown. It is important to note that loss of hepatocyte viability after the times quoted was not due to spontaneous degeneration of the hepatocytes themselves but was brought about deliberately by withdrawal of the peroxisome proliferator or in order to end the experiment. Indeed, hepatocyte cultures have actually been maintained in nafenopin for as long as 18 months (Cliff Elcombe, Zeneca CTL, personal communication). This prolonged maintenance of viability of cells that would normally die is fascinating and must have some relevance to the hepatocarcinogenicity of the peroxisome proliferators.

In the absence of a peroxisome proliferator, primary hepatocyte monolayers degenerated within around 8 days of their establishment. Analysis of these degenerating monolayers with Ho258 revealed the presence of cells with chromatin condensation and nuclear fragmentation patterns typical of apoptosis (Fig. 4.2, page 138). These apoptotic cells were only present in small numbers in spontaneously degenerating hepatocyte monolayers (<1%), reflecting the short half life of apoptotic cells (Fig. 4.7, page 146). However despite this, nafenopin-treated hepatocyte monolayers consistently exhibited significantly fewer apoptotic cells at any one time than DMF-treated solvent controls ($p < 0.05$). In one preliminary experiment, fewer apoptotic cells were also detected in hepatocyte monolayers treated with Wy-14,643 compared to solvent-treated controls

(Fig. 4.9, page 148). Since this experiment was only performed once, it is not possible to determine whether the difference between the number of apoptotic cells detected in solvent-treated controls and Wy-14,643-treated monolayers is statistically significant. However, since the result obtained using Wy-14,643 was similar to that demonstrated with nafenopin, it is probable that further repetitions of this experiment would yield a similar result.

The presence of nafenopin and Wy-14,643 did not completely prevent apoptosis in hepatocyte monolayers. Although the viability of hepatocyte monolayers was maintained by treatment with either of the peroxisome proliferators, in both cases some degeneration was seen, usually in areas of the monolayer where cells were not confluent after seeding. This cell degeneration is likely to account for the small numbers of apoptotic cells detected in hepatocyte monolayers treated with peroxisome proliferators. The preferential maintenance of viability of hepatocytes in a confluent monolayer is interesting and suggests that the presence of a peroxisome proliferator may not be the sole factor responsible for maintenance of hepatocyte viability. It is likely that additional determinants such as maintenance of cell-cell contacts and functional cell-surface receptors are also important for maintenance of an intact and viable monolayer. It has been demonstrated elsewhere that loss of cell-cell contacts (Bates *et al.*, 1994) and cell-matrix contacts (Meredith *et al.*, 1993; Frisch and Francis, 1994) is a trigger for apoptosis in some epithelial cell systems. The importance of cell-cell contacts in the maintenance of hepatocyte viability by peroxisome proliferators is highlighted by the observation that hepatocyte monolayers containing cells at a lower density than used routinely in this project were not maintained as satisfactorily as those which were fully confluent at the time of drug addition. It is likely that cells at a sub-optimal density, with fewer cell contacts are under more stress than confluent cells and as such, they may well lose differentiated liver function sooner after isolation than confluent cells. For example, a loss of markers of differentiated function, such as PPAR could prevent the low-density hepatocytes from responding to peroxisome proliferators as rapidly as confluent cells.

Despite the partial loss of hepatocyte viability detected in the presence of the two peroxisome proliferators, results presented here suggest that maintenance of hepatocyte viability by nafenopin (and potentially Wy-14,643) *in vitro* is the result of suppression of

hepatocyte apoptosis. This proposal is supported further by the observation that withdrawal of nafenopin from primary hepatocyte cultures, maintained by this peroxisome proliferator for 6 weeks, led to the degeneration of the cultures (Fig. 4.5c, page 143). Moreover, the degeneration that followed nafenopin withdrawal was associated with an increase in the number of apoptotic cells detected (Fig. 4.8, page 147). The number of apoptotic cells detected in these long-term hepatocyte monolayers was lower than seen in hepatocytes that spontaneously degenerated after their isolation. As a result, the difference in number of apoptotic cells detected in cultures from which nafenopin had been withdrawn compared to cultures maintained in nafenopin was only statistically significant at one time point measured. It is important to note that hepatocytes maintained in long-term culture by nafenopin existed as an island of viable cells in the middle of the culture flask. Monitoring the degeneration of such an island of cells was often quite difficult considering the reduced number of cells present. However, it would appear from the results obtained that degeneration of hepatocyte monolayers following nafenopin withdrawal was occurring via apoptosis.

4.4.3.2 Effect of Nafenopin on Spontaneous Fao Apoptosis.

In contrast to the hepatocyte system, spontaneous Fao apoptosis was not inhibited or retarded by the presence of the peroxisome proliferator nafenopin. Nafenopin did not prevent either the detachment of Fao cells from the monolayer as the cultures approached confluence or the confluent density of the cells. From this result it would appear that transformed hepatoma cells do not mirror the response of primary cells to peroxisome proliferators. However, Fao cells were shown in Chapter 3 to respond to the peroxisome proliferator nafenopin, both by induction of cytochrome P4504A1 and by enhanced mitogenesis. There is thus no reason to assume that the survival response is absent from these cells. An alternative explanation is that the protective effect of peroxisome proliferators can only be invoked under certain circumstances. Although the apoptosis detected in both primary hepatocyte and Fao cultures was spontaneous, the stimulus for cell death in the two systems was quite different. Primary hepatocyte death occurred as a result of an absence of certain factor(s) required by the cells for survival. Thus, suppression of hepatocyte cell death could be

mediated by peroxisome proliferators without any accompanying transformation of the cells. In contrast Fao cell death occurred as the cells approached confluence. In order for nafenopin to maintain the viability of Fao cells, it would have to alter the normal behaviour of the cells considerably, forcing them to grow on top of one another. Such behaviour is typical of highly transformed cells and if it occurred would suggest that nafenopin has a strong transforming activity. This would not conform with the idea of peroxisome proliferators as tumour promoters. In order to examine further the nature of the survival advantage afforded by peroxisome proliferators, the effect of nafenopin on chemically-induced Fao apoptosis was examined.

4.4.4 Effect of Nafenopin on Apoptosis Induced by DNA-Damaging Agents.

The two agents used to investigate the ability of nafenopin to protect liver cells from chemically-induced apoptosis were etoposide and hydroxyurea. Etoposide is an inhibitor of the mammalian enzyme topoisomerase II, which is involved in the regulation of chromatin configuration (Pommier and Kohn, 1989). Topoisomerase II induces the cleavage and resealing of double stranded DNA to allow the passage of a second DNA strand. Etoposide and other similar topoisomerase II inhibitors act by preventing the resealing of DNA, inducing the formation of protein-associated DNA double strand breaks (Glisson and Ross, 1987). Hydroxyurea is an inhibitor of ribonucleotide reductase, which induces the formation of single strand breaks in DNA by preventing the synthesis of DNA bases (Skog *et al.*, 1987).

Both etoposide and hydroxyurea induced apoptosis rapidly in Fao hepatoma cells (Fig. 4.10 and 4.11, pages 150-151). Once again, cell death was associated with detachment of Fao cells from the monolayer and it was these detached cells that exhibited chromatin condensation and non-random DNA fragmentation patterns typical of apoptosis . There was no evidence of primary or secondary necrosis as defined by the morphology of detached cells and the low percentage of cells with membranes permeable to TB. Treatment of Fao cells with etoposide (10 μM) and hydroxyurea (1 mM) led to the detachment and death of over 60% of the cells within 48 hours. However the onset of cell death induced by these DNA damaging agents was not inhibited or retarded by co-addition of nafenopin (Fig. 4.12, page 153).

The inability of nafenopin to suppress either spontaneous or chemically-induced Fao apoptosis raises further questions as to the nature of the survival signal afforded by peroxisome proliferators. A possible explanation for this finding is that the extent of cellular damage following exposure to genotoxic agents such as etoposide and hydroxyurea is too extreme to be prevented by nafenopin. Alternatively it may be that peroxisome proliferators such as nafenopin interfere with specific physiological cell death signals with the result that only certain forms of cellular damage can be prevented. It is also fair to question whether transformed hepatoma cells such as Fao have retained a survival response at all to peroxisome proliferators.

4.4.5 Differences in Expression of Bcl-2 in Primary Hepatocytes and Fao Hepatoma Cells.

The *bcl-2* oncogene and its product have been implicated in the suppression of apoptosis in a number of cell systems (Vaux *et al.*, 1988; Mah *et al.*, 1993; Deng and Podack, 1993; see page 70). This led to speculation that maintenance of primary hepatocyte viability by peroxisome proliferators is mediated by *bcl-2*. Western blot analysis of primary hepatocytes revealed that freshly isolated primary hepatocytes do not express detectable levels of Bcl-2 protein and that no induction of Bcl-2 expression occurs in response to the peroxisome proliferator nafenopin (Fig. 4.13b, page 154). However, low Bcl-2 expression was detected in hepatocyte monolayers 4 days after seeding. It is possible that this increase in Bcl-2 expression was the result of a proliferation of fibroblasts expressing Bcl-2 in the cultures rather than an up-regulation of Bcl-2 by the hepatocytes themselves. Immunocytochemical analysis of degenerating hepatocyte monolayers with an antibody to Bcl-2 would verify this hypothesis. Interestingly, Fao cells differ from primary hepatocytes in that they do express Bcl-2 protein (Fig. 4.13a, page 154), suggesting that up-regulation of Bcl-2 may occur during hepatocyte transformation. However, as with the primary hepatocytes, the presence of nafenopin did not affect the level of Bcl-2 expression in Fao cells. This result is perhaps not surprising. De-regulated expression of *bcl-2* has been shown to protect cells from apoptosis induced by DNA damaging agents including etoposide (Collins *et al.*, 1992; Dole *et al.*, 1994). However, results presented in this chapter demonstrate that

peroxisome proliferators do not protect cells from apoptosis induced by the DNA damaging agents etoposide and hydroxyurea.

Results presented here suggest that the effects of the peroxisome proliferators are not mediated by transcriptional up-regulation of Bcl-2 itself. However, it would be interesting to see whether peroxisome proliferators alter the level of expression of Bcl-2 binding partners or other members of the Bcl-2 family such as Bax and $Bclx_l$, which are implicated in the co-regulation of *bcl-2* and in suppression of apoptosis (Oltvai *et al.*, 1993; Boise *et al.*, 1993).

4.4.6 Summary.

This chapter demonstrates the ability of two peroxisome proliferators, nafenopin and Wy-14,643 to suppress spontaneous hepatocyte apoptosis. Since these two peroxisome proliferators can suppress apoptosis it is possible that this ability is a feature of all peroxisome proliferators.

In contrast to the results from the primary hepatocyte system, the peroxisome proliferator nafenopin did not protect Fao hepatoma cells from either the spontaneous apoptosis of confluent cells or from chemically-induced apoptosis. Since Fao cells have been shown to respond to nafenopin by up-regulation of cytochrome P4504A1 and by enhanced mitogenesis, it is unlikely that the lack of suppression of apoptosis is due to an inability of Fao cells to respond to peroxisome proliferators. A more likely explanation is that peroxisome proliferators can only protect cells from apoptosis induced by certain 'physiological' inducers such as growth factor withdrawal. More extreme methods of inducing cell death, while producing the same endpoint, may act through different signalling pathways which are not affected by peroxisome proliferators. A better understanding of the risk posed by the ability of peroxisome proliferators to suppress apoptosis requires knowledge of the signals involved in hepatocyte cell death *in vivo*. It has been proposed that hepatocyte apoptosis *in vivo* is mediated by the negative growth regulator $TGF\beta_1$. The effects of peroxisome proliferators on $TGF\beta_1$-induced cell death are discussed in Chapter 5.

Chapter 5.

Effect of Peroxisome Proliferators on TGFβ_1-Induced Liver Cell Death.

5.1 Introduction.

5.1.1 Signals for Hepatocyte Cell Death and Survival.

A number of different growth modulators have been implicated in the control of hepatocyte proliferation, death and survival. These fall into three categories: 1] complete hepatocyte mitogens, such as EGF, transforming growth factor α (TGFα) and hepatocyte growth factor (HGF), which are categorised by their ability to stimulate DNA synthesis and mitosis in quiescent hepatocyte populations; 2] co-mitogens, such as insulin, norepinephrine and vasopressin, which positively control hepatocyte proliferation, but do so indirectly; 3] growth inhibitors, such as TGFβ and IL1-β, which can inhibit mitogenesis and / or induce apoptosis. It is likely that liver homeostasis is controlled by the balance and interaction of these different growth factors. For example, TGFβ inhibits adult hepatocyte mitogenesis induced by EGF and HGF (Carr *et al.*, 1986). Similarly, norepinephrine enhances the mitogenic effects of EGF and reduces the mito-inhibitory effects of TGFβ (Cruise *et al.*, 1986; Houck *et al.*, 1988).

Changes in expression of EGF, HGF, TGFα and TGFβ have been implicated in the control of liver regeneration (Michalopoulos, 1990). Furthermore, the tumour promoter, phenobarbital alters the pathway of EGF and $TGF\beta_1$ signalling in normal hepatocytes (Jirtle and Meyer, 1991). It is possible therefore, that peroxisome proliferators also exert their effects on hepatocyte mitogenesis and tumour promotion via changes in the expression or response of hepatocytes to some or all of these growth factors. The focus of this chapter is the negative liver growth regulator, $TGF\beta_1$. Since $TGF\beta_1$ has been implicated in the control of hepatocyte apoptosis, the ability of peroxisome proliferators to suppress spontaneous hepatocyte apoptosis may be mediated via effects on $TGF\beta_1$ signalling.

5.1.2 TGFβ.

TGFβ belongs to a superfamily of cytokines that includes activins, inhibins and bone morphometric proteins (Barnard *et al.*, 1990). Three distinct molecular forms of TGFβ have been identified in mammals and have been named $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$ (Ohta *et al.*, 1987; Cheifetz *et al.*, 1990). Of these, $TGF\beta_1$ is the most abundant in mammalian cells and is the main focus of this chapter.

All three isoforms of TGFβ are synthesised initially as large, inactive precursor proteins which are then processed to yield the biologically active protein (Jirtle *et al.*, 1991). Following synthesis, $TGF\beta_1$ is cleaved proteolytically to yield pro- and mature forms of the molecule (Sha *et al.*, 1989). The pro-region of $TGF\beta_1$ is glycosylated and phosphorylated, the latter occurring on two mannose side chains, forming mannose-6-phosphate (Purchio *et al.*, 1988). In this form, the pro-region binds non-covalently to mature $TGF\beta_1$, forming a latent $TGF\beta_1$ complex which is secreted by cells (Lyons *et al.*, 1990). In order for mature $TGF\beta_1$ to regain its biological activity, the pro-region must be removed. Such activation of $TGF\beta_1$ can be induced by a number of conditions including heat, pH changes, urea and the action of proteases such as plasminin (Lyons *et al.*, 1990; Massague, 1990). The pro-region of $TGF\beta_1$ itself is believed to play a role in $TGF\beta_1$ activation, by binding to the mannose-6-phosphate receptor (M-6-PR / IGFIIR; Dennis and Rifkin, 1991). It has been proposed that M-6-PR mediates both the transportation of latent $TGF\beta_1$ into cells and the subsequent activation of the mature protein by directing the latent complex to acidic intracellular endosomal compartments where the pro-region can be removed (Jirtle *et al.*, 1991; Dennis and Rifkin, 1991).

5.1.2.1 TGFβ Receptors.

A number of putative receptors for TGFβ have been identified (reviewed in Massague *et al.*, 1990; Yin and Lodish, 1993). The most widely distributed of these are the type I and type II TGFβ receptors (TβR-I and TβR-II), which are transmembrane serine/threonine kinases (Massague *et al.*, 1990; Kingsley, 1994). Both of these receptors are required in order for a cell to initiate a signalling response to TGFβ. It has been demonstrated recently, that the TβR-II receptor is a constitutively active kinase to which TGFβ binds directly (Wrana *et al.*, 1994). While TβR-I does not recognise free TGFβ, it can recognise TGFβ bound to TβR-II. It remains to be established whether recognition of the TβR-II/TGFβ complex by TβR-I occurs as a result of a conformational change in TGFβ or whether TβR-I recognises a TβR-II receptor/TGFβ protein interface. However, recruitment of TβR-I into the TβR-II /TGFβ complex results in phosphorylation of TβR-I by TβR-II. This phosphorylation is essential for the subsequent propagation of

downstream signalling events and represents the first step of the TGFβ signalling cascade (Wrana *et al.*, 1994).

5.1.2.2 Biological Effects of TGFβ.

The effects of TGFβ *in vivo* and *in vitro* are extremely diverse. Although first identified by its ability to stimulate the proliferation of fibroblasts in soft agar (Assoian *et al.*, 1983), TGFβ is now recognised to be a potent growth inhibitor for the majority of cell types (reviewed in Barnard *et al.*, 1990). In addition to inhibition of proliferation, roles for TGFβ have also been demonstrated in the production of extracellular matrix and bone (Ignotz and Massague, 1986; Barnard *et al.*, 1990), cell differentiation (Masui *et al.*, 1986; Torti *et al.*, 1989), wound healing (Mustoe *et al.*, 1987) and modulation of immune responses (Kehrl *et al.*, 1986). $TGF\beta_1$ has also been implicated in the induction of apoptosis. Expression of $TGF\beta_1$ mRNA has been detected during the regression of the prostate following castration (Kyprianou *et al.*, 1990, Tenniswood *et al.*, 1992), involution of the mammary gland after weaning (Strange *et al.*, 1992) and during regression of MCF-7 breast cancer in ovariectomized animals *in vivo* (Kyprianou *et al.*, 1991). Similarly, levels of $TGF\beta_1$ mRNA increase in MCF-7 cells exposed to anti-oestrogens *in vitro* (Butta *et al.*, 1991). Direct induction of apoptosis by $TGF\beta_1$ has also been demonstrated in a variety of epithelial cell lines *in vitro* including cultured uterine epithelial cells (Rotello *et al.*, 1991) and gastric carcinoma cells (Yanagihara and Tsumuraya, 1992).

5.1.2.3 Effects of $TGF\beta_1$ in the Liver.

It has been proposed that $TGF\beta_1$ may function as an autocrine or paracrine inhibitor of regenerative liver hyperplasia, with the role of preventing uncontrolled liver growth (Russell *et al.*, 1988). $TGF\beta_1$ inhibits the regenerative hepatocyte DNA synthesis that follows a partial hepatectomy *in vivo* (Russell *et al.*, 1988). In addition, $TGF\beta_1$ inhibits DNA synthesis *in vitro* in hepatocytes from normal and regenerating liver (Strain *et al.*, 1987) and can suppress EGF-induced hepatocyte mitogenesis (Carr *et al.*, 1986). A role for $TGF\beta_1$ in preventing liver hyperplasia is supported further by the finding that $TGF\beta_1$ mRNA and protein accumulates transiently in hepatocytes following

a partial hepatectomy (Braun *et al.*, 1988; Jirtle *et al.*, 1991) or CCl_4 treatment (Armendariz-Borunda *et al.*, 1993). The increase in TGFβ_1 expression following partial hepatectomy is accompanied by an elevation in expression of the M-6-PR gene (Jirtle *et al.*, 1991). This concomitant increase in TGFβ_1 and M-6-PR supports the theory that M-6-PR is involved in the recruitment and subsequent activation of TGFβ_1 by hepatocytes (Jirtle *et al.*, 1991).

Suppression of hepatocyte DNA synthesis by TGFβ_1 is associated with the induction of apoptosis. It has been demonstrated *in vitro* that, at concentrations sufficient to inhibit DNA synthesis, TGFβ_1 also induces hepatocyte apoptosis (Oberhammer *et al.*, 1991; Bursch *et al.*, 1993). The ability of TGFβ_1 to induce liver cell apoptosis *in vitro* has also been shown using Hep3B and McA-RH7777 hepatoma cells (Lin and Chou, 1992; Fukuda *et al.*, 1993). *In vivo*, TGFβ_1 significantly augments the onset of hepatocyte apoptosis induced by withdrawal of the hepatic mitogen cyproterone acetate (Oberhammer *et al.*, 1992). Moreover, cytoplasmic latent TGFβ_1 protein is up-regulated in apoptotic hepatocytes following withdrawal of cyproterone acetate from the hyperplastic liver (Bursch *et al.*, 1993). Although it is the mature form of TGFβ_1 that induces apoptosis, immunostaining for the latent form of the protein is much more pronounced in apoptotic hepatocytes (Oberhammer *et al.*, 1991). It has been proposed that latent TGFβ_1 is either produced in hepatocytes primed to die by apoptosis or is taken up by hepatocytes from non-parenchymal cells (Bursch *et al.*, 1993). It is likely that although activation of mature TGFβ_1 induces the death of the cells, it is degraded rapidly so that few cells expressing mature TGFβ_1 can be detected (Bursch *et al.*, 1993). It is not yet apparent whether up-regulation of TGFβ_1 in apoptotic hepatocytes is controlled at the pre- or post-translational level. However, these data certainly support the theory that TGFβ_1 plays a significant role in the induction of apoptosis in the liver.

5.1.2.4 TGFβ_1 and Hepatocarcinogenesis.

A possible mechanism for the uncontrolled growth of tumours is that tumour cells lose the normal response to growth inhibitors during transformation (Goustin *et al.*, 1986). A loss of sensitivity of preneoplastic hepatocytes to TGFβ_1 could therefore explain the reduction in apoptosis detected in liver foci exposed to tumour promoters

(Schulte-Hermann *et al.,* 1990). This theory is supported by the finding that transformed hepatocytes are resistant to the growth inhibitory effects of TGFβ_1. Rat liver epithelial cells transformed by either AFB1 or transfection with the oncogene *Ha-ras*, and initiated hepatocytes from the livers of diethylnitrosamine-treated rats are all resistant to the growth inhibitory effects of TGFβ_1 (McMahon *et al.*, 1986; Houck *et al.*, 1989; Stenius, 1993). However, a number of transformed hepatoma cell lines can be induced to enter apoptosis by treatment with TGFβ_1 *in vitro* (Lin and Chou, 1992; Fukuda *et al.*, 1993). A possible explanation for these conflicting observations is that the response of hepatoma cells to TGFβ_1 *in vitro* is affected by the different micro-environment of the cell. The composition of growth media and the presence or absence of growth factors in the serum could all affect the response of a cell to TGFβ_1 *in vitro*.

The role of TGFβ_1 during tumour promotion has been investigated quite extensively using the liver tumour promoter, phenobarbital (Jirtle and Meyer, 1991). It is proposed that the clonal expansion of preneoplastic cells during tumour promotion involves a reduced sensitivity of preneoplastic cells and an increased sensitivity of normal cells to the effects of growth inhibitors (discussed in Chapter 4, page 129). The ability of phenobarbital to inhibit the proliferation of normal hepatocytes is believed to be mediated by changes in sensitivity of the cells to TGFβ_1 (Jirtle and Meyer, 1991). In normal liver, hepatocytes express only low levels of TGFβ_1, whereas the non-parenchymal and endothelial cells express high levels of the protein (Carr *et al.*, 1989). In contrast, following chronic exposure to phenobarbital, periportal hepatocytes stain intensely with antibodies to both the latent and the mature form of TGFβ_1 (Jirtle and Meyer, 1991). Thus it appears that administration of phenobarbital enhances either the production of TGFβ_1 by hepatocytes or the rate of uptake of TGFβ_1 by hepatocytes from non-parenchymal cells (Jirtle and Meyer, 1991). This increase in TGFβ_1 latent protein expression is proposed to contribute to phenobarbital-induced suppression of normal hepatocyte proliferation (Jirtle and Meyer, 1991). Indeed, TGFβ_1 has been shown to be several times more effective at inhibiting the proliferation of normal hepatocytes from phenobarbital-treated rats than untreated controls (Jirtle and Meyer, 1991). In contrast, putative preneoplastic hepatocytes in phenobarbital-treated rats have been shown to express lower levels of TGFβ_1 than normal hepatocytes (Jirtle and Meyer, 1991). Similar

studies have not been carried out extensively using peroxisome proliferators. However, it is likely that the difference in TGFβ_1 expression between normal and preneoplastic cells has a significant impact on the selective growth of preneoplastic foci.

5.1.3 Aims of Chapter.

The ability of the peroxisome proliferators nafenopin and Wy-14,643 to suppress spontaneous hepatocyte apoptosis was demonstrated in Chapter 4. The aim of this chapter is to investigate further how this suppression may be mediated. The physiological, negative growth regulator, TGFβ_1 has been implicated in the regulation of hepatocyte cell death (Bursch *et al.*, 1993). It is possible therefore that the spontaneous death of hepatocytes in primary culture is also mediated by TGFβ_1. The ability of peroxisome proliferators to interfere with TGFβ_1-induced apoptosis would thus provide a mechanism for maintenance of hepatocyte viability.

In this chapter, the ability of nafenopin and Wy-14,643 to suppress TGFβ_1-induced primary hepatocyte and Fao hepatoma cell death has been examined. This first required a thorough examination of the kinetics of TGFβ_1-induced apoptosis in both cell systems. In Chapter 4 it was found that peroxisome proliferators did not suppress spontaneous or chemically-induced Fao cell death. The effect of peroxisome proliferators on TGFβ_1-induced Fao cell death was examined to determine whether transformed liver cells maintain any survival response to peroxisome proliferators.

Hepatocytes may up-regulate TGFβ_1 latent protein via a number of mechanisms, including increased synthesis of TGFβ_1 mRNA or protein or increased uptake of the protein by M-6-PR from non-parenchymal cells. Expression of TGFβ_1 and M-6-PR mRNA has been examined in spontaneously degenerating hepatocyte monolayers. Furthermore, in order to examine the mechanism by which peroxisome proliferators may exert their protection against primary hepatocyte cell death, the effect of peroxisome proliferators on TGFβ_1 and M-6-PR mRNA expression has also been assessed.

5.2 Methods.

Throughout this chapter, culture of primary hepatocytes and Fao hepatoma cells, treatment of cells with nafenopin and Wy-14,643, cell counts and viability measurements were carried out as described in the general methods section (pages 77-87).

5.2.1 Determination of the Effect of TGFβ_1 on Primary Hepatocyte and Fao Hepatoma Cell Viability in the Presence and Absence of Peroxisome Proliferators.

Freshly isolated hepatocytes were seeded as described (page 78). Fao hepatoma cells were seeded at 3 x 10^5 cells / 25 cm^3 flask in Ham's F12, medium (4 ml) containing charcoal-stripped FCS (10%). After 24 hours, hepatocyte and Fao monolayers were washed with the appropriate serum-free medium and medium was replaced with either serum-free William's E medium or Ham's F12 medium containing BSA (0.2%). After a further 24 hours, medium was removed by aspiration and replaced with serum-free medium containing TGFβ_1 (1 ng/ml or 5 ng/ml from a 2 µg/ml stock in HCl (4 mM)). Control flasks were treated with HCl to the same final concentration (1.6 µM). Nafenopin (50 µM), Wy-14,643 (1-50 µM) or DMF alone (0.25% $^v/_v$) was added to primary hepatocyte and Fao cultures at the same time as TGFβ_1. The viability of primary hepatocytes was assessed by Ho258 staining 24 and 48 hours after TGFβ_1 addition. Fao cell viability was assessed by TB and AO staining and by CAGE over 3 days following TGFβ_1 addition.

In addition to assessment of the number of Fao cells remaining attached to the monolayer after TGFβ_1-treatment, the rate of cell detachment from the monolayer was also examined. Fao cells were seeded at 1 x 10^6 / 75 cm^3 flask in Ham's F12 medium (10 ml) containing charcoal-stripped FCS (10%). Cultures were serum-starved for 24 hours and treated with TGFβ_1 (1 ng/ml) and nafenopin (50 µM) or DMF alone (0.25% $^v/_v$) as described above. At defined 2 hour intervals over the following 30 hours, medium containing cells that had detached over the previous 2 hours was removed from the flasks and transferred to a universal tube. Cells were pelletted by centrifugation (200 x g, 5 min.), the supernatant was removed and replaced in the appropriate flasks which were

returned to the incubator. Cell number and viability were determined by TB and AO staining as described.

5.2.2 Northern Blot Analysis of Primary Hepatocytes.

5.2.2.1 Preparation of Reagents.

Solutions used at all stages of mRNA extraction and analysis were prepared in sterile diethylpyrocarbonate (DEPC)-treated ddH_2O. DEPC (500 µl) was added to ddH_20 (500 ml) and left to stand at room temperature for 24 hours. DEPC-treated ddH_2O was then sterilised by autoclaving. All plastics and glassware used for mRNA preparation and analysis were also sterilised before use by autoclaving. The composition of all buffers and solutions used during mRNA extraction and analysis are given in Appendix 2 (page 213).

5.2.2.2 Extraction of mRNA.

Primary rat hepatocytes were seeded and treated with nafenopin (50 µM) or DMF alone (0.25% $^{v}/_{v}$) as described (page 133). Total mRNA was extracted using RNAzol (Biogenesis) from freshly isolated and 1-day old hepatocytes, and from nafenopin- and DMF alone-treated hepatocyte monolayers 2 and 4 days after seeding. All stages of RNA extraction were performed on ice using the protocol supplied with RNAzol. Approximately $1x10^7$ cells were removed from hepatocyte monolayers by gentle pipetting in RNAzol (1.6 ml) and aliquots (800 µl) were transferred to an eppendorf. Chloroform (80 µl) was added to each tube and samples were incubated for 5 min. Aqueous and organic phases were then separated by centrifugation (15, 000 x g, 15 min., 4°C). The upper, aqueous phase, containing mRNA and DNA, was transferred to a fresh eppendorf and an equal volume of isopropanol (~ 500 µl) was added to each sample. Samples were incubated for at least 15 min. to remove DNA. The mRNA was pelletted by centrifugation (15 000 x g, 15 min., 4°C) and the supernatant removed by aspiration. Samples were then dehydrated by incubation for 15 min. with 70% and then 95% ethanol. Following each incubation, mRNA was pelletted by centrifugation (15 000 x g, 5 min., 4°C). Pellets were air-dried, resuspended in 5-10 µl DEPC-treated water and stored at -70°C until required.

5.2.2.3 Denaturing Gel Electrophoresis.

Prior to electrophoresis, RNA concentration was measured using a GeneQuant RNA/DNA calculator (Pharmacia). Electrophoresis was then conducted following the protocol in Sambrook *et al.* (1989). RNA samples (20 μg) were diluted in 4.5 μl DEPC-treated water and were then incubated with sample buffer (16 μl) at 65°C for 15 min. Samples were centrifuged briefly to remove condensation from the eppendorf lids and placed on ice. Loading buffer (2 μl) was added to each sample before loading into a 1% denaturing agarose gel flooded with northern running buffer. mRNA was subjected to electrophoresis at 15 V for 16 hours. Following electrophoresis, the gel was soaked for 2 hours in DEPC-treated water to remove formaldehyde. The mRNA was then visualised by UV illumination to ensure equal loading. The gel was photographed using Polaroid 667 film.

5.2.2.4 Northern Blotting.

The gel and nylon membrane (Hybond-N) of equivalent size were soaked in 2 x SSC for 5 min. A raised support was placed in a dish containing 20 x SSC and overlaid with a glass plate covered with 2 pieces of Whatmann filter paper (3 mm) large enough to dip into the SSC at either side. The filter paper acted as a wick, drawing SSC up onto the support. Once the filter paper was completely soaked in SSC, the gel was inverted onto the support and overlaid with the wet nylon membrane. The sides of the gel were surrounded with Nescofilm to avoid dehydration of the gel and further pieces of SSC-soaked and dry pieces of filter paper placed on top. These were followed by several dry pieces of blotting paper and another glass plate. A heavy weight was placed on top of the plate and the mRNA was left to transfer by capillary action onto the nylon membrane overnight. Following transfer, mRNA was cross-linked onto the nylon filter using a Spectrolinker XL 1500 (Spectronics Co.). Filters were wrapped in foil and stored at -20°C prior to hybridisation.

5.2.2.5 Radioactive Labelling of cDNA Probe.

TGFβ_1 and M-6-PR cDNA probes (gifts from Mark Ferguson, Manchester University and Pete Lord, Paterson Institute respectively) were labelled using Rediprime

$TGF\beta_1$ and M-6-PR cDNA probes (gifts from Mark Ferguson, Manchester University and Pete Lord, Paterson Institute respectively) were labelled using Rediprime (Amersham), a random primed labelling method. The cDNA (50 ng) was denatured by heating for 10 min. at 95°C. TE was added to make a final volume of 45 µl which was then added to the Rediprime labelling mixture and mixed thoroughly. ^{32}P-labelled dCTP (50 µCi) was added and the reaction left for 10 min. at 37°C. The reaction was then stopped by addition of EDTA (5 µl of a 0.2 M solution). Radiolabelled probe was separated from un-incorporated isotope by passing through a NucTrap sephadex G50 push-probe purification column (Stratagene) as follows: the column was equilibrated using TE (70 µl). Radiolabelled probe was then mixed with TE to a final volume of 70 µl and passed through the column. A further aliquot of TE (70 µl) was pushed through the column, followed by a final volume of air. The radiolabelled probe was collected at the base of the column in an eppendorf tube.

5.2.2.6 Prehybridisation and Hybridisation of Filter.

Hybridisation of the filter with the radiolabelled $TGF\beta_1$ or M-6-PR cDNA probe was carried out using Quickhyb hybridisation fluid (Stratagene). The filter was washed in DEPC-treated water and placed into a hybridisation tube. Quickhyb (5 ml at 65°C) was added and the tube placed in a hybridisation oven at 65°C for 15-30 min. Salmon sperm DNA (1 mg denatured at 95°C) was added to the probe, which was then mixed with Quickhyb (400 µl at 65°C). The probe and Quickhyb were added to the hybridisation tube which was returned to the hybridisation oven for 1 hour at 65°C.

Following hybridisation, the filter was washed twice in 2 x SSC (500 ml containing 0.1% SDS) for 15 min. at 45°C. The filter was monitored using a Geiger counter and if still hot, a third high stringency wash was performed using 0.1 x SSC (500 ml containing 0.1% SDS). The filter was then wrapped in a plastic bag and exposed to X-ray film for 24-48 hours at -70°C.

5.3 Results.

5.3.1 Effect of TGFβ_1 on Primary Hepatocyte Viability.

Treatment of primary hepatocyte cultures in serum-free medium with TGFβ_1 resulted in the rapid onset of hepatocyte degeneration and cell death. Within 48 hours of exposure of a confluent hepatocyte monolayer to TGFβ_1 (5 ng/ml), the majority of hepatocytes had degenerated and detached from the monolayer. Fluorescence microscopy of these TGFβ_1-treated hepatocyte monolayers, stained with Ho258, revealed a significant increase in the number of hepatocytes exhibiting condensed and fragmented nuclei typical of apoptosis compared to untreated controls. Following a 24-hour exposure of hepatocytes to TGFβ_1 (5 ng/ml) the number of apoptotic cells detected in hepatocyte monolayers was 5-6 times greater than in untreated controls (n=3; $p \leq 0.001$; Fig. 5.1). Withdrawal of serum from hepatocyte cultures and treatment with the TGFβ_1 solvent HCl alone (1.6 μM) had no significant effect on hepatocyte viability over the course of the experiment (Fig. 5.1).

Analysis of the DNA from control and TGFβ_1-treated primary hepatocyte monolayers by CAGE yielded only high molecular weight DNA (Fig. 5.2). No DNA 'ladder', indicative of internucleosomal DNA fragmentation, was apparent. FIGE of DNA from control and TGFβ_1-treated hepatocyte monolayers yielded a smear, indicative of non-specific DNA fragmentation (data not shown).

5.3.2 Effect of TGFβ_1 on Fao Hepatoma Cell Viability.

Treatment of serum-free Fao cultures with TGFβ_1 (1-5 ng/ml) led to the rapid induction of cell death, accompanied by detachment of cells from the monolayer (Fig. 5.3). For example, a 48 hour exposure of Fao cells to TGFβ_1 (5 ng/ml) resulted in the loss of 89% of cells from the monolayer (n=3, se=1.2). Following TGFβ_1 treatment, 70-80% of the Fao cells that detached from the monolayer collected at 2 hour intervals and stained with AO, exhibited chromatin condensation patterns typical of apoptosis (n=8, x=72, se=2.03; Fig. 5.4 a and b). If cells were collected at such regular intervals, there was no evidence of primary or secondary necrosis, since only 1-4% of detached cells allowed uptake of TB (n=8, x=2.4, se=0.34). Of the cells that remained attached to the monolayer after TGFβ_1 administration, there was no evidence of apoptosis determined

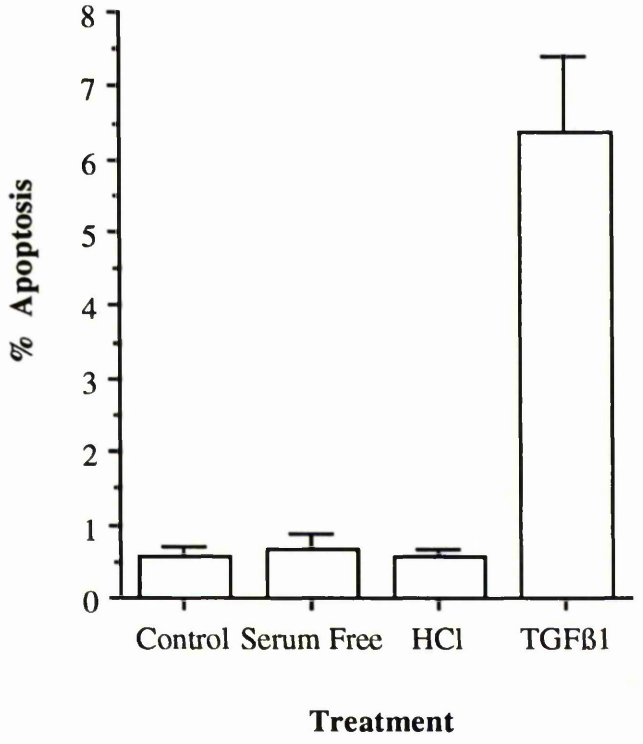


Figure 5.1. Percentage of cells with an apoptotic morphology detected by Ho258 staining of primary rat hepatocyte monolayers 24 hours after treatment with TGFβ_1 (5 ng/ml). Data points represent the mean and standard error of 3 independent experiments in which at least 1000 cells were counted per treatment.

Lanes:

1: Untreated viable cells

2: "

3: TGFβ_1-treated cells

4: "

5: Hae III size markers.

Figure 5.2. CAGE of DNA from TGFβ_1 (5 ng/ml) treated primary hepatocyte monolayers. Gel is representative of 3 independent experiments.

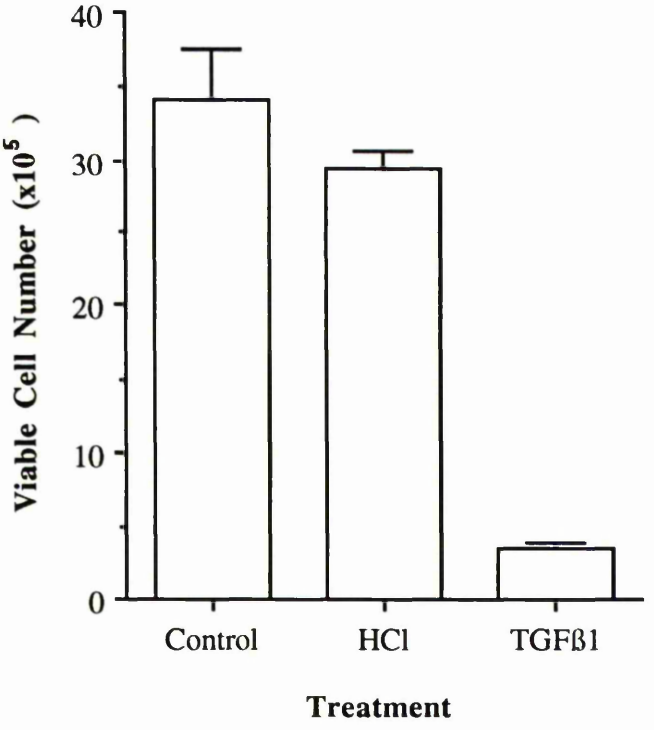


Figure 5.3. Effect of TGFβ_1 (5 ng/ml) on the viability of Fao Cells. Data points represent the mean and standard error of 3 independent experiments in which the number of viable cells remaining attached to the monolayer was counted 48 hours after TGFβ_1 treatment.

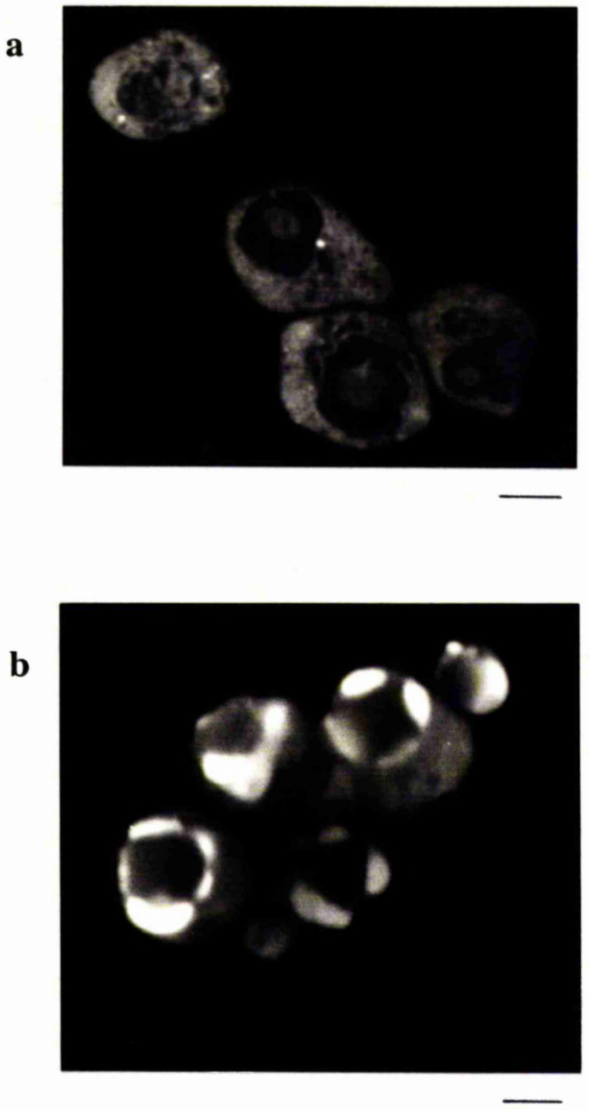


Figure 5.4. Fluorescent photograph demonstrating the morphology of **(a)** viable attached and **(b)** apoptotic detached Fao cells following treatment with TGFβ_1 (5 ng/ml). Apoptotic cells which detached from the monolayer were collected 2 hours after addition of fresh medium to the flask. Bar= 5μM. Photographs are representative of 3 independent experiments.

by AO staining and only 1-2% allowed uptake of TB (n=3, x=1.1, se=0.5). HCl alone (1.6 μM) had no effect on Fao cell viability (Fig. 5.3).

Following TGFβ$_1$ treatment, CAGE of DNA from detached Fao cells, collected at 2 hour intervals, revealed a 180-200 base pair 'ladder' of DNA fragments indicative of the internucleosomal DNA cleavage reported for apoptosis in many cell types (Fig. 5.5). In contrast viable monolayer cells yielded only high molecular weight DNA. Once again, FIGE of DNA from Fao cells yielded a smear indicative of non-specific DNA fragmentation (data not shown).

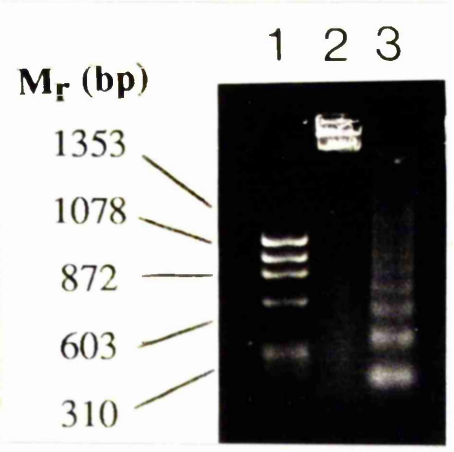
5.3.3 Effect of Peroxisome Proliferators on TGFβ$_1$-Induced Hepatocyte Apoptosis.

5.3.3.1 Nafenopin.

Co-addition of nafenopin (50 μM) to primary hepatocyte cultures treated with TGFβ$_1$ (5 ng/ml), significantly reduced the number of apoptotic hepatocytes detected compared with cultures treated with TGFβ$_1$ alone (Fig. 5.6). Following a 24 hour exposure to TGFβ$_1$, there was a significant 40% reduction in the number of apoptotic cells counted in those cultures treated with nafenopin compared with cultures treated with TGFβ$_1$ alone (n=3; p≤0.1). Co-addition of DMF alone (0.25% $^{v}/_{v}$) to TGFβ$_1$-treated hepatocyte cultures had no significant effect on the number of apoptotic cells detected (Fig. 5.6).

5.3.3.2 Wy-14,643.

Co-addition of Wy-14,643 (1, 10 and 50 μM) to primary hepatocyte monolayers treated with TGFβ$_1$ (5 ng / ml) also resulted in a decrease in the number of apoptotic cells detected compared to cultures treated with TGFβ$_1$ alone (Fig. 5.7). The decrease was more statistically significant in the presence of 1 and 10 μM Wy-14, 643 (n=3; p≤0.05) than in the presence of 50 μM Wy-14, 643 (n=3; p≤0.1). Once again treatment with DMF alone (0.25% $^{v}/_{v}$) had no significant effect on the viability of the cells (Fig. 5.7).



Lanes:

1: Hae III size marker

2: Viable attached cells

3: Apoptotic TGFβ_1-treated detached cells

Figure 5.5. CAGE of DNA from viable and apoptotic Fao cells 24 hours after treatment with TGFβ_1 (5 ng/ml). Gel is representative of 3 independent experiments.

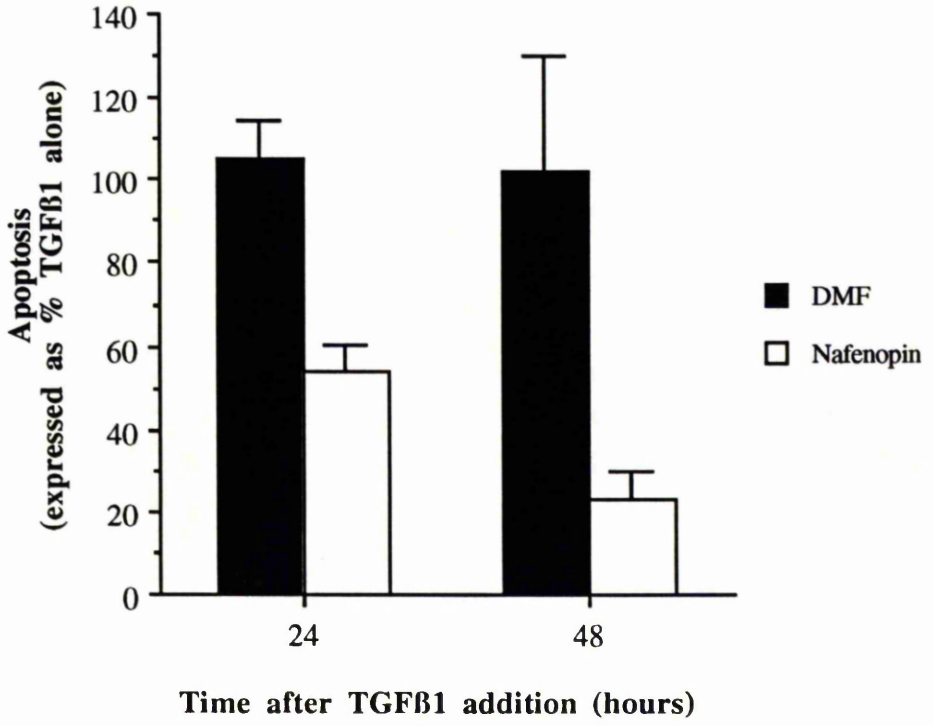


Figure 5.6. Percentage of apoptotic cells detected in primary rat hepatocyte monolayers treated with TGFβ_1 (5 ng/ml) in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). Data points are expressed as a percentage of TGFβ_1-treated controls and represent the mean and standard error of 3 independent experiments in which at least 800 cells were counted at 1 time-point per day.

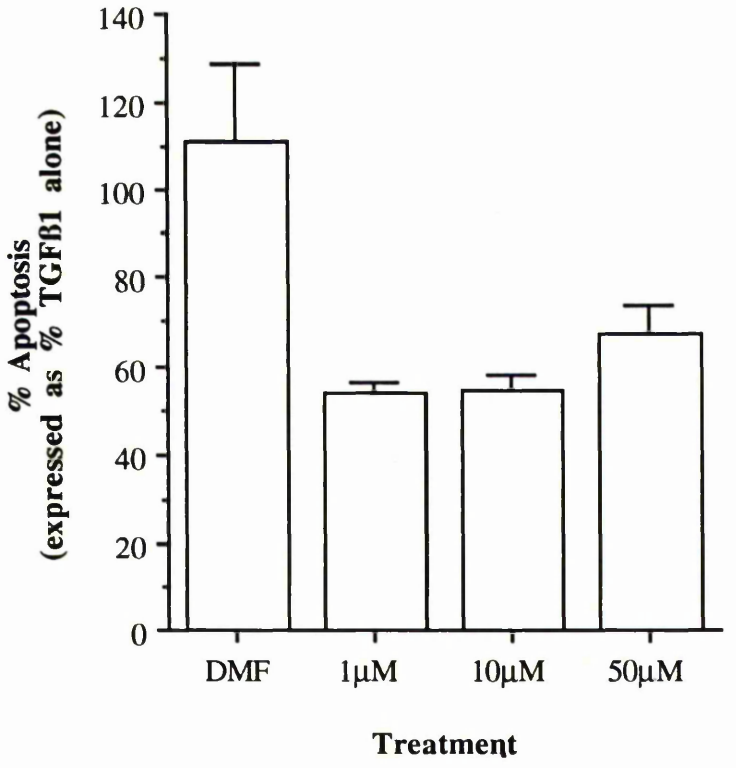


Figure 5.7. Percentage of apoptotic cells detected in primary rat hepatocyte monolayers treated with TGFβ_1 (5 ng/ml) in the presence of Wy-14,643 (1, 10 or 50 µM) or DMF alone (0.25% $^{v}/_{v}$). Data points are expressed as a percentage of TGFβ_1-treated controls and represent the mean and standard error of 3 independent experiments in which at least 800 cells were counted 24 hours after TGFβ_1 treatment.

5.3.4 Effect of Peroxisome Proliferators on TGFβ_1-Induced Fao Apoptosis.

5.3.4.1 Nafenopin.

The effect of nafenopin on TGFβ_1-induced Fao cell death mirrored that seen in primary hepatocytes. Co-addition of nafenopin (50 μM) to Fao cultures treated with TGFβ_1 (1-5 ng/ml) significantly increased the number of viable cells remaining attached to the monolayer above the number counted in cultures treated with TGFβ_1 alone ($p \leq 0.05$). Fig. 5.8a represents the effect of nafenopin on viable cell number in Fao cultures treated with TGFβ_1 (1 ng/ml, n=5). The increase in cell number seen in the presence of nafenopin represents a rescue of 10-20% of total cells from cell death by apoptosis. Co-addition of DMF alone (0.25% $^{v}/_{v}$) to TGFβ_1-treated cultures had no significant effect on the viability of Fao cells (Fig. 5.8a). The increase in viable cell number remaining attached to TGFβ_1-treated Fao monolayers in the presence of nafenopin was found to be accompanied by a significant decrease in the number of cells detaching from the monolayer (n=3; $p \leq 0.05$; Fig. 5.8b). Again, DMF alone (0.25% $^{v}/_{v}$) had no significant effect on the rate of cell detachment from the monolayer (Fig. 5.8b).

5.3.4.2 Wy-14, 643.

An increase in the number of viable Fao hepatoma cells remaining attached to the monolayer after treatment with TGFβ_1 (1 ng/ml) was also detected in the presence of Wy-14,643 (n=3; Fig. 5.9). The increase in cell number was most apparent 48 hours after TGFβ_1 treatment with Wy-14,643 concentrations of 1μM and 10μM, although the extent of the increase in viable cell number was not as great as that seen with nafenopin (50 μM). Co-addition of 50 μM Wy-14,643 actually reduced the number of viable cells counted compared with cultures treated with TGFβ_1 alone (Fig. 5.9). Once again DMF alone had no effect on TGFβ_1-induced Fao cell death (Fig. 5.9).

5.3.5 Expression of TGFβ_1 mRNA by Primary Hepatocytes.

Northern blot analysis revealed that TGFβ_1 mRNA is expressed in freshly isolated primary hepatocytes and in primary hepatocytes in culture (Fig. 5.10a). Little change in TGFβ_1 mRNA expression was detected in primary hepatocytes over 4 days following their isolation although a slight increase in TGFβ_1 expression was detected in

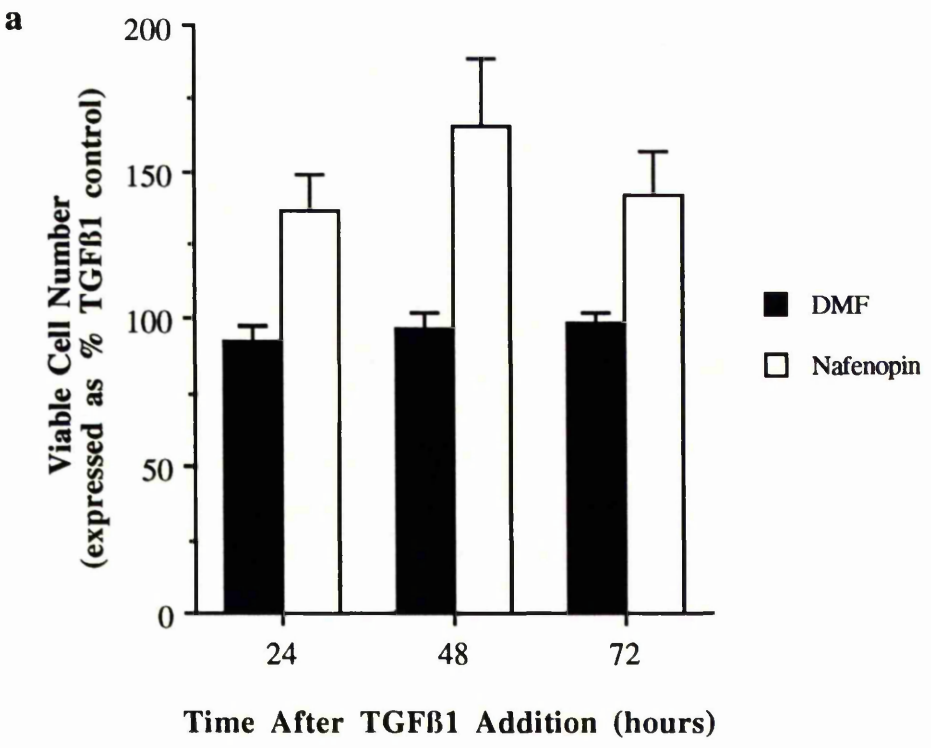


Figure 5.8. (a) Effect of nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) on Fao cell viability in the presence of TGFβ_1 (1 ng/ml). Data points represent the mean and standard error of 5 independent experiments in which the number of viable cells remaining attached to the monolayer after TGFβ_1 addition were counted at 1 time-point per day. Results are presented as a percentage of the cell number in cultures treated with TGFβ_1 alone.

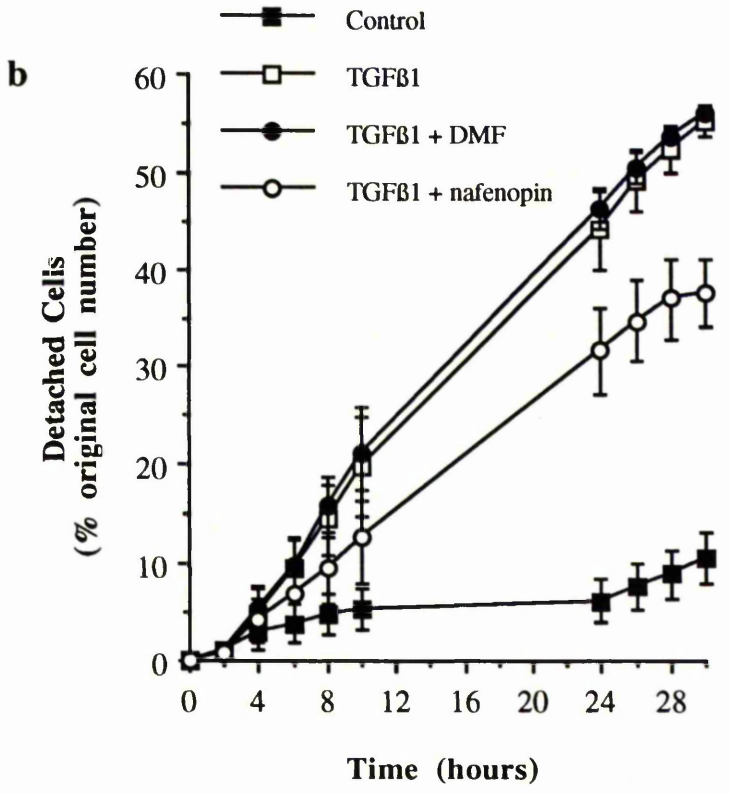


Figure 5.8. (b) Effect of nafenopin (50 μM) and DMF alone (0.25% $^{v}/_{v}$) on the detachment of Fao cells from the monolayer following treatment with TGFβ_1 (1 ng/ml). Data points represent the mean and standard error of 3 independent experiments in which the number of detached cells was counted at defined 2-hour intervals over 30 hours after drug addition. Results are presented as a cumulative percentage of the total cell number present on addition of TGFβ_1.

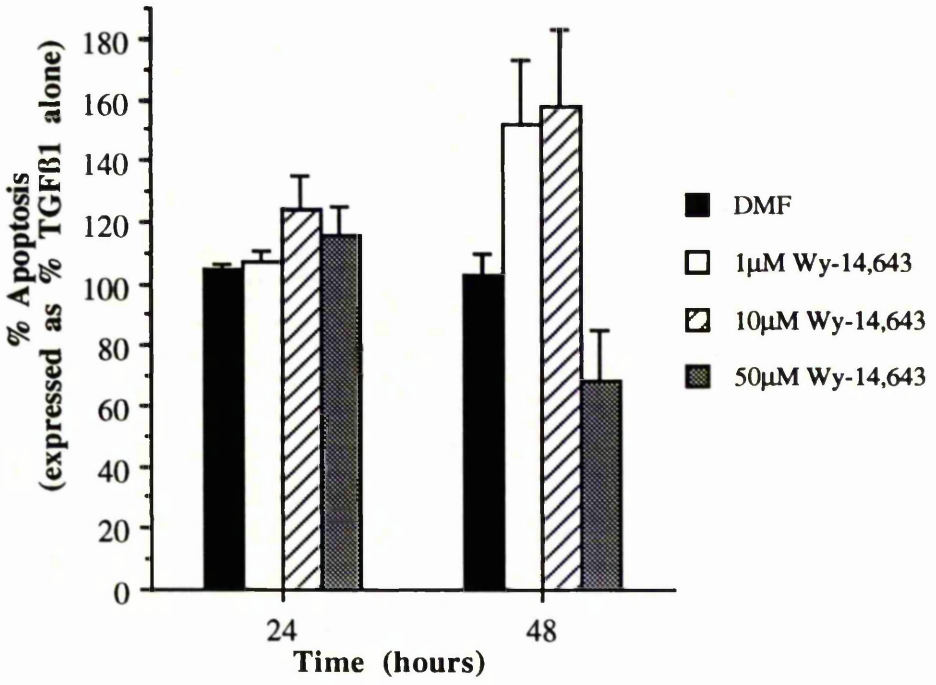


Figure 5.9. Effect of Wy-14,643 (1-50 μM) and DMF alone (0.25% $^{v}/_{v}$) on Fao cell viability following treatment with TGFβ_1 (1 ng/ml). Data points represent the mean and standard error of 3 independent experiments in which the number of viable cells remaining attached to the monolayer after TGFβ_1 addition were counted 24 hours after drug addition. Results are presented as a percentage of the cell number in cultures treated with TGFβ_1 alone.

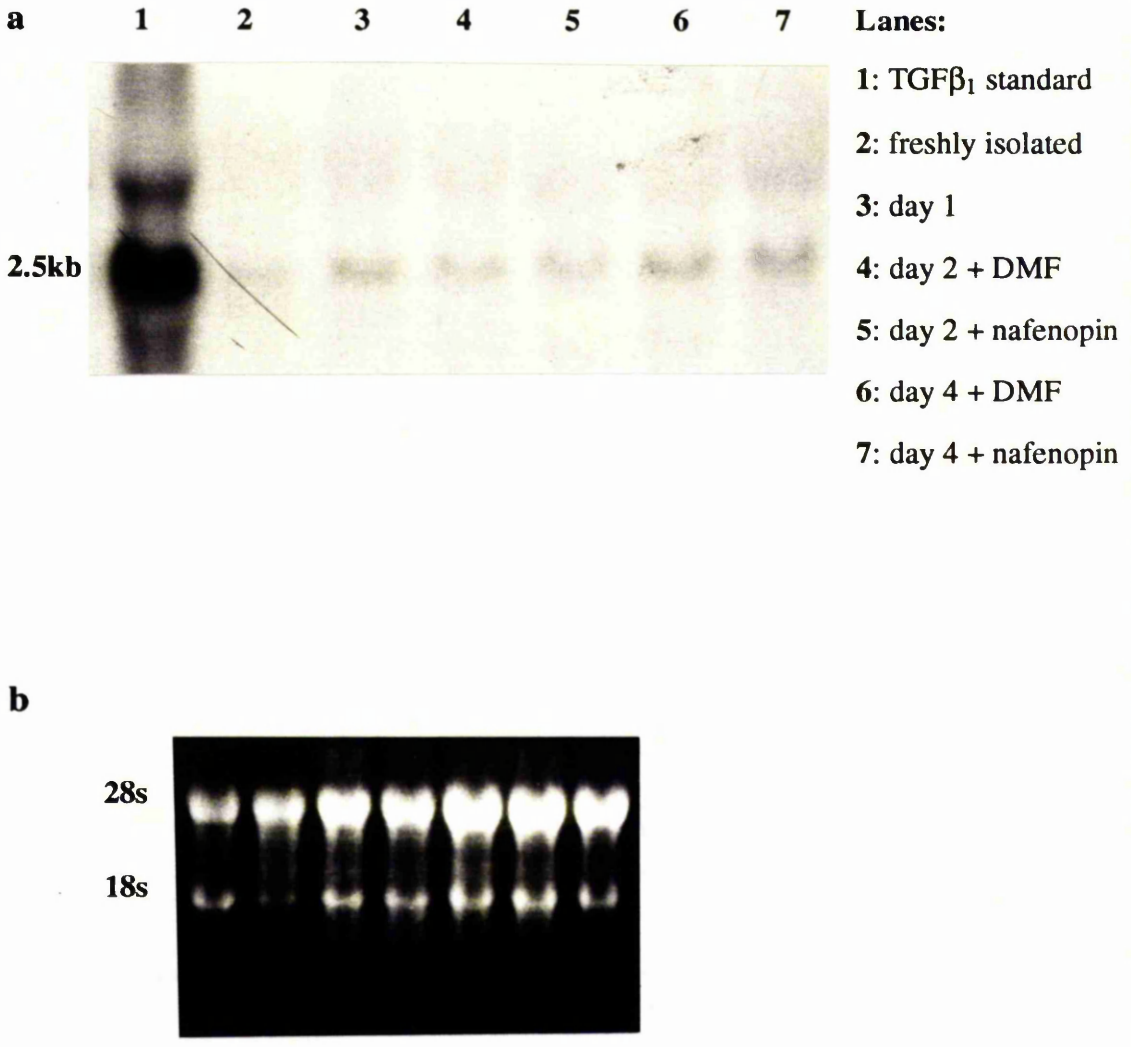


Figure 5.10. **(a)** Northern blot analysis of primary hepatocytes demonstrating TGFβ$_1$ mRNA expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). 20 μg mRNA was loaded in each lane. TGFβ$_1$ standard was obtained from rat palate cells (absolute mRNA concentration unknown). Northern is representative of 3 independent experiments.

(b) Ethidium bromide-stained gel demonstrating loading of hepatocyte mRNA. Gel is relevant for Figure 5.10 and 5.11.

hepatocyte cultures after 4 days. Furthermore, the presence of nafenopin on primary hepatocyte cultures did not alter the level of TGFβ_1 expression in these cells over the time-course of the experiment (Fig. 5.10a). Ethidium bromide staining of RNA revealed equal loading in each lane (Fig. 5.10b).

5.3.6 Expression of M-6-PR mRNA by Primary Hepatocytes.

Northern blot analysis and hybridisation with M-6-PR cDNA revealed a clear band at approximately 2.1 kb (Fig. 5.11). The intensity of this band increased dramatically in primary hepatocytes cultured for 24 hours compared to freshly isolated cells and then decreased with time in culture (Fig. 5.11). The size of the band detected did not match the published size of M-6-PR (9.1 kb; Jirtle and Meyer, 1991). The relevance of this finding is discussed on page 197.

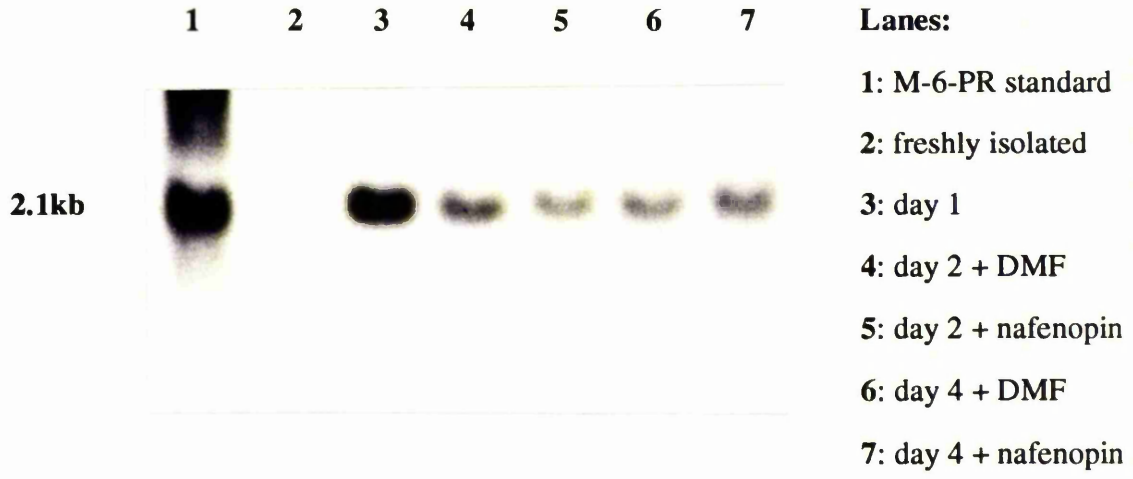


Figure 5.11. Northern blot analysis of primary hepatocytes demonstrating M-6-PR mRNA expression in the presence of nafenopin (50 μM) or DMF alone (0.25% $^{v}/_{v}$). 20 μg mRNA was loaded in each lane. M-6-PR standard was obtained from rat palate cells (absolute mRNA concentration unknown). Northern is representative of 3 independent experiments.

5.4 Discussion.

The effect of peroxisome proliferators on the response of hepatocytes to TGFβ_1 has not been investigated previously. In this chapter the effect of the peroxisome proliferators, nafenopin and Wy-14,643 on TGFβ_1-induced primary hepatocyte and Fao hepatoma cell death has been assessed. This first required an understanding of the kinetics of TGFβ_1-induced hepatocyte and Fao hepatoma cell apoptosis.

5.4.1 Induction of Apoptosis by TGFβ_1 in Primary Hepatocytes and Fao Hepatoma Cells.

In the absence of a peroxisome proliferator, hepatocyte and Fao degeneration occurred within 48 hours of TGFβ_1-treatment. The rapid onset of primary hepatocyte cell death was reflected in the increased percentage of apoptotic cells (3-8%) present in the monolayer at any one time compared to untreated controls ($p \leq 0.001$; Fig. 5.1, page 177). However, no internucleosomal DNA fragmentation was detected in these cells (Fig. 5.2, page 178). In Chapter 4 it was suggested that the apparent absence of internucleosomal DNA fragmentation during spontaneous hepatocyte apoptosis may be attributed to the small number (<1%) of apoptotic cells present at any one time for analysis (page 157). While significantly higher numbers of apoptotic hepatocytes were present in TGFβ_1-treated hepatocyte monolayers, the level of apoptosis was still relatively low compared to Fao samples, which did yield a DNA ladder (Fig. 5.5, page 182). It has been reported that internucleosomal DNA fragmentation does not occur during TGFβ_1-induced hepatocyte apoptosis (Oberhammer *et al.*, 1993a). However, this finding is difficult to reconcile with other reports which demonstrate clearly the occurrence of DNA fragmentation in apoptotic hepatocytes (Sanchez *et al.*, 1992; Shinagawa *et al.*, 1991). The use of a more sensitive detection method such as Southern blotting (Csernansky *et al.*, 1994) may clarify whether internucleosomal DNA fragmentation is indeed absent during TGFβ_1-induced hepatocyte apoptosis, or whether the apparent absence of a DNA ladder is due to the insensitivity of CAGE.

Cleavage of DNA into high molecular weight fragments (50-300 kb) is proposed to be a universal biochemical event during apoptosis (Walker *et al.*, 1991; Oberhammer *et al.*, 1993b). However, during this study, numerous attempts at FIGE of DNA from

both viable control and TGFβ_1-treated hepatocyte monolayers yielded only a smear indicative of non-specific DNA degradation. As discussed in Chapter 4 (page 157), it is possible that the induction of protease activity as a result of sample preparation was responsible for this non-specific fragmentation. Thus, although cleavage of DNA into high molecular weight DNA fragments may be a universal biochemical hallmark of apoptosis, it appears from these results that the methodology for detection of such fragments may not yet be universally suitable for the rapid detection of apoptosis in all cell types.

TGFβ_1 also rapidly induced Fao cell death which, as seen during spontaneous Fao apoptosis, was accompanied by detachment of cells from the monolayer (Fig. 5.3, page 179) In contrast to detached hepatocytes which were always highly degraded, detached Fao cells initially excluded TB and exhibited chromatin condensation patterns and non-random DNA fragmentation patterns typical of apoptosis (Fig. 5.4 and 5.5, pages 180 and 182). At any 2 hour time-point after TGFβ_1 (5 ng/ml) administration, 70-80% of the detached cells exhibited an apoptotic morphology. This strongly suggests that the mode of cell death induced by TGFβ_1 was apoptosis and not necrosis. Apoptosis was induced in Fao hepatoma cells by both 1 ng/ml and 5 ng/ml TGFβ_1. However, since a similar level of cell death was seen with both concentrations of TGFβ_1, the lower concentration was used in subsequent experiments.

5.4.2 Suppression of TGFβ_1-Induced Hepatocyte and Fao Hepatoma Cell Apoptosis by Peroxisome Proliferators.

In the primary hepatocyte system, TGFβ_1-induced apoptosis was significantly reduced by co-addition of nafenopin or Wy-14,643 (Fig. 5.6, 5.7, pages 183-184). The solvent DMF alone had no effect on cell viability. Suppression of TGFβ_1-induced hepatocyte apoptosis was achieved using Wy-14,643 at one fifth of the concentration used routinely for nafenopin. However, in contrast to the effect on spontaneous hepatocyte apoptosis, the level of protection afforded by the maximum concentration of Wy-14,643 (50 μM) against TGFβ_1-induced apoptosis, was not as great as that detected using lower concentrations. This finding is interesting and suggests that at a concentration of 50 μM and above, Wy-14,643 becomes toxic to hepatocytes. The lack

of any observed Wy-14,643 toxicity in spontaneously degenerating hepatocyte cultures indicates that at 50 μM, Wy-14,643 alone may be only marginally toxic to the cells. However, in the presence of an additional insult such as TGFβ$_1$ the toxic effect of Wy-14,643 appears to be enhanced.

Fao cells also were protected from TGFβ$_1$-induced apoptosis by co-addition of a peroxisome proliferator. In the presence of nafenopin, significantly more viable Fao cells remained attached to the monolayer following TGFβ$_1$ treatment ($p \leq 0.05$; Fig. 5.8a, page 186). The rate of Fao cell detachment following TGFβ$_1$ treatment was significantly reduced by nafenopin ($p \leq 0.05$; Fig. 5.8b, page 187) indicating that the increase in viable Fao cell number seen in nafenopin-treated cultures was the result of decreased cell death rather than increased cell proliferation. In contrast to results from primary hepatocyte studies, the protective effect of Wy-14,643 against TGFβ$_1$-induced Fao apoptosis was not as marked as that seen with nafenopin (Fig. 5.9, page 188). It could be concluded from this observation that Wyeth-14,643 does not have the same capacity for suppression of apoptosis as nafenopin. However, the results of experiments using primary hepatocytes suggest that this is not the case. An alternative explanation is that the Fao cells used in the two experiments differed phenotypically. The effect of nafenopin on TGFβ$_1$-induced apoptosis was investigated 6-8 months prior to studies using Wy-14,643 and therefore involved the use of a different stock of cells. Fao cells were typically passaged up to 25 times after which early passage cells were revived from cryostorage. However it is possible that Fao cells became altered at the time of cryostorage, revival or with time in culture, losing some of their differentiated status. Even a small change in Fao cell biochemistry could explain the reduced response to Wy-14,643. This phenomenon of 'drift' in culture is always a problem when using cell lines and is a strong incentive to use cells of a low passage number. Interestingly, combined treatment of Fao cells with TGFβ$_1$ and 50 μM Wy-14,643 again led to a marked decrease in cell viability compared to cultures treated with TGFβ$_1$ alone. This finding corresponds with the results of experiments conducted using primary hepatocytes and adds further support to the theory that at concentrations of 50 μM and above, Wy-14,643 becomes toxic to liver cells.

Results presented in Chapter 4 led to the hypothesis that transformed cells may not exhibit the survival response of primary cells to peroxisome proliferators (see page 163). No suppression of either spontaneous or chemically-induced Fao apoptosis was detected in the presence of nafenopin or Wy-14,643. However, in this chapter both peroxisome proliferators have been shown to suppress $TGF\beta_1$-induced Fao cell death. This ability of peroxisome proliferators to suppress only $TGF\beta_1$-induced Fao apoptosis suggests that the stimulus for cell death is critical to peroxisome proliferator-mediated cell survival. This hypothesis assumes that neither spontaneous Fao apoptosis nor apoptosis induced by the DNA-damaging agents etoposide and hydroxyurea occurs via a $TGF\beta_1$-based mechanism.

While $TGF\beta_1$-induced hepatocyte and Fao cell death were prevented by nafenopin and Wy-14643, this protection was only a partial effect. In the Fao system, for example, the presence of nafenopin resulted in a rescue of only 10-20% of the cells from $TGF\beta_1$-induced cell death. Nevertheless, suppression of cell death in only a small percentage of hepatocytes *in vivo* could be relevant to hepatocarcinogenesis, particularly if any surviving cells have, or are likely to accumulate genetic mutations. This phenomenon, would be enhanced by the sustained hyperplasia induced in liver cells by peroxisome proliferators demonstrated in Chapter 3 (page 120). The prolonged viability of potentially tumourigenic cells could allow the accumulation of further transforming lesions and ultimately the development of liver tumours. Interestingly, nafenopin is unable to enhance the survival of more transformed hepatoma cell lines (Ruth Roberts, Zeneca CTL, personal communication). Thus, the tumour-promoting effects of peroxisome proliferators are likely to occur in the early stages of hepatocyte transformation.

5.4.3 Mechanism of Suppression of Apoptosis by Peroxisome Proliferators.

Results presented in this chapter and in Chapter 4 demonstrate that peroxisome proliferators can suppress liver cell apoptosis. How is this effect mediated? One obvious suggestion, addressed in Chapter 4 is that peroxisome proliferators alter the expression of *bcl-2* or a member of the *bcl-2* family of genes (Korsmeyer *et al.*, 1993). However, as

discussed in Chapter 4, no effect of nafenopin on expression of the *bcl-2* protein product was detected in either hepatocytes or Fao hepatoma cells (page 163).

An alternative possibility is that peroxisome proliferators exert their protective effect via specific changes in expression or activation of $TGF\beta_1$. This would not be surprising since the liver tumour promoter phenobarbital has been shown to alter $TGF\beta_1$ mRNA expression in hepatocytes (Jirtle and Meyer, 1991). It has been demonstrated that apoptotic hepatocytes *in vivo* up-regulate expression of $TGF\beta_1$ latent protein (Bursch *et al.*, 1993). There are a number of mechanisms by which this increase in hepatocyte $TGF\beta_1$ levels could be achieved. Firstly, hepatocytes themselves may up-regulate synthesis of $TGF\beta_1$. This could be at the level of mRNA and / or protein. Alternatively, hepatocytes could increase their uptake of $TGF\beta_1$ protein from non-parenchymal cells. The uptake and subsequent activation of $TGF\beta_1$ protein from non-parenchymal cells is reported to be mediated by the M-6-PR (Jirtle *et al.*, 1991). During liver regeneration, increases in hepatocyte M-6-PR expression have been shown to correlate with the increase seen in $TGF\beta_1$ protein (Jirtle *et al.*, 1991). It is possible therefore that expression of M-6-PR is also up-regulated during hepatocyte apoptosis. Whichever of these mechanisms is responsible for the increase in $TGF\beta_1$ latent protein seen in apoptotic hepatocytes, it represents a potential target for the action of peroxisome proliferators.

In this chapter, the effects of the peroxisome proliferator nafenopin on $TGF\beta_1$ mRNA and M-6-PR mRNA expression in primary hepatocyte cultures has been investigated. A low level of $TGF\beta_1$ mRNA expression was detected in freshly isolated primary hepatocyte cultures and this increased slightly with increasing time in culture (Fig. 5.10, 189). Two possible conclusions can be drawn from this result. Firstly primary hepatocytes may normally express low levels of $TGF\beta_1$ mRNA but up-regulate this expression during cell death. Alternatively it is possible that the $TGF\beta_1$ mRNA detected was expressed not by the hepatocytes but by non-parenchymal cells isolated during hepatocyte perfusions. The increase in $TGF\beta_1$ expression detected with time in culture could thus be attributed to either proliferation of non-parenchymal cells within the cultures or enhanced synthesis of $TGF\beta_1$ mRNA by these cells. Current dogma suggests that the latter hypothesis is correct (Jirtle *et al.*, 1991). The presence of nafenopin in

primary hepatocyte cultures had no apparent effect on expression of TGFβ_1 mRNA. Thus if the effect of peroxisome proliferators is mediated through expression of TGFβ_1, this regulation is not mediated at the level of mRNA. Preliminary experiments were conducted to examine expression of TGFβ_1 protein in control and nafenopin-treated hepatocyte cultures. However, the TGFβ_1 antibody used was polyclonal making attempts to identify TGFβ_1 using this antibody inconclusive.

Unfortunately the results of northern blot analysis conducted to investigate the expression of M-6-PR mRNA in hepatocyte cultures were also not conclusive. The M-6-PR cDNA used in this investigation highlighted a strong band at 2.1 kb (Fig. 5.11, page 191). However, this does not correspond with the published size of the M-6-PR (9.1 kb). Interestingly a 2.1 kb mRNA has been detected previously using M-6-PR cDNA from a different source (C. Brunet, personal communication). It is possible therefore that the 2.1 kb mRNA detected is a commonly occurring fragment of the M-6-PR. Expression of this mRNA increased substantially in primary hepatocytes 24 hours after their isolation. If the 2.1 kb mRNA does represent a cleavage product of M-6-PR, the increase in expression detected may represent an adaptive stress response of the cells as they attach to plastic. However, since the identity of the mRNA detected has not been established, this conclusion is speculative.

While the work described in this chapter was in progress, a report was published demonstrating the short-term effects of peroxisome proliferators on TGFβ_1 and M-6-PR mRNA expression in rat liver *in vivo* (Rumsby *et al.*, 1994). The results demonstrate that following a 7 day feeding regimen with the peroxisome proliferators nafenopin, Wy-14,643 and methylclofenapate, TGFβ_1 and M-6-PR mRNA expression is elevated in rat liver. It is not apparent whether this increase in expression occurs in hepatocytes or in non-parenchymal cells. However, in light of results published elsewhere on the effect of phenobarbital on these two genes (Jirtle and Meyer, 1991), it is likely that the increase in TGFβ_1 mRNA at least is confined to the non-parenchymal cells. It is also unknown whether expression of TGFβ_1 protein mirrors the increase in gene expression. However, the authors propose that the increase in TGFβ_1/ M-6-PR detected occurs in an attempt by the liver to combat the initial hyperplastic response of hepatocytes to the peroxisome proliferators. It would be interesting to see whether following a longer exposure to the

peroxisome proliferators, the increase in TGFβ_1 expression remains confined to the normal hepatocytes or whether, as shown by Jirtle *et al.* (1991) preneoplastic cells lose this response to TGFβ_1.

5.4.4 Summary.

This chapter presents evidence that maintenance of hepatocyte viability by peroxisome proliferators may be mediated via their effects on the function of TGFβ_1. Results presented here demonstrate the ability of two peroxisome proliferators to suppress TGFβ_1-induced primary hepatocyte and Fao apoptosis. In both cases protection was only partial. However, maintenance of viability of only a proportion of hepatocytes could have serious ramifications *in vivo* if preneoplastic cells are protected from cell death. The results also provide further evidence of a correlation between the carcinogenicity of a peroxisome proliferator and its ability to suppress apoptosis. A lower concentration of Wy-14,643 than nafenopin was required to afford the same level of protection of primary hepatocytes from TGFβ_1-induced apoptosis. Furthermore, when equivalent concentrations (50 μM) of the two peroxisome proliferators were used, Wy-14,643 was toxic to the cells.

Results presented in Chapter 4 demonstrate that spontaneous and chemically-induced Fao apoptosis is not prevented by peroxisome proliferators. The finding that Fao cells were protected from TGFβ_1-induced apoptosis suggests that the signalling pathway involved in TGFβ_1-induced cell death represents a potential target for the action of the peroxisome proliferators. In order to examine this hypothesis further, expression of TGFβ1 and M-6-PR mRNA was examined in spontaneously degenerating and nafenopin-treated hepatocytes. No significant change in TGFβ_1 mRNA expression was detected in the presence of nafenopin. Expression of an mRNA detected by M-6-PR cDNA increased in primary hepatocytes with time in culture. Interpretation of this result is purely speculative, but it is possible that up-regulation of M-6-PR occurs in hepatocytes undergoing apoptosis. Once again, nafenopin did not change the level of mRNA expressed. In conclusion therefore, it appears that while peroxisome proliferators are capable of suppressing TGFβ_1-induced liver cell death, the protective effect is most

likely to be mediated at the post-translational level or further down the TGFβ_1 signalling cascade (see general discussion, pages 200-209).

Chapter 6.

General Discussion.

6. General Discussion.

Current knowledge of chemically-induced carcinogenesis has been derived mainly from studies of the effects of genotoxic carcinogens on experimental animals (Purchase, 1994). Less is known about how non-genotoxic carcinogens such as peroxisome proliferators contribute to the development of tumours. In contrast to genotoxic agents, non-genotoxic carcinogens cannot be identified on the basis of their chemical structure or effects on DNA (Ashby, 1992). Therefore, if the risk posed by these chemicals to humans is to be fully assessed, the mechanism(s) by which they exert their effects must be more clearly understood.

There is no unifying hypothesis to explain how non-genotoxic carcinogens cause cancer. Potential contributory factors include the activation of specific receptors, such as PPAR and the dioxin receptor (Issemann and Green, 1990; Whitlock *et al.*, 1993), enhanced expression of specific genes such as *c-myc* or *c-Ha-ras* (reviewed by Green, 1991), changes in the production and sensitivity of cells to growth factors such as EGF and TGFβ_1 (Eckl *et al.*, 1988; Jirtle and Meyer, 1991) and indirect induction of DNA damage (Reddy and Rao, 1986). However it is believed that, in general, non-genotoxic carcinogens act as tumour promoters, 'fixing' cells which have previously sustained genetic mutations into a cell population, where further transforming events can then occur (Green, 1991). It has long been assumed that this promotion of preneoplastic cells occurs by sustained hyperplasia (Cohen and Ellwein, 1990). Nevertheless, there are cases where the ability of a non-genotoxic carcinogen to stimulate mitogenesis does not correlate with its carcinogenicity (Yeldandi *et al.*, 1989; Eacho *et al.*, 1991). Thus, although considerable emphasis has been placed on investigating the importance of mitogenesis in non-genotoxic carcinogenesis, it is unlikely to be the only factor involved.

In recent years, a new dimension has been added to previously established theories of tumour promotion. This change in perspective has come from studies, not of proliferation, but of the cell death pathway of apoptosis. In healthy tissue, proliferation and apoptosis work in tandem to maintain homeostasis. The role of apoptosis in this equation appears to be the removal of cells produced in excess, cells whose function is no longer required or cells that have sustained some form of damage (Ellis *et al.*, 1991).

The anomalous survival of these cells, particularly those carrying a genetic mutation, could have serious implications. Therefore, it has been proposed that suppression of apoptosis may be as important as sustained hyperplasia in tumour promotion by non-genotoxic carcinogens (Bursch *et al.*, 1992). However, at the outset of this thesis, conclusive evidence of a role for suppression of apoptosis in non-genotoxic hepatocarcinogenesis was elusive. Quantitative studies of the occurrence of apoptosis in the liver *in vivo* were hampered by the rapid removal of apoptotic hepatocytes from the liver. The objectives of this thesis were first to examine the effect of the peroxisome proliferator class of non-genotoxic carcinogens on liver cell apoptosis *in vitro* and, if the hypothesis of peroxisome proliferator-mediated cell survival was confirmed, to then examine the molecular mechanisms involved.

6.1 Achievements and Success of this Thesis.

Following a systematic assessment of a range of hepatoma cell lines, the Reuber hepatoma cell line, Fao, was validated as a suitable model system for investigating the mechanism(s) of action of the peroxisome proliferators (Chapter 3, Bayly *et al.*, 1993). Fao cells are well differentiated, respond to the peroxisome proliferator nafenopin by induction of cytochrome P4504A1 and are easy to manipulate in culture. However, as with many transformed cell lines, Fao cells are susceptible to phenotypic change with time in culture, and therefore passage number is an important determinant in their behaviour. This was a limitation which was recognised at the outset of this project and some problems with cell line 'drift' were later encountered. However, use of the Fao cell line did allow detailed assessments of the effects of peroxisome proliferators on apoptosis and thus the advantages of using the cell line outweighed its limitations.

Apoptosis was seen to occur spontaneously in primary hepatocyte and Fao monolayers. Furthermore, this could be induced more rapidly in both systems using $TGF\beta_1$. In addition, Fao cells underwent apoptosis in response to treatment with the DNA damaging agents etoposide and hydroxyurea. A number of differences between hepatocyte and Fao apoptosis were highlighted, irrespective of the stimulus used to induce cell death. Apoptotic Fao cells always detached from the monolayer prior to loss of membrane integrity and exhibited internucleosomal DNA fragmentation patterns. In

contrast, apoptotic primary hepatocytes remained attached to the plastic substratum, perhaps reflecting the more extensive cell-matrix interactions of primary cells. Furthermore, primary hepatocytes did not exhibit any apparent internucleosomal DNA fragmentation. It remains to be established whether the differences in behaviour of primary cells and hepatoma cells highlighted in this thesis reflect inherent differences in the late stages of apoptosis in the two cell types. However, the fact that hepatoma cells did not exactly mirror the pathway of primary hepatocyte cell death, indicates the need for caution in the interpretation of results based solely on experiments using cell lines.

The peroxisome proliferators nafenopin and Wy-14,643 suppressed spontaneous hepatocyte apoptosis and TGFβ_1-induced hepatocyte and Fao apoptosis (Chapter 5, Bayly *et al.*, 1994). Interestingly, no protection was given against Fao apoptosis induced by DNA damaging agents. Furthermore, protection from spontaneous apoptosis was not mediated by Bcl-2, since treatment of hepatocytes and Fao cells with nafenopin did not result in a detectable change in the level of expression of the Bcl-2 protein. This protein was examined because de-regulated expression has been shown to protect cells from apoptosis in other systems (Collins *et al.*, 1992; Dole *et al.*, 1994).

These findings led to speculation that peroxisome proliferators may interfere specifically with TGFβ_1-induced cell death. TGFβ_1 is reported to regulate liver homeostasis *in vivo*. It is possible therefore that spontaneous hepatocyte apoptosis *in vitro* is mediated also by TGFβ_1. Unfortunately, determination of TGFβ_1 protein expression in degenerating hepatocyte cultures was inconclusive. Interestingly, induction of a putative M-6-PR cleavage product was detected in hepatocytes following their isolation. This agrees with previous reports suggesting that hepatocytes increase their expression of TGFβ_1 via increased M-6-PR-mediated uptake from neighbouring non-parenchymal cells (Jirtle and Meyer, 1991). On examination of TGFβ_1 mRNA levels in degenerating primary hepatocyte monolayers, only minimal induction was detected. It was not apparent whether expression occurred in the hepatocytes themselves or the surrounding non-parenchymal cells.

It is possible that peroxisome proliferators alter TGFβ_1 protein expression in hepatocytes. However, no change in the level of expression of TGFβ_1 mRNA or the transcript detected using the M-6-PR probe in hepatocytes treated with nafenopin was

demonstrated. Therefore, on the basis of results presented in this thesis, no firm conclusion can be drawn regarding the importance of TGFβ_1 regulation in peroxisome proliferator-induced suppression of apoptosis. While these investigations into the molecular mechanisms by which peroxisome proliferators suppress liver cell apoptosis were inconclusive, the results obtained point the way forward for future studies.

6.2 Future Directions for this Project.

Data presented in this thesis strongly suggest that peroxisome proliferators suppress hepatocyte apoptosis and that this suppression appears to be more specific to physiologically-induced cell death. A point of conjecture is that peroxisome proliferators may affect only TGFβ_1-induced apoptosis. In order to validate this hypothesis conclusively, it would be necessary to determine where in the TGFβ_1 signalling pathway peroxisome proliferators exert their effects. One possibility is that peroxisome proliferators prevent the up-regulation of TGFβ_1 protein in degenerating hepatocytes. Preliminary results presented in this thesis indicate that if this is the case up-regulation is unlikely to occur at the mRNA level. Use of antibodies specific to TGFβ_1 mature and latent protein would reveal whether any changes in TGFβ_1 protein expression occur in peroxisome proliferator-treated hepatocytes.

Even, if peroxisome proliferators do not alter the level of TGFβ_1 protein expression in hepatocytes, they may still disrupt later events in the TGFβ_1 signalling pathway. Such disruption could be mediated by the peroxisome proliferator itself, or by a protein whose expression is regulated by peroxisome proliferators. It has been established that the first step in the TGFβ_1 signalling cascade involves the TGFβ receptors, TβR-I and TβR-II (Wrana *et al.*, 1994). TGFβ_1 binds TβR-II which then complexes with and phosphorylates TβR-I. The signals downstream of TGFβ_1 receptor binding are not so well characterised and what little is known pertains to TGFβ_1-induced growth arrest rather than apoptosis. Inhibition of DNA synthesis in adult hepatocytes and rat liver epithelial cell lines by TGFβ_1 occurs in G_1, shortly before the G_1/S border (Thoresen *et al.*, 1992). In rat liver epithelial cell lines, this inhibition is associated with suppressed phosphorylation of the product of the retinoblastoma gene, pRb (Whitson and Itakura, 1992). Similar results have been reported for mink lung epithelial cells and

keratinocytes (Laiho *et al.*, 1990). In normal cells, phosphorylation of pRb is believed to be required for progression of cells from G_1 to S-phase (DeCaprio *et al.*, 1989). pRb is normally phosphorylated at the G_1/S border and is de-phosphorylated in late M / early G_1 (Ludlow *et al.*, 1989; Buchkovich *et al.*, 1989). This phosphorylation is reported to be mediated by the cyclin D-associated kinases CDK4 and CDK6 (Meyerson and Harlow, 1994; Bates *et al.*, 1994) which are inhibited by the protein $p16^{INK4}$ (Hannon and Beach, 1994). It has been proposed recently that TGFβ may cause cell cycle arrest via suppression of these cyclin-dependent kinases (Hannon and Beach, 1994). This is supported by the finding that human keratinocytes, treated with TGFβ, express high levels of a newly characterised CDK4 and CDK6 inhibitor $p15^{INK4B}$ (Hannon and Beach 1994). The gene encoding this new kinase inhibitor is adjacent to the P16 gene (Hannon and Beach, 1994).

Another putative regulatory protein in $TGF\beta_1$-induced growth arrest has been identified in mink lung epithelial cells. This protein, $p27^{kip1}$, inhibits the formation of cyclin E-CDK2 complexes (Polyak *et al.*, 1994a). It is proposed that $p27^{kip1}$ blocks CDK2 activation by raising the threshold level of cyclin E needed to activate CDK2 above intracellular cyclin E levels (Polyak *et al.*, 1994a). However, although $p27^{kip1}$ has been isolated from arrested cells following treatment with TGFβ, its expression is not enhanced in either keratinocytes or mink lung epithelial cells in response to TGFβ (Hannon and Beach, 1994; Polyak *et al.*, 1994b). Thus if $p27^{kip1}$ does play a role in TGFβ-induced growth inhibition, its effects must be mediated at the post-translational level.

It is not yet known if $p15^{INK4B}$ and $p27^{kip1}$ are both involved universally in TGFβ-induced growth arrest. However, the proposal that $TGF\beta_1$-induced growth arrest is mediated by inhibitors of cyclin-dependent kinases is compelling and certainly warrants further investigation. It also remains to be determined whether $TGF\beta_1$-induced apoptosis is mediated via the similar signalling events or shares components of this signalling pathway. It would not be surprising if the two pathways did share common elements, since precedents for shared events in the control of apoptosis and mitosis exist. For example, expression of the *p53* tumour suppresser gene can cause growth arrest and / or apoptosis depending on the presence of additional signalling factors and the genetic

context of the cell in question (Kastan *et al.*, 1991; Yonish-Rouach *et al.*, 1991). If $TGF\beta_1$-induced apoptosis is mediated by a similar pathway to growth arrest, peroxisome proliferators may disrupt any one of the signalling events involved. Peroxisome proliferators may suppress phosphorylation of TβR-I, or alternatively may suppress the activity of $p21^{kip1}$ or $p15^{INK4B}$.

A number of genes have been implicated in the regulation of apoptosis. It would be interesting to determine whether the ability of peroxisome proliferators to inhibit liver cell apoptosis is related to a change in expression of any of these genes or their protein products. In this thesis, no change in expression of the Bcl-2 protein was detected in hepatocytes treated with nafenopin. However, this in no way rules out the possibility that *bcl-2* is involved in peroxisome proliferator-mediated survival. Peroxisome proliferators may affect the level of expression of *bcl-2* binding partners such as *bax* or may affect the expression of other *bcl-2* family members such as $bcl\text{-}x_L$.

Another intriguing possibility involves the *c-Ha-ras* oncogene. Levels of *c-Ha-ras* expression are reported to be elevated in rat liver following treatment with the peroxisome proliferators clofibrate, ciprofibrate and BR-931 (Cherkaoui-Malki *et al.*, 1991; Hsieh *et al.*, 1991; Hegi *et al.*, 1993). Since rat liver epithelial cells and hepatocytes transfected with a mutated form of *Ha-ras* are also resistant to the growth inhibitory effects of $TGF\beta_1$, it is interesting to speculate that the protective effect of peroxisome proliferators may stem from this induction of *c-Ha-ras*.

A number of recent studies have focused on the role of the tumour suppressor gene *p53* as a regulator of apoptosis. Many hepatocellular carcinomas carry mutations of *p53* (Bressac *et al.*, 1991; Murakami *et al.*, 1991; Hsu *et al.*, 1993). Mutations in *p53* can also be induced in hepatocytes by exposure to hepatocarcinogens such as AFB1 and hepatitus B virus (Beasley *et al.*, 1981, Ozturk *et al.*, 1991). However, it has been demonstrated that hepatocellular carcinomas induced in rats by the peroxisome proliferator BR-931 do not carry mutations in the *p53* gene (Smith *et al.*, 1993).Whether the same is true of other peroxisome proliferators remains to be established.

Throughout this thesis, suppression of hepatocyte and Fao cell death by peroxisome proliferators was only partial. Thus, while the ability of peroxisome proliferators to suppress hepatocyte apoptosis is likely to be a contributory factor in

peroxisome proliferator-induced hepatocarcinogenesis, it is unlikely to be the only factor involved. Other proposed mechanisms of peroxisome proliferator-induced hepatocarcinogenesis include activation of PPAR (Issemann and Green, 1990), stimulation of mitogenesis (Marsman *et al.*, 1988) and induction of oxidative DNA damage (Reddy and Rao, 1989). Suppression of apoptosis may operate in tandem with any one of these effects to promote the development of preneoplastic cells.

It would be interesting to determine whether PPAR activation is critical for suppression of hepatocyte apoptosis. This would reveal whether the suppression of apoptosis is dependent specifically on the peroxisome proliferator signalling pathway. The role of PPAR in suppression of apoptosis could be examined using hepatoma cells transfected with high levels of PPAR. Alternatively, the use of anti-PPAR antibodies could abrogate PPAR-induced signalling events. Another approach would be to investigate whether enhancement of PPAR activation using agents such as 9-cis retinoic acid results in an increase in the level of suppression of cell death by peroxisome proliferators. A possible drawback of this latter study would be that retinoic acid has been shown to suppress apoptosis in non-liver cell systems (Attencia *et al.*, 1994) and therefore any effect seen may not be the result of PPAR activation.

It would be important also to correlate the combined ability of a peroxisome proliferator to suppress apoptosis and induce mitosis, with its carcinogenicity. It is likely that chemicals with the potent ability to affect both sides of growth regulation (mitosis and apoptosis) will be more potent carcinogens than those which induce one or other response. For example, the peroxisome proliferator DEHP can cause the development of liver tumours in rats in the absence of a sustained increase in mitosis (Marsman *et al.*, 1988). It is possible that this carcinogenicity is due mainly to suppression of apoptosis. However, peroxisome proliferators that can exert both effects, like nafenopin and Wy-14,643 are more potent carcinogens. It is tempting to assume that since nafenopin and Wy-14,643 can both suppress apoptosis, the phenomenon is common to all peroxisome proliferators. However, both chemicals used are hypolipidaemic agents and it would therefore be necessary to screen a variety of other chemicals before making such an assumption.

Finally, if suppression of apoptosis is universally important in peroxisome proliferator-induced hepatocarcinogenesis, it would be important to establish which cells are protected from cell death. An interesting theory is that those cells whose viability is maintained after exposure to peroxisome proliferators may have sustained DNA damage not by previous mutations but indirectly following peroxisome proliferator-induced oxidative stress. This would explain why peroxisome proliferators can act as complete carcinogens (Reddy and Lalwani, 1983).

6.2 Wider Implications of Suppression of Apoptosis by Non-Genotoxic Carcinogens.

It remains to be established whether the ability to suppress apoptosis is common to all non-genotoxic carcinogens. Results of experiments conducted *in vivo* demonstrate that the liver tumour promoters phenobarbital and cyproterone acetate can suppress apoptosis during regression of the hyperplastic liver (Bursch *et al.*, 1984; Bursch *et al.*, 1986). It is possible that these chemicals may also mediate their effects via TGFβ_1. Indeed, phenobarbital has been shown to down-regulate TGFβ_1 expression in preneoplastic hepatocytes (Jirtle and Meyer, 1991). However, these results are contradicted by a recent report demonstrating that cyproterone acetate actually induces apoptosis at certain concentrations in hepatocytes (Oberhammer *et al.*, 1994). It remains to be established whether this effect occurs at concentrations above those which have been reported previously to suppress hepatocyte apoptosis (Bursch *et al.* 1986).

The major incentive for understanding the mechanism of carcinogenicity of non-genotoxic carcinogens is to facilitate an assessment of the risk these chemicals pose to man. Some of the biological effects induced by these agents, such as peroxisome proliferation, appear to be limited to rodents. If the molecular mechanisms of carcinogenicity of these chemicals stems from such species-specific effects, it is unlikely that the chemicals will be carcinogenic in humans. However, the ability of these compounds to induce a more universal response, such as suppression of apoptosis, suggests that a hazard may exist for humans. More detailed studies of non-genotoxic carcinogens may reveal that many of these chemicals are harmless to man. However since humans are currently exposed to a whole range of non-genotoxic carcinogens on a

daily basis, this is a dangerous assumption. The ability of xenobiotics to control the balance between apoptosis and mitosis is not only relevant to peroxisome proliferator-induced hepatocarcinogenesis but may provide a paradigm with widespread implications for molecular toxicology research.

Appendix.

Appendix 1. Solutions Used in SDS-PAGE and Western Blotting.

1A. 2 x Laemmli Sample Buffer.

Tris-HCl (80 mM, pH 6.8) containing	SDS (4% $^{w}/_{v}$)
	glycerol (20% $^{v}/_{v}$)
	bromophenol blue (0.004% $^{w}/_{v}$)
	mercaptoethanol (1% $^{v}/_{v}$)

1B. Composition of Polyacrylamide Gels.

Separating gel:	**8%**	**14%**
Tris base pH 8.8	0.375 M	0.5 M
SDS	0.1% $^{w}/_{v}$	0.1% $^{w}/_{v}$
polyacrylamide	8% $^{v}/_{v}$	14% $^{v}/_{v}$
ammonium persulphate	0.5 mg/ml	0.5 mg/ml
TEMED	0.004% $^{v}/_{v}$	0.04% $^{v}/_{v}$

Stacking Gel:	**3 %**	**5 %**
Tris base pH 6.8	0.125M	0.05M
SDS	0.1% $^{w}/_{v}$	0.1% $^{w}/_{v}$
polyacrylamide	3% $^{v}/_{v}$	5% $^{v}/_{v}$
ammonium persulphate	0.5 mg/ml	1 mg/ml
TEMED	0.04% $^{v}/_{v}$	0.04% $^{v}/_{v}$

1C. SDS-PAGE Running Buffer.

dd H_2O containing	Tris base (20 mM, pH 8.6)
	glycine (190 mM)
	SDS (0.01% $^{w}/_{v}$)

1D. SDS Transfer Buffer.

dd H_20 containing	Tris base (20 mM, pH 8.6)
	glycine (0.192 mM)
	SDS (0.01% $^{w}/_{v}$)
	methanol (20% $^{v}/_{v}$)

1E. TBS

ddH_20 containing	Tris base (25 mM, pH 8.2)
	NaCl (144 mM)

Appendix 2. Solutions Used in Northern Blot Analysis.

1A. 5 x Formaldehyde Running Buffer.

0.1 M MOPS (pH 7) in DEPC-treated water containing:

40 nM sodium acetate

5 mM EDTA (pH 8)

1B. Gel Composition.

DEPC-treated water containing: 1% agarose

20% $^{v}/_{v}$ 5 x formaldehyde running buffer

2.2 M formaldehyde

1C. Sample Buffer.

Volumes for 4.5 µl RNA: 2 µl 5x formaldehyde running buffer

3.5 µl formaldehyde

10 µl formamide

12.4 µg /ml ethidium bromide

1D. Northern Loading Buffer.

Sterile DEPC-treated water containing: 50% glycerol

1 mM EDTA (pH8)

0.25% bromophenol blue

0.25% xylene cyanol FF

1E. Northern Running Buffer.

Sterile DEPC-treated water containing: 20% 5 x formaldehyde running buffer

2.2 M formaldehyde

1F. 20 x SSC.

Sterile DEPC-treated water containing: 3 M sodium chloride

0.3 M sodium citrate

1G. TE Buffer.

10 mM Tris chloride (pH 8) containing 1mM EDTA

References.

References.

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