The induction of liver growth by peroxisome proliferators

by

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Abstract

Peroxisome Proliferators (PPs) are a class of chemicals that cause a programme of augmentative liver growth, however, the mechanism which regulates the induction of hepatic DNA synthesis as a result of exposure to peroxisome proliferators is currently uncharacterized. This study sets out to characterise the induction of DNA synthesis in mouse by peroxisome proliferators, as a prerequisite for investigating and identifying the genes that are responsible for induction of DNA synthesis to control liver growth.

Administration of BrdU in drinking water can reduce mouse body weight; an optimized protocol was devised, which does not lead to body weight loss, and which enables reliable measurement of DNA synthesis.

Male 129S4/SvJae mice were treated with a single dose of ciprofibrate (100-400 mg kg^{-1}) or methylclofenapate (25 mg kg^{-1}) for two days. Although liver to body weight ratios increased significantly at all doses, no induction in DNA synthesis was observed within 2 days. Subsequent time course studies with ciprofibrate (100 mg $kg^{-1}day^{-1}$) or methylclofenapate (25 mg $kg^{-1}day^{-1}$) showed that liver-to-body weight ratio was significantly increased in treated groups by day 2, but that the induction of DNA synthesis was increased significantly only after three days of treatment, for both compounds. No induction of hepatic DNA synthesis was observed in PPARα null mice after treatment with ciprofibrate (100mg $kg^{-1}day^{-1}$) for 2 or 6 days, showing that the effect required the PPARα.

A dose-response study with 0,1,3,10,30,100 or 200 mg kg^{-1} day^{-1} ciprofibrate for 3 days, or with 0,10,30,100 mg kg^{-1} day^{-1} ciprofibrate for 4 days revealed that liver to body weight ratios were significantly increased in 129S4/SvJae mice treated with 10mg $kg^{-1}day^{-1}$ and greater ciprofibrate at 3 and 4 days, whereas hepatic labelling index was significantly increased at 100 mg kg^{-}

^{1} day^{-1} ciprofibrate at 3 days after dosing, with progressive increases at doses of 30 and 100 mg kg^{-1} day^{-1} ciprofibrate at 4 days after dosing.

In order to explain the early time course of induction of DNA synthesis reported by Styles [113] [164] in Alderley Park mice, a time course study was performed between 1-4 days in Alderley park mice using methylclofenapate (25mg kg^{-1}day^{-1}). The study showed that liver growth was induced by day 2, but DNA synthesis was significantly induced only after 3 days of dosing.

To evaluate species differences, the time-course of induction of DNA synthesis was examined in F-344 rats treated with ciprofibrate (50mg kg^{-1}day^{-1}) for 1-4 days. The liver-to-body weight ratio was significantly increased in all time points, but DNA synthesis was significantly increased after 2 days of dosing.

These finding demonstrate that there was a delay in induction of DNA synthesis by peroxisome proliferators in mouse by at least 48 hours. This delay in response is not due to strain differences. Moreover, induction of DNA synthesis in rat was earlier than those in mouse, which makes rats a feasible experimental model to study the immediate early genes/ proteins induced by peroxisome proliferators to induce liver growth.

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Dedication

This thesis is dedicated to:

My mother,

My wife, Khloud,

My daughters, Sarah and Nouf,

My sons, Mohammed and Abdulaziz.

Finally, this thesis is dedicated to the memories of my brother-in-law, Abdulaziz Alkholaifi, who always encourage me to be a scientist

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Abbreviations

2-AAF	2-Acetylaminofluorene
8-OHdG	8-Hydroxydeoxyguanoside
9c-RA	9-Cis-Retinoic Acid
AF-1	Activation Function Domain-1
ALT	Alanine Aminotransferase
ANOVA	Analysis Of Variance
AP	Alderley Park
APE	Apurinic/Apyrimidinic (Abasic) Endonuclease
AST	Aspartate Aminotransferase
ATP	Adenosine 5'-Triphosphate
BER	Base Excision Repair
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
C3a and C5a	Complement Factors 3a and 5a
CA	Chromosomal Aberration
CAR	Constitutive Androstane Receptor
CDK	Cyclin-Dependent Kinase
CHO	Chinese hamster ovary
CIP	Ciprofibrate
CoA	CoenzymeA
COX	Cyclooxygenase
DAB	3,3' Diaminobenzidine Tetrahydrochloride
DBD	DNA-Binding Domain
DEHP	Diethylhexylphthalate
DEN	Diethylnitrosamine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DPX	Distyrene, Plasticiser, And Xylene

DR1	Direct Repeat Element With A Single Nucleotide Spacer
ENU	Ethylnitrosourea
ER	Estrogen Receptor
F-344	Fischer-344
FC	Flow Cytometry
G0	Gap 0
G1	Gap 1
G2	Gap 2
H_2O_2	Hydrogen Peroxide
HGF	Hepatocyte Growth Factor
HIER	Heat Induced Epitope Retrieval
HREs	Hormone Response Elements
HRP	Horseradish Peroxidase
HSC	Haematopoietic Stem Cell
ICAMs	Intercellular Adhesion Molecules
IHC	Immunohistochemistry
IL-6	Interleukin-6
I.P.	Intraperitoneal
KO mouse	Knockout Mouse
LBD	Ligand-Binding Domain
LBW	Liver-To-Body Weight
LD50	Lethal Dose, 50%
LI	Labelling Index
LOEL	Lowest Observable Effect Level
LPS	Lipopolysaccharide
LRF-1	Liver regeneration factor-1
MCP	Methylclofenapate
MEHP	Mono(2-Ethylhexyl)Phthalate
MN	Micronuclei
M-phase	Mitotic Phase
NA	No Data

NBF	Neutral Buffered Formalin
NF-kB	Nuclear Factor-Kappa B
NHRs	Nuclear Hormone Receptors
OGG1	8-Oxoguanine DNA Glycosylase/Apurinic Lyase
PHx	Partial Hepatectomy
PI	Propidium Iodide
Pol	Polymerase
PP	Periportal
PPARα	Peroxisome Proliferators Activated Receptor
PPRE	Peroxisome Proliferators Response Element
PPs	Peroxisome Proliferators
PV	Perivenous
QMC	Queen Medical Center
RAR	Retinoic Acid Receptor
RBW	Relative Body Weight
RXR	Retinoid X Receptor
S/N	Signal-to-Noise
SCE	Sister Chromatid Exchange
S-phase	Synthesis Phase
STAT3	Signal Transducer And Activator Of Transcription-3
T_4	Thyroid Hormone
TBS	Tris Buffered Saline
TCPOBOP	1,4-Bis[2-(3,5-Dichloropyridyloxy)] Benzene
TdR	[^{3}H]thymidine
TGFα	Transforming Growth Factor ?
THR	Thyroid Hormone Receptor
TNF	Tumor Necrosis Factor
UDS	Unscheduled DNA Synthesis
WT mouse	Wild Type Mouse
WY	4-Chloro-6-(2,3-Xylidino)-2-Pyrimidinylthio Acetic Acid (WY14,643)

Chapter 1 Introduction

Section 1.1 Liver

Liver is the largest mass of glandular tissue and the second largest organ after skin in the body [1]. It represents about 2-5% of the body weight in man and other mammals such as the rat and mouse (human liver: 2%; rat liver 4%; mouse liver 5%) [2] [3] [4] [5]. The liver has the ability to regulate its growth in humans and animals The liver is the main detoxifying organ of the body and may be injured by ingested toxins. Loss of hepatic tissue by surgical removal (partial hepatectomy), or liver cell loss caused by viral or chemical injury triggers a mechanism by which hepatocytes begin to divide, continuing until the original mass of tissue is restored; text; indicates, that liver "knows" when to start and when to stop growing during liver regeneration. This is mediated via activation of several genes such as c-fos, c-jun and c-myc or and inhibition the process by other factors such as TGFβ [6]. The set point for liver growth is the ratio between liver mass and body mass. This ratio enables the liver to perform the amount of metabolic work needed to meet the functional requirements of the body. Thus, any surgical removal of tissue or hepatic functional deficiency will induce sequential changes in gene expression in the hepatocyte to restore the liver mass/body mass ratio. This condition induces the liver cell to move from quiescent state to the replicative state. On the other hand, when liver mass exceeds the body's functional needs, in the case of drug-induced liver growth, the liver loses mass to restore the optimal liver mass/body mass ratio upon cessation of drug treatment [7].

Section 1.1.1 Physiology of the liver

In mammals, the liver is composed of different cell types; parenchymal cells or hepatocytes which constitute about 90% of the liver by volume and nonparenchymal cells which constitute mainly sinusoidal lining cells and hemopoietic, bile duct and blood vessels wall cells. The sinusoidal lining cells consist of four types of cells: endothelial, Kupffer, fat-storing and pit (like

natural killer) cells [2]. Unlike most organs, the liver receives blood from two sources, the hepatic portal vein from the gastrointestinal tract which accounts for 75% of the liver's blood supply and the hepatic artery, which supplies 25% of the liver's blood. The liver consists of several lobes and each lobe contains thousands of six-sided units called a lobule, which is a structural and functional unit of the liver [2]. Liver has a number of essential roles in the body including glycogen storage, plasma protein synthesis and it plays an important role in metabolism of protein and lipid [4]. The liver also has an important role in detoxification and breaking down substances such as xenobiotic chemicals and metabolic waste; there are two types of metabolism, phase I, whereby the liver is using Cytochrome P-450 mixed function oxidase enzyme pathway to oxidise the potential toxicant and phase II, the conjugation pathway, whereby the hepatocytes add another substance (e.g. glutathione or a sugar) to a toxic chemical or drug to make it water-soluble, so it can then be excreted from the body via watery fluids such as bile or urine [9].

There are three alternative ways to describe the structure of the liver in terms of a functional unit: the classic lobule, the portal lobule, and the liver acinus. The classic lobule is the traditional way that explains the structural organization of the liver (Figure 1.1). It is based on the concept that blood flows from the periphery (portal triads) to the centre of the lobule (central vein). In the portal lobule concept, the major exocrine function of the liver is bile secretion. Thus, this concept defined the portal lobule as a triangular region with the portal triad as the centre and the central veins as the apices of the triangle (Figure 1.1). Recently, the hepatic acinus unit has been used to describe the structure of liver (Figure 1.1). It is the structural unit that provides the best correlation between blood perfusion, metabolic activity, and liver pathology [10]. However, the liver acinus, unlike the classic lobule, is difficult to visualize, but represents a unit that is of more relevance to hepatic function because it is oriented around the afferent vascular system. The hepatocytes in each liver acinus are arranged in three ellipsoidal zones: Zone 1, 2 and 3.

Those hepatocytes closest to the arterioles (zone 1or periportal, PP) are best oxygenated because they encircle the portal canals where the oxygenated blood from hepatic arteries enters the liver. Oxygen tension decreases toward the centrolobular region, zone 3 [4]. Moreover, zone 1 shows higher β-oxidation of fatty acids than zone 3. Zone 2 has no sharp boundaries, but is intermediate to both zones 1 and zone 3. Zone 3 is the region surrounding the central vein, so called centrilobular or perivenous (PV). Zone 3 is characterised by higher basal levels of cytochrome P450, ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase, the key enzymes of lipogenesis as compared to zone 1 [11]. This arrangement also means that cells in the centre of the acinus (zone 1) are the first to be exposed to, and potentially absorb, blood-borne toxins absorbed into portal blood from the small intestine

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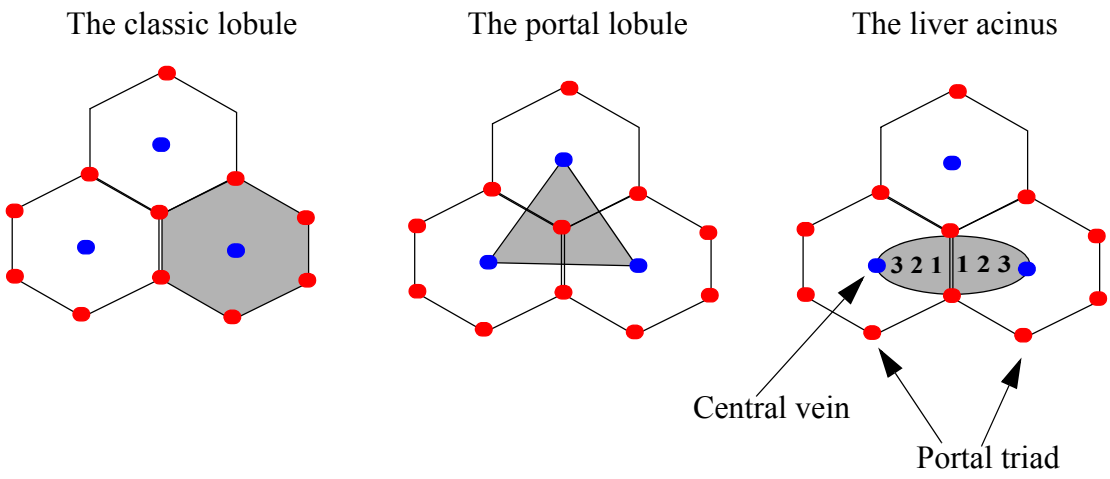


Figure 1.1 Schematic drawing illustrating the different organization of liver lobule. Liver lobule can be defined by three different ways. The classic lobule, which is hexagon-shaped and the lobule is filled by cords of hepatocytes that radiate the central vein (left). The portal lobule has a portal triad in the centre and the central veins at the peripheral angles of the lobule (center). The liver acinus, which was described as diamond or elliptical shaped, divided into three zones; zone 1 (periportal, PP) is the closest to the axis (is the short axis between two portal triad); zone 3 (perivenous, PV) is the furthest from the short axis; zone 2 is the area between zone 1 and 3. (Modified from Michael, 2003 [10])

Section 1.1.2 Liver growth

Living cells have the ability to multiply by replication of genetic material. This multiplication of a single cell can be divided into two essential processes: (1) the replication of DNA which

involves the incorporation of nucleotide precursors into DNA that can be detected by exposing the cell to a DNA analogue, such as bromodeoxyuridine (BrdU); and (2) the cell splitting itself into two daughter cells by the cell division process which consist of nuclear division (mitosis) and cytoplasmic division (cytokinesis) [12]. In general, the cycle of proliferating cells is divided into four stages G1, S, G2, and M phase. S phase is characterised by DNA synthesis and the amount of DNA in the cell doubles. At the end of the S-phase, the number of chromosomes have been replicated to 4n in diploid organisms and 2n in haploid organisms [13]. M (mitotic) phase is characterised by two processes; mitosis (prophase, metaphase, anaphase, telophase) and cytokinesis to generate two identical daughter cells. G1(first gap) is the period between the M-phase and the S-phase. In the middle of G1, cyclin-CDKs start to prepare cells for DNA replication in the S-phase. G2 (second gap) is the phase between S- and M-phase. In G2 stage, significant protein synthesis occurs notably the proteins for remodeling of microtubules to form the spindle apparatus [13]. There is another stage which is the non-proliferating state of cells, the G0 phase [14]. In normal rat liver, hepatocytes are in the G0 phase, or quiescent phase, of the cell cycle [15]. When induced to regenerate (for example, by partial hepatectomy), hepatocytes enter G1 phase, which is the initial stage in the cell cycle. They proceed through the cell cycle (G1-S-G2-M), and at the end of M phase, cell division occurs. Normally, hepatocytes may have one or two nuclei which contain 2n, 4n, 8n and even greater DNA amounts. The existence of binucleated hepatocytes is a consequences of DNA synthesis and mitosis with no of cell division [16]. There is a large proportion of polyploid nuclei in hepatocytes, and they are tetraploid or occasionally octaploid (8n) at G0 phase [14]. For example, normal adult rat liver is constituted from 85-90% tetraploid, 8-10% diploid and 1-3% octaploid cells. Among the tetraploid cells, 65% of the cells are mononucleate and 20% are binucleate hepatocytes [17]. Since cell division is rarely seen in hepatocytes and DNA synthesis activity in young adult rodent is infrequent (1% of the nuclei were in the S phase), then liver regeneration, (resection of the liver)

becomes a useful model for studying liver growth [18] [19]. Liver growth can be induced in two different patterns: compensatory regeneration (compensatory) and primary or direct hyperplasia (mitogen-induced liver growth). In compensatory regeneration, the DNA synthesis in the liver is induced by a loss of liver cells such as that seen during partial hepatectomy or cell necrosis, whereas in direct hyperplasia, DNA synthesis is induced without preceding cell loss [17] [20] [19]. The regenerative capacity of the liver was first clearly described by the two-thirds partial hepatectomy model in rodents by Higgins and Anderson (1931). They showed that when two thirds of the liver is surgically removed, the remaining liver enlarges until the original liver mass is restored at approximately 1 week after surgery when the regenerative process stops. Two main distinct changes happened during liver regeneration. Adaptive changes, where dramatic metabolic and circulatory perturbations were enforced due to liver mass loss and mitogenic changes, where the 95% of hepatic cell, which are normally quiescent, rapidly re-enter a replicative state [19]. In rat liver, the rate of DNA synthesis in parenchymal cells begins to increase after about 12 hours and peaks around 24 hours, whereas in non-parenchymal cells such as Kupffer and biliary epithelial cells it begins around 48 hours, and at ~96 hours for endothelial cells. The onset of DNA synthesis is synchronized in hepatocytes, starting in cells that surround the portal triad (periportal hepatocytes) of the liver lobule and then proceeding towards the central vein, pericentral hepatocytes [21]. However, the incidence of mitotic figures (M phase) is lower than the number of hepatocytes that undergo DNA synthesis during liver regeneration [19]. This is because partial hepatectomy leads to hepatic polyploidy, which increase nuclear DNA content. Moreover, cells with greater ploidy exhibit reduced mitogenic activity and increase the probability of cell death [22], and the ploidy of hepatocytes and percentage of binucleate cells increases with successive rounds of DNA synthesis, which ultimately limits further regeneration [22].

Section 1.1.3 Control of liver cell growth

Liver has the ability to regulate its growth through controlling a complex array of signal and transcription factors and on interaction of hepatocytes with non-parenchymal cells. Studies of liver growth in hepatocyte culture have limited relevance to *in vivo*, since *in vitro* study frequently does not mimic the liver: this may be because *in vitro* studies do not take into account the extracellular growth factors or systemic factors which might be crucial for liver growth [6]. For this reason, much of the investigation on the mechanism of hepatic growth has been done in partially hepatectomised animals. After partial hepatectomy (PH), a large number of genes are either newly expressed or increase their expression. These genes are believed to be responsible for the transit of quiescent hepatic cells into replicative cells through to two stages: the "competence stage" where the cell gets the ability to enter cell cycle (G0 to G1 phase) and the "progression stage" where liver cell progresses from G1 phase to undergo DNA synthesis (G1 to S phase). The G0/G1 transition involves the activation of certain genes termed immediate early genes (primary response), whereas during the G1/S phase transition, another set of genes is involved termed delayed response genes [6] [18] [23]. The priming phase, characterised by the expression of immediate early genes, takes up 4 hours after partial hepatectomy, whereas in the second phase (G1/S), hepatic cells are less synchronized than in the priming phase, since the variation in the length of G1 time varies in cells located in different areas of liver lobule [7]. The mechanism of liver growth during liver regeneration is quite complex. Several potential signaling stimuli are released in the liver or in circulation after the loss of hepatic cells. These signals can be classified into two main pathways: cytokine-dependent and a cytokine-independent pathway. Cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), norepinephrine, are not mitogens, but they do enable activation of an intracellular signaling cascade in the hepatocytes to allow the cell to passage from G0 to G1 of the cell cycle [24] [19]. In the cytokine dependent pathway, after partial hepatectomy (PHx) or liver injury, immune system components

such as lipopolysaccharide (LPS), complement factors C3a and C5a and intercellular adhesion molecules (ICAMs,) activate kupffer cells to produce tumour necrosis factor-α (TNF-α) [25] [26] [27] [19], which in turn up-regulates interleukin-6 (IL-6) transcription by the nuclear factor (NF)-kB pathway [19]. TNF-α and IL-6 activate neighbouring hepatocytes to produce the transcription factors, signal transducer and activator of transcription-3 (STAT3). NF-kB and STAT3 are proteins that bind to target genes in specific recognition sites to initiate or enhance their transactivation after partial hepatectomy within 30 min for NF-kB and 1-2 h for STAT3. STAT3 activates transcription of ~ 36% of immediate early genes [19]. In addition to the cytokine-dependent pathway, the growth-factor-mediated pathway is a second pathway involved in liver regeneration. It has been reported that IL-6 is not a powerful hepatocyte mitogen in the absence of partial hepatectomy [28] [19]. So, growth factors play a crucial role to drive the cell from G1- to S-phase. Transforming growth factor (TGFα) and hepatocyte growth factor (HGF) are considered to be the most important growth factors [29]. HGF is a paracrine factor synthesised by non-parenchymal cells, particularly stellate cells. TGFα is an autocrine growth factor produced from an active hepatocyte [30]. Both factors HGF and TGFα regulate various processes in the liver, as well as being direct stimulants of hepatocyte proliferation [7]. It has been reported that neither of these growth factors are capable of increasing DNA synthesis in quiescent hepatocytes *in vivo* [6]. In contrast, HGF and TGF increase DNA replication during the progression phase and help cells to move beyond a restriction point in G1 phase [7]. Many genes were identified and defined as immediate early genes. By using DNA microarray analysis, there are more than 100 immediate early genes which have been identified during liver regeneration [31] [19]. Some of these genes show transient up-regulation, whereas others were involved in protein synthesis and cell growth such as fos and jun family, egr-1, LRF-1, c-myc [7] [32]. Finally, when liver is restored and restructured, the liver growth and DNA synthesis eventually stop. The size of the liver is highly regulated and controlled, but, the signals that are controlling these

events are poorly understood. However, the most well-known inhibitor of hepatocyte proliferation is transforming growth factor-β (TGFβ) and related TNFβ-family member such as activin [33]. TNFβ is produced mainly by hepatic stellate cells and it is upregulated during liver regeneration. It has been reported that TNFβ mRNA increase within 3 to 4 hours after PHx, reaching a plateau in the liver at 48 to 72 hours [24]. Therefore, it has been postulated that the increased concentration of TGFβ is responsible for the termination of DNA synthesis.

Section 1.2 Peroxisome proliferators

Section 1.2.1 Peroxisomes

Peroxisomes are single-membrane bound organelles with peroxidative function. The term 'peroxisome' was introduced by de Duve and Baudhuin in 1965 [34]. They are present in most mammalian cells specially tissues active in lipid metabolism such as the liver. Peroxisomes contain oxidases and catalase enzymes and oxidise a variety of substances in the liver [35]. In rat hepatocytes, peroxisomes are normally an oval shape and comprise around 2% of the total cytoplasmic volume and cellular protein [36]. These organelles are easily identifiable in several species of animal, but not human, due to the presence of urate oxidase crystalline deposits in the matrix of these organelles. Peroxisomes have important metabolic functions, including catabolic pathways such as a breakdown of polyunsaturated medium-, long- and very long-chain fatty acids and some xenobiotics [37] and anabolic pathways such as cholesterol synthesis and dolichol synthesis [38]. Certain chemicals are capable of increasing the size and number of peroxisomes, and are designated as peroxisome proliferators [36] [35] [39] [5].

Section 1.2.2 Peroxisome proliferators

Reddy and Krishnakantha were the first people who used "peroxisome proliferators" to describe the hypolipidemic drug clofibrate and other structurally diverse chemicals causing hepatic peroxisome proliferation [39]. Peroxisome proliferators are a diverse group of chemicals that do

not necessarily have similar structure but all induce characteristic effects in the liver of treated rats and mice. The initial hepatic response in rodents is a dramatic increase in the size and number of peroxisomes up to 25% of hepatocyte cytoplasmic volume in association with the induction of some enzymes such as Cytochrome P450 4A [35]. In addition, these compounds cause a marked increase in liver weight (hepatomegaly) of susceptible species in the form of both hyperplasia and hypertrophy. Hepatocyte hypertrophy is a result of an increase in the size of liver cells with an increase in the numbers and size of subcellular organelles such as peroxisomes, mitochondria and smooth endoplasmic reticulum [36]. In contrast, hyperplasia is an increase of in the number of liver cells due to replicative DNA synthesis. Peroxisome proliferators include hypolipidemic drugs, plasticizers and organic solvents used in the chemical industry, herbicides, and naturally occurring hormones such as dehydroepiandrosterone. Peroxisome proliferators are excellent models for the study of liver growth and liver tumorigenesis because the liver is a target organ of many non-genotoxic compounds including PP and these chemicals regulate gene transcription through a receptor, PPARα, which is expressed in hepatocytes [40] [41].

Section 1.2.3 Chemical structure of peroxisome proliferators

Efforts have been made to reveal the structure-activity relationship of compounds causing proliferation of peroxisomes. For example, many peroxisome proliferators contain an acidic function [36]. This acidic function is normally a carboxyl group, either present in the parent structure or generated by metabolism such as clofibrate, which is metabolized to clofibric acid. On the other hand, Reddy and Lalwai (1983) classified these compounds into several groups; clofibrate and its analogues, plasticizers, other compounds, and dietary and other factors [5]. Bentley reported that compounds and physiological factors, which have been shown to induce peroxisome proliferation can be arranged into groups based on chemical structure (Table 1.1). Recently, it has been concluded that a lipophilic anion is the ultimate structure to induce peroxisome prolif-

eration [42].

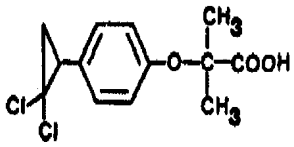
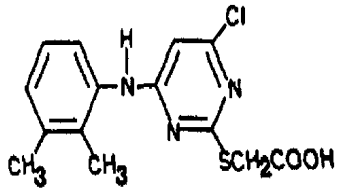
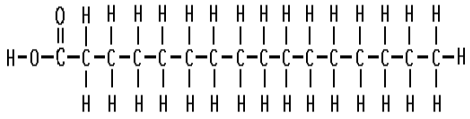
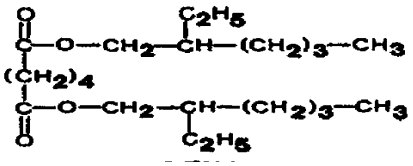
Group	Example	Structure
2-Phenoxyacetic acids	Ciprofibrate	
2-Phenoxyacetic acid and analogues	WY-14,643	
n-alkylcarboxylic acids	Palmitic acid	
n-alkylcarboxylic acids and their precursors	Di-(2-ethylhexyl)adipate	
Long-chain aklyldicar-boxylic acid and their precursors	Hexadecanedioic acid	$HOOC-(CH_2)_{14}-COOH$

Table 1.1 Classification of peroxisome proliferators based on chemical structure.

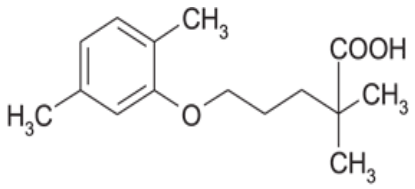
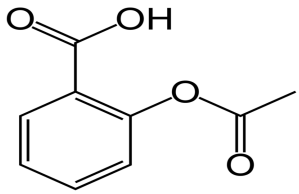
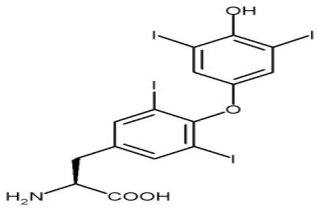
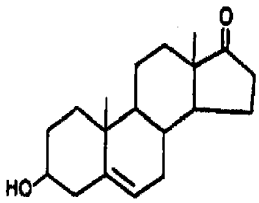
Group	Example	Structure
'Alkyl-aryl' carboxylic acids and their precursors	Gemfibrozil	
O-substituted benzoic acids and their precursors	Aspirin	
Non-carboxylic acids and their precursors	Thyroid hormone (T_4)	
Other compound and physiological factors	Dihydroepiandrosterone acetate	

Table 1.1 Classification of peroxisome proliferators based on chemical structure.

Section 1.2.4 Effects of non-genotoxic agents

Chemical carcinogens can be classified into two classes based on their mechanisms of action: genotoxic carcinogens and non-genotoxic (epigenetic) carcinogens. Genotoxic carcinogens are either immediately electrophilic or are metabolically activated by endogenous enzymes to highly reactive electrophilic compounds that will covalently interact with DNA to cause mutation, and ultimately cell transformation [43]. In contrast, non-genotoxic (epigenetic) carcinogens cause cell transformation through mechanisms that do not involve direct DNA damage [43].

Non-genotoxic agents constitute a diverse group of chemicals. Some of these group cause acute liver injury followed by regenerative hyperplasia such as carbon tetrachloride [44], whereas other groups cause cell proliferation without tissue damage such as peroxisome proliferators class [45]. In the liver, tissue damage is indicated by an increase in liver-specific enzyme such as alanine aminotransferase (ALT), in the serum [46]. Other examples of non-genotoxic hepatocarcinogens are phenobarbital and TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)] benzene). These compounds are ligands of the constitutive androstane receptor (CAR), a member of the nuclear hormone receptor superfamily [47] [48]. Phenobarbital and TCPOBOP have been reported to increase DNA synthesis in hepatocytes [206] [49].

Section 1.2.5 Peroxisome proliferators are non-genotoxic carcinogen

Peroxisome proliferators (PPs) are one of the most widespread of non-genotoxic (non-mutagenic) class of carcinogen. Unlike genotoxic carcinogens, peroxisome proliferators do not give a positive result with mutagenicity tests but are still able to perturb hepatocyte proliferation through altered gene expression or interruption of growth-regulatory signal transduction pathways [50]. In addition, several years ago, data has been generated to support the concept that a large fraction of carcinogens are not mutagenic and do not directly damage DNA [51]. Current estimates are that about half of all rodent carcinogens are mutagens and that the other half is not mutagenic [52]. Several tests for genotoxicity of peroxisome proliferators have failed to detect any damage to DNA. *In vivo*, the peroxisome proliferator, WY 14,643, failed to induce unscheduled DNA synthesis in rat hepatocytes following treatment at a dose which induces liver cancer [53]. Galloway *et al.* (2000) reported no evidence of chromosome aberration induction with nafenopin in Chinese hamster ovary (CHO) cells at dose up to the level that are toxic to CHO cells. Moreover, an extensive literature review for 18 peroxisome proliferators concluded that peroxisome proliferators are predominantly non genotoxic [51]. Some peroxisome proliferators

show a weak positive response in the genotoxicity test. For example, ciprofibrate shows a positive effect with genotoxicity tests, sister chromatid exchange (SCE), chromosomal aberration (CA), and micronuclei (MN) in rat hepatocytes [54]. However, ciprofibrate gave negative result with unscheduled DNA synthesis (UDS) and Ames tests [55]. Nilsson *et al*. (1991) who examined the ability of ciprofibrate to induce chromosomal damage in Wistar rats after treatment with 750 mg $kg^{-1}day^{-1}$ ciprofibrate for 14 days. Hepatocyte were then isolated and examined for micronuclei and chromosomal aberration. Ciprofibrate did not show any increase in chromosomal damage as compared to control [56]. The highly sensitive ^{32}P-postlabeling assay was employed to detect peroxisome proliferator-DNA adducts in male F-344 rats treated with clofibrate, ciprofibrate, Wy-14643 or di(2-ethylhexyl)phthalate *in vivo* and *in vitro*. No adducts were detected by this assay in the DNA isolated from the livers of rats treated *in vivo* or *in vitro* with any of the peroxisome proliferators [57].

Section 1.3 The nuclear receptor superfamily

Nuclear receptors are a class of proteins located inside cells that are responsible for mediating the action of hormones and certain other molecules. They have the ability to directly bind to DNA at specific sites and regulate the expression of adjacent genes, hence these receptors are classified as transcription factors. So they serve as on-off switches for transcription within the cell nucleus. The nuclear receptor superfamily includes nuclear hormone receptors (NHRs) and orphan nuclear receptors. NHRs are receptors for which hormonal ligands have been identified, whereas orphan receptors have a similar structure to other identified receptors but their ligands are unknown, at least at the time the receptor is identified [58]. Manglesdorf *et al*. (1995) proposed four categories of nuclear receptors; Class I receptors, which are located in the cytosol when ligands bind to it before they translocate from the cytosol to nucleus. They work as homodimers binding to half-site hormone response elements (HREs) inverted repeats. Class I has subfamily estrogen-like receptors, that include estrogen receptor (ER) group. Class II receptors,

in contrast to class I, exist in the nucleus before binding to ligands, and bind as heterodimers with RXR receptor partners and function in a ligand dependent manner. Class II has subfamily thyroid hormone receptor-like that include different receptors such as thyroid hormone receptor (THR), and retinoic acid receptors (RAR), constitutive androstane receptor (CAR), and peroxisome proliferator receptors (PPAR). Class III nuclear receptors are similar to class I receptors in that both classes bind to DNA as homodimers, but class III in contrast to class I nuclear receptors, bind to direct repeat instead of inverted repeat HREs. Class III has subfamily retinoid X receptor-like which include Retinoid X receptor (RXR). Class IV are the nuclear receptors that bind either as monomers or dimers, but only a single DNA binding domain of the receptor binds to a single half site HRE. The last two classes are considered to be orphan receptors [59].

The regulation of gene expression by nuclear receptors is ligand dependent. In other words, nuclear receptors normally are only active in the presence of ligand. More specifically, ligand binding to a nuclear receptor results in a conformational change in the receptor which in turn activates the receptor resulting in up-regulation of gene expression [60]

Section 1.4 Mechanism of Peroxisome proliferation: receptor-mediated mechanism

It was hypothesized that effects induced by peroxisome proliferators were the result of a receptor-mediated mechanism [5]. This hypothesis remained until the receptor was cloned from mouse liver by Issemann and Green in 1990 and called peroxisome proliferator-activated receptor α (PPARα) [61]. To date, three different isoforms of PPAR were cloned: PPARα, PPARβ/δ and PPARγ. The different PPARs appear to perform distinct physiological functions. PPARγ was cloned from several mammalian species including human. PPARγ is expressed at high levels in adipose tissue, and is an important regulator of adipocyte differentiation and lipid metabolism [62]. In mouse, there are two PPARγ isoforms have been described, that is, γ1 and γ2 [63]. PPARγ1 is found mainly in liver, whereas PPARγ2 is expressed exclusively in adipose tissue

[64]. PPARβ/δ is widely expressed in a wide range of tissues and cells, with relatively higher levels expression in adipose tissues, brain, skin, heart, kidney, and digestive tract [62]. PPARα has critical roles in regulation of fatty acid metabolism. It mediates the expression of genes encoding both peroxisomal and mitochondrial fatty acid metabolizing enzymes at both the constitutive and inducible level in tissues such as liver, kidney, heart, and intestinal mucosa [43] [61].

PPAR isoforms	Action
PPARα	- Serve as receptor for diverse compounds, e.g. fibrates that induce hepatic peroxisome proliferation, hepatomegaly, hepatocarcinogenesis, and activate expression of several enzymes such as CYP4A in rodent. - Plays a critical roles in the β-oxidation fatty acid and lipid metabolism. - Induce expression of the fatty acid transport protein. - Upregulates transcription of long chain fatty acid acetyl-CoA synthase [65]. - Regulation of inflammation [64]
PPARβ/δ	- Developmental and physiological roles in adipose tissues, brain, skin, heart, kidney, and digestive tract tissues [62] - Play a critical role in the control of keratinocyte proliferation and helping in rapid healing of a skin wound in mouse [66] - Play an important role in adipocyte proliferation and differentiation [67] [62]

Table 1.2 The physiological actions of PPAR isoforms .

PPAR isoforms	Action
PPARγ	- Regulates the expression of numerous genes involved in lipid metabolism, including acyl-CoA synthase and lipoprotein lipase [65] - Plays an important role in the differentiation, proliferation, and programmed cell death of adipocytes. - Participating in the regulation of glucose and lipid homeostasis, immune function, and cell growth regulation [62].

Table 1.2 The physiological actions of PPAR isoforms .

PPARα target genes encodes for the microsomal CYP4A family (CYP4A1 and 4A3) and several cell recognition surface proteins including CD24 and CD39 [35]. After murine PPARα was cloned, several PPARs have been cloned from different species such as frog [68], rat [69], rabbit [70], and human [40].

Section 1.4.1 Mode of action of PPARs

PPAR receptors, like other nuclear receptors, have four functional domains: the ligand-independent transcriptional activation function domain (AF-1) that works in a cell-specific manner; the highly conserved DNA-binding domain (DBD) holding two zinc finger DNA binding motifs that target the receptor to specific DNA sequences called PP response elements PPRE in responsive genes; the ligand-binding domain (LBD), is responsible for interacting with a broad range of ligands and responsible for conversion of PPAR from an inactive form to a form that can bind DNA.[64]. Ligand-dependent activation function domain (AF-2) is involved in the generation of the receptor'coactivator binding site Figure 1.2. In rodents, PPARα regulates transcription of a number of genes by binding to a specific DNA regulatory element located in the upstream promoter region of these genes. In the beginning, a peroxisome proliferator binds to a specific site in PPARα which then heterodimerises with an other receptor, the retinoid X receptor, the re-

ceptor for 9-cis-retinoic acid (9c-RA). The ligand induces allosteric changes within the LBD that regulate interactions with coactivator and corepressor complexes, which can further control the transcriptional activities of the nuclear receptors [71]. The heterodimer, PPAR–RXR, binds to the consensus PPRE as a direct repeat of two AGGTCA X AGGTCA. Interaction between the PPAR–RXR heterodimer and other transcription factors such as coactivators or corepressors leads to either increases or decreases in transcription of target genes Figure 1.3. Gene regulated by PPRE's may contain sequences that are not uniform, as they may differ by five nucleotides at maximum. For example, Rat CYP4A1 has a PPRE sequence TCCCCT C TGACCT [72], Rat acyl-CoA oxidase has PPRE sequence TGACCT T TGTCCT [73], and human fatty acyl-CoA has PPRE sequence: AGGTCA G CTGTCA [74].

Figure 1.2 Functional domain of peroxisome proliferator activated receptor (PPARs). As any nuclear receptor, PPAR protein has five domains, activation function domain (AF-1) in N-terminal. DNA-binding domain (DBD) consisting of two highly conserved zinc-finger motifs that bind the receptor to specific DNA sequences. a hinge region that allows flexibility of the nuclear receptors to dimerise to other nuclear receptors and binding to DNA. a large region containing the ligand-binding domain (LBD). Finally a second ligand dependent activation function domain (AF-2) at the c-terminal.(Modified from Li and Palinski 2006 [212])

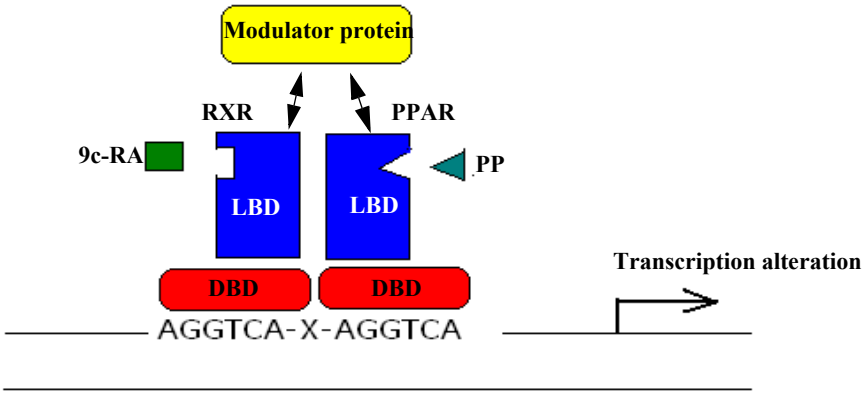


Figure 1.3 Mechanism of gene transcription by a peroxisome proliferator-activated receptor. peroxisome proliferator (PP) binds to PPAR via LBD to activate the receptor, and then heterodimer with the retinoid X receptor (RXR), the receptor for 9-cis-retinoic acid (9c-RA). The PPAR-RXR heterodimer, through their DNA-binding domains (DBD), binds to the consensus sequence 5'-AGGTCA-X-AGGTCA-3'. Interactions between the PPAR-RXR heterodimer, modulator proteins, and the transcription machinery initiate transcription process. (Modified from Corton 2000 [64])

Section 1.4.2 The Genetic variation in PPARα

Tugwood has reported that there are variants in the human PPARα gene. These included Thr^{71} to Met, Lys^{123} to Met, Ala^{268} to Val, Gly^{296} to Ala, and Val^{444} to Ala [75]. It has been proposed that the amounts of PPARα mRNA expression in the liver were behind the difference between responsive and non-responsive species since there was an estimation that human hepatocytes express around 5-10% of the levels found in rodent hepatocytes [76]. However, despite the low levels of mRNA, humans are still able to respond to PPs by decreasing serum lipid levels [77].

Section 1.4.3 PPARα knockout mice

PPARα KO mice are very informative to study relationship between the PPARα receptor and liver's response to peroxisome proliferators. Although mPPARα was shown to be activated by peroxisome proliferators [78] [79] [80], it was not known if peroxisome proliferation can also be stimulated by other receptor isoforms (mPPARδ, mPPARγ and mPPARβ). It was also un-

clear whether the retinoid X receptor (RXR) is required for mPPARα activity *in vivo*. The generation of PPARα null mice has added much information in understanding the role of this receptor in peroxisome proliferation and lipid metabolism. Gonzalez has developed a line of mPPARα-deficient mice as a model to test the hypothesis that PPARα mediates the pleiotropic response of peroxisome proliferators by disrupting the ligand-binding domain of the isoform of mouse PPAR (mPPARα) by homologous recombination [81]. PPARα mutant mice are fertile and healthy, but do not display the pleiotrophic effects of hepatomegaly, peroxisome proliferation or hepatocarcinogenesis that appear on the wild-type mice [81]. Evidence for the involvement of PPARα in replicative DNA synthesis and hepatocarcinogenesis comes largely from work with PPARα-null mice following dietary exposure to WY 14,643. In these studies, it was shown that wild-type (129S4/SvJae) mice showed increased hepatic labeling by bromodeoxyuridine (BrDU) compared to controls while no increase in hepatic labeling index was observed in the Sv129 ter PPARα null mice. Also, chronic treatment with WY14,643 resulted in a 100% incidence of hepatocellular neoplasia in wild type mice while the null mice were unaffected [82]. Further analyses revealed that the basal expression of seven mitochondrial enzymes in the liver, including very-long-chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, long chain acyl-CoA synthetase, and short chain-specific 3-ketoacyl-CoA thiolase, are lower in Sv/129 PPARα-null mice as compared to wild-type animals [83]. This latter study emphases the importance of PPARα for the constitutive level of mitochondrial β-oxidation. In contrast, the constitutive expression of enzymes involved in peroxisomal β-oxidation of very long-chain fatty acids, lignoceric acid, was unaffected in PPARα null mice (Sv/129), suggesting that constitutive expression of enzymes involved in peroxisomal β-oxidation is independent in PPAR-null mouse livers. Thus, it is clear that this receptor plays a necessary role in PPARα-ligand-induced peroxisome proliferation and cell proliferation in the liver, and subsequent increases in hepatocellular tumors.[83] [64].

Section 1.5 Induction of hepatic DNA synthesis by peroxisome proliferators

Section 1.5.1 Acute effects of peroxisome proliferators

Exposure to peroxisome proliferators in short term causes marked changes in liver characteristics. These changes are associated with hepatocyte hypertrophy and hyperplasia and induction of many peroxisomal, mitochondrial enzymes and cytochrome p450 which are involved in fatty acid metabolism [84] [42]. Several reports have been published showing that liver enlargement in male rats occurs within a few days of administering the peroxisome proliferators and reaches a steady-state level within 10 to 14 days [85] [5]. The liver weight returns to normal usually within 10 to 14 days after the cessation of administration of a peroxisome proliferator [5]. The comparison of acute and chronic effects of peroxisome proliferators methylclofenapate (MCP) rat liver was studied by Barrass *et al.*[86]. After acute (1 week) and chronic (26 week) treatment with MCP, the relative liver weight was significantly increased to 170% of control and to 231% of control respectively. Peroxisome proliferation as assessed by palmitoyl CoA oxidation was also increased 17 and 12- fold over controls. Hepatic DNA replication and labelling indices were also increased 5 and 5.7-fold over controls. The hepatomegaly is maintained for as long as the PP is administered. This hyperplastic or mitogenic effect is maximum during the first week of the treatment as assessed by Bromodeoxyuridine (BrdU) incorporation with immunohistochemistry technique [87].

Sex differences studies on acute effect of peroxisome proliferators have been published. Nakajima *et al.* (2000) reported that the peroxisome proliferator trichloroethylene (0.75g/kg for 2 weeks by gavage) did not show any sex differences in peroxisome proliferation in SV/129 mice, but the induction of PPARα protein and mRNA was greater in males than females [88]. In addition, other studies have reported that peroxisome proliferators perfluoro-octanoic acid [89], and dehydroepiandrosterone [90] cause a greater induction of peroxisome proliferation and per-

oxisomal β-oxidation in male rat than in female.

Section 1.5.2 Chronic effects of peroxisome proliferators

Chronic exposure of rodents to peroxisome proliferators not only induces cell proliferation, but will lead to tumour growth [5]. Long-term administration of peroxisome proliferators to rodents is associated with increased risk of hepatocarcinogenesis [43]. This incidence of tumorigenesis depends on PP potency, species, and the dose [36]. Many peroxisome proliferators have been reported to produce liver tumors, including hepatocellular carcinoma in life-long feeding of rats or mice, although to a different extent. For example, potent compounds (e.g. Ciprofibrate, Wy-14,643) may produce 100% incidence of tumors within a short period (40-60 weeks), whereas weaker agents (e.g. Clofibrate, DEHP) may require two years of administration to produce hepatocellular carcinomas [91] [36] [92]. Long term administration of the PPARα agonist, Wy 14,643, for 11 months caused a 100% incidence of liver tumors in Sv 129 wild type mice, whereas PPARα-null mice were refractory to this effect [82]. In conclusion, marked compound potency differences are known to exist. The mechanism by which peroxisome proliferators cause liver cancer is not currently understood [93]. Several mechanisms have been proposed to account for peroxisome proliferator-induced hepatocarcinogenesis. These hypotheses include oxidative stress and promotion of liver preneoplastic lesions (see Section 1.9.3).

Section 1.6 Species differences in response to peroxisome proliferators

Many studies have investigated species differences in hepatic peroxisome proliferation [77] [94] [45] [5] [95]. Clearly the rat and mouse are the most sensitive species to peroxisome proliferators [96] [97] [94] [36] [98] [99], whereas the hamster shows an intermediate response [100] and guinea pig, monkey, and human appear to be relatively insensitive comparing to the mouse and rat [51] [101] [102] [77] [103] [98]. Several studies have examined the effect of the PPARα agonist, diethylhexylphthalate (DEHP), on different species both *in vivo* and *in vitro*.

In an *in vivo* study, after 4 days of exposure to DEHP, F-344 rats exhibited induction in peroxisomal β-oxidation, DNA synthesis and suppression of apoptosis. In contrast, there was no response of guinea pig liver to DEHP [104]. In rat hepatocytes *in vitro*, mono (2-ethylhexyl)phthalate (MEHP), a principal metabolite of DEHP, induced peroxisomal β-oxidation, DNA synthesis and suppressed apoptosis. In contrast to the pleiotropic response noted in rat hepatocytes, there was no response of human hepatocytes to MEHP [105] [104]. More studies with a variety of compounds including ciprofibrate, methylclofenapate, DEHP, and nafenopin have demonstrated that the Syrian hamster exhibit an intermediate response, whereas in most studies the guinea pig is either nonresponsive or refractory [101] [94] [106] [98] [107] [108]. Other studies have evaluated species differences in cell replication and hepatocarcinogenesis. Although nafenopin and Wy-14,643 were potent mitogens in rat liver, they do not appear to stimulate replicative DNA synthesis in Syrian hamster hepatocytes after either acute or chronic treatment [109] [110].

Lake *et al.* (2000) have evaluated species differences of rats (Male Sprague–Dawley), hamsters (Syrian) and guinea pigs (Dunkin–Hartley) in the hepatic effects of three potent rodent peroxisome proliferators, namely methylclofenapate (MCP), ciprofibrate (CIP) and Wy-14,643 (WY), on hepatic peroxisomal and microsomal fatty acids enzyme activities, CYP4A mRNA level, replicative DNA synthesis and transforming growth factor-β1 (TGF-β1) gene expression. He showed that relative liver weights were significantly increased in treated rats and hamsters, but not in guinea pigs. In addition, peroxisomal palmitoyl-CoA oxidation and microsomal lauric acid 12-hydroxylase fatty acid oxidising enzyme activities and CYP4A isoform mRNA levels were significantly increased in rats and Syrian hamsters, whereas only minor effects were observed in the guinea pig. Labelling indices were increased by MCP in the rat, but neither MCP, CIP nor WY produced any significant effect on replicative DNA synthesis in the Syrian hamster

and guinea pig [111]. Similarly, no peroxisome proliferating was found in human liver of a group males and female received fenofibrate for between 6 to 36 months [112]. However, humans are still able to respond to PPs by lowering serum lipid levels [77]. These results provide further evidence for distinct species differences in response to peroxisome proliferators

Section 1.7 Strain differences in induction of DNA synthesis

It is well known that peroxisome proliferators exhibited species differences in PP-induced pleiotropic effects (Section 1.6), with maximal effects in rats and mice e.g. [113]. However, little information is available about strain differences in the effect of peroxisome proliferators either in mice or rats. Some mouse strains show variation in susceptibility to liver-tumor induction by genotoxins. For example, mouse strain C3H/HeJ are approximately 50-fold more susceptible to liver-tumor induction by diethylnitrosamine (DEN) or ethylnitrosourea (ENU) than C57BL/6J mice [114] [115]. However, bioassays have not been performed with peroxisome proliferators to determine if there is a strain difference in peroxisome proliferator-induced tumorigenesis. A study by Dwivedi *et al.* (1989) [3] examined that the hepatic effect of ciprofibrate over 2 weeks in nine stains of mice, C3H/HeN, B6C3F1, CFW, CF-1, CB6F-1, CBA/Ca, BALB/cAnN, DBA/2N, C3FeB6F1/J. The study revealed that C3H/HeN mice showed no significant induction of hepatic DNA synthesis, but all other stains showed approximately the same levels of DNA induction. However, the C3H/HeN mouse strain showed a trend for DNA induction. Dwivedi *et al*. concluded there was no significant strain difference in ciprofibrate-induced hepatic pleiotropic response across the mice strains [3].

In rats, Biegel *et al*.(1992) [116] demonstrated the strain differences in peroxisome proliferation and hepatic cell replication in rat strain; F344 and CD BR (CD). He showed that basal β-oxidation activity, a metabolic marker of peroxisome proliferation and hepatic cell replication were about twofold higher in CD than F344. After treatment with WY-14,643 (1000ppm), peroxi-

some proliferation and DNA synthesis in CD rats were significantly higher (~2-fold) than those in F344 rats [116]. Ciprofibrate induced a ninefold increase in peroxisomal fatty acyl-CoA oxidase in Sprague-Dawley, Wister and F344 strains, whereas a marked 35-fold was noted in Long-Evan strain [117].

Section 1.8 The biology of hepatocarcinogenesis

Cell proliferation plays a fundamental role in the initiation of liver carcinogenesis and DNA replication is required to sustain a permanent mutation. Columbano *et al.* (1981) showed that rats treated with nonnecrogenic dose of a genotoxic carcinogen have shown very few or no foci. However, when the genotoxic carcinogen was coupled with a cell-proliferative stimulus such as partial hepatectomy, foci of preneoplastic hepatocytes were detected [118].

Cancer is a diverse class of diseases resulting from failure of mechanism that control the growth and proliferation cells [13]. Cancer differs widely in their causes and biology. During normal development, cells are in balance between cell division and death. However, in cancer cells, this balance is interrupted and the cancer cells start to grow without respect to normal limits [13]. For this reason, the cancer cells could invade and destroy adjacent tissues, and may spread to distant anatomic sites through a process called metastasis. So, cancer can be classified in to two types: benign or malignant tumor. Benign tumors are characterised by an inability to spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures [119]. Moreover, benign tumor are encapsulated usually by a fibrous capsule, which make it distinct from malignant tumor [13]. In contrast, cells composing a malignant cancer are differentiated from benign tumors in that they grow and divide rapidly, and invade nearby tissue and other organs, spread to distant locations (metastasis) and become life threatening [13]. Cancer may affect different organs in the body such as lung, breast, stomach and liver [119]. Liver cancer is the sixth most common cancer worldwide in terms of numbers

of cases (626,000 or 5.7% of new cancer cases). It is the third most common cause of death from cancer [120]. Basically, liver tumors can be classified either as primary (which originate in the liver itself) or secondary where the cancer started in another part of the body and has spread to the liver by a process called metastasis. Liver is consider to be a common site for metastasis from other organs. The most common types of benign liver tumors, include hemangiomas (a swelling or cluster of blood vessel cells), focal nodular hyperplasia and adenomas (a cluster of hepatocytes, often encapsulated). The most common type of diagnosed malignant liver tumors is hepatocellular carcinoma, HCC [121] [122], these tumors arise from hepatocytes. The second most common tumor in the liver is the cholangiocarcinoma (7-15%), these tumors arise form bile duct cells [123]. Rare types of malignant liver tumors include, angiosarcomas (from blood vessel endothelial cell tumor) and lymphomas (from Kupffer cells). Liver carcinogenesis is characterized by the sequential stages defined as initiation, promotion and progression [124]. The initial stage is irreversible cellular alteration caused by a carcinogen resulting in the generation of mutation. Some initiators of liver tumour are able to interact directly with DNA such as methylnitrosurea (MNU), while many chemical carcinogens need to be enzymatically activated to electrophilic analogs mainly by microsomal cytochrome systems before they act as attack DNA such as Benzo[a]pyrene [125]. It has been published that treatment of rats with single dose of DEN (10 to 30 mg kg^{-1}) produces irreversible liver foci and if the single dose DEN treatment is preceded by 2/3 partial hepatectomy 20 to 24h earlier, the number of irreversible foci is increased tenfold [126]. The second stage is promotion which causes pre-neoplastic cells to proliferate. The promotion can be reversible or irreversible [124]. In the rat liver, tumor promotors have been found to stimulate proliferation as well as to inhibit apoptosis both in normal hepatocytes, foci, and nodules. In addition the response is much stronger in the preneoplastic islands [211]. Some promoters exhibit specificity and differentiation on the stimulation of initiated cells. For example, 2-acetylaminofluorene (2-AAF) can promote the outgrowth of foci and nod-

ules without stimulating normal liver growth of rat [127]. It has also reported that phenobarbital promotes the outgrowth of preneoplastic rat liver foci initiated by DEN, but has no effect on foci initiated by aflatoxin B_1 [128]. Cattley *et al.* (1994) also has examined the hypothesis that promoting activity for peroxisome proliferators depends on selection of the initiating agent by treating the rats with WY-14,643 or clofibrate after rats were initiated with either 2-acetylaminofluorene (2-AAF) or diethylnitrosamine (DEN). Homogeneous basophilic foci were observed in PP-treated rats initiated with DEN, but not with PP-treated rats initiated with 2-AAF [129]. Non-genotoxic carcinogens are believed in many cases to play a role in inducing hyperplasia resulting in tumour promotion [130]. Peroxisome proliferators as non-genotoxic carcinogens promote the development of preneoplastic foci following initiation with DEN. Moreover, administration of peroxisome proliferator without initiation can also lead to formation of focal lesions after 5 to 6 months, neoplastic nodules after 6 to 12 months, and hepatocellular carcinoma after 1 to 2 years [131]. The final step in the hepatocellular process is progression which is transformation of initiated cells to the fully malignant phenotype. In this stage preneoplastic foci transform into neoplastic nodules after a few months after the start of the carcinogen treatment. Like preneoplastic foci, nodules have been shown to be clonal in origin and have elevated proliferative activity. Neoplastic nodules may be divided into at least two distinct subgroup, reversible and persistent nodules [124].

Section 1.9 Peroxisome proliferator-induced hepatocarcinogenesis in rodents

Several mechanisms have been proposed to describe the action of peroxisome proliferator in induction of hepatocarcinogenesis in rodents. These hypotheses include: (a) The oxidative stress model, (b) enhanced cell replication, and (c) the promotion of spontaneous preneoplastic lesions.

Section 1.9.1 Oxidative stress hypothesis

The oxidative stress is hypothesized to be a common pathway for many non-genotoxic chemical carcinogen [132]. However, the role of oxidative stress has been questioned. The oxidative stress hypothesis is based on the hypothesis that long term administration of peroxisome proliferators produces a sustained oxidative stress in rodent hepatocytes due to an imbalance in the production and degradation of hydrogen peroxide [133] [134]. In rodent liver, peroxisome proliferators markedly increase peroxisomal fatty acid β-oxidation and the H_2O_2-generating enzyme acyl-CoA oxidase but only yield a small increase in catalase activity [42]. Thus, the hypothesis is that excess production of hydrogen peroxide from the β-oxidation pathway is not destroyed by peroxisomal catalase will, and diffuse easily across the peroxisome membrane to the cytoplasm where it will be degraded by other enzymes, such as glutathione peroxidase. However, cytosolic selenium-dependent glutathione peroxidase activity and other enzymes such as superoxide dismutase and glutathione S-transferase, cytoplasmic enzymes capable of degrading H_2O_2, are often reduced by PPs [109] [134]. Thus, excess production of H_2O_2 could result in H_2O_2 in the cytoplasm, where it can attack membranes and DNA either directly or via other reactive oxygen species [43] [36] [109]. However, this hypothesis does not match with other findings. Tamura *et al*.(1990) [135] has examined whether H_2O_2 has a role in PP-induced hepatocarcinogenesis *in vivo* and *in vitro* using F-344 rat fed clofibrate, bezafibrate and DEHP for up to 78 weeks. Hepatic hydrogen peroxide increased slightly by these chemicals. The rate

of leakage of peroxisomal H_2O_2 into cytosol increased 2.5-4-fold. In contrast, the hepatocellular contents of H_2O_2 were not affected by the induction. Moreover, the H_2O_2 leaked from peroxisome into cytosol would be quickly decomposed. Thus, peroxisomal H_2O_2 does not appear to play an important role in liver tumour [135].

Weak evidence exists for the hypothesis that increasing H_2O_2 in PP-treated animals leads to DNA adduct formation and hepatocarcinogenesis. Conway has reported no increase in exhalation of ethane, a sensitive indicator of oxidative damage, on F-344 rats fed WY 14,643 for 23 to 345 days [136]. Formation of DNA adducts after exposure to peroxisome proliferators is also very low. Oxygen radicals can attack DNA to form a variety of modified DNA bases including 8-hydroxydeoxyguanoside (8-OHdG). These adducts can lead to mutation during DNA replication. Treatment with a number of peroxisome proliferators has been reported to increase levels of 8-OHdG in rat hepatic DNA [137] [138]. However, such increases are small and do not correlate with chemical potency of peroxisome proliferators. For example, F-344 rats fed either ciprofibrate (0.025%), DEHP (1.2%) or DEHP(2.5%) exhibited little difference in the level of 8-OHdG [137] [138] but substantial differences in carcinogenic potency. Another study reported that 8-OHdG did not increase in rats treated with WY-14,643, (0.1% or 0.005%); di(2-ethylhexyl)phthalate (1.2%); clofibric acid (0.5%) or phenobarbital (0.05%) for 3 or 11 weeks [139]. Most studies of 8-OHdG have utilized whole liver homogenate which contain nuclear and mitochondrial DNA, and normal levels of 8-OHdG in mitochondrial DNA are 16-fold greater than in nuclear DNA due to the extensive oxygen metabolism in these organelles [140]. Isolated hepatic nuclei also exhibited no increase in adduct 8-OHdG in PP-treated rats [139].

Recently, a new approach was introduced to assess oxidative DNA damage by Rusyn *et al*. (2000) [141]. The base excision repair (BER) system is a multiple enzyme system responsible for repairing oxidative DNA damage [141]. BER enzymes are induced *in vivo* and *in vitro* by

oxidative stress. The primary pathway for 8-OHdG removal is the enzymes OGG1, a glycosylase/lyase, AP endonuclease (APE), polymerase (Pol) and ligase [142]. Rusyn et al. have reported that PPs with varying carcinogenic potency induce the BER system in both rat and mouse liver in a dose-, time-, and carcinogenic potency-dependent fashion. This work provide evidence for the role of oxidative stress in PP-mediated carcinogenesis [141]

Although there is much speculation in the literature that oxidative stress may play a role in PP-induced hepatocarcinogenesis through increasing in hydrogen peroxide after chronic administration of PP, the evidence does not support the oxidative stress hypothesis as a model to explain the mechanism of PP-induced hepatocarcinogenesis. For example, the evidence is poor that there is mutation of DNA when animals are given peroxisome proliferators. Wy 14,643 at carcinogenic levels had no effect on unscheduled DNA synthesis [53].

Section 1.9.2 Enhanced cell replication

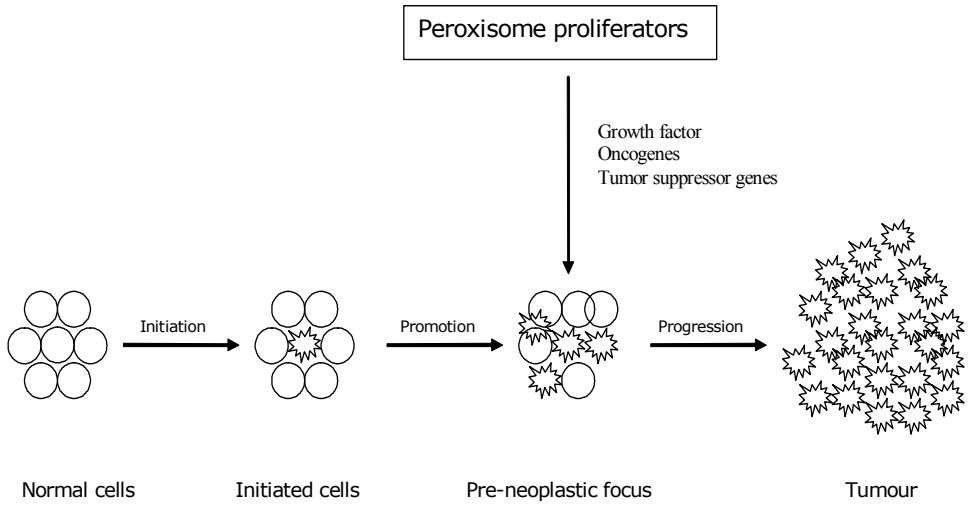
Cell division plays a critical role in the initiation stage of carcinogenesis. It has been proposed that any agent that causes persistent cell replication can be indirectly mutagenic because cell replication can increase the frequency of spontaneous mutations and the probability of converting DNA adducts into mutations before they can be repaired [143]. Cell replication is also an important factor in the promotion and progression of initiated cells into tumors. Peroxisome proliferators are known to increase cell replication in rodent hepatocytes during the first few days of treatment [144] [145] [45]. Continuous infusion of DNA precursor rather than pulse-labelling for detection of DNA replication in PP-treated animals is more sensitive and necessary for detection of chronic replicative DNA synthesis [36]. For example, Popp *et al*. (1993) observed a correlation between increases in chronic replicative DNA synthesis and hepatocarcinogenesis in F-344 rats treated with DEHP and WY 14,643 for up to 365 days. All rats fed Wy-14,643 for 365 days had numerous grossly visible nodules in comparison to none in the livers

of DEHP-fed or control rats. Rats implanted with 7-day osmotic pumps containing tritiated thymidine produced 5- to 10-fold increase in replicative DNA synthesis after treatment with WY 14,643 for 18 to 365 days as compared to DEHP-fed rats or controls [145]. Apart from compound potency, dose also plays an important role in sustained stimulation of cell proliferation. Wada *et al*. [146] showed that hepatocellular replication in rats dosed with 5 and 10 ppm WY-14,643 returned to control levels after three weeks. In contrast, rats dosed with 50, 100, and 1000 ppm WY-14,643s had sustained increases in cell replication for up to 13 weeks [146]. While there is a strong correlation between increases in cell replication and hepatocarcinogenesis, there was no relationship between the induction of peroxisome proliferation and hepatocarcinogenesis [84]. Parzefall et al reported that in purified hepatocyte cultures, WY-14,643 and nafenopin had no effect on DNA synthesis, whereas they did increase ACO activity [147]. In addition, other study showed a poor correlation between peroxisome proliferation and hepatocarcinogenesis after treating the rats with DEHP and WY-14,643 [145].

Section 1.9.3 Promotion of liver preneoplastic lesions.

The oxidative stress hypothesis does not explain well PP-induced hepatocarcinogenicity, which suggests other potential mechanisms are important for how these PPs induce carcinogenicity. One approach to characterization of the mechanism of PP-induced hepatocarcinogenicity is to evaluate the stage of tumour development through the initiation-promotion system [148]. Limited evidence suggests that PP fail to initiate hepatocarcinogenesis in rats following *in vivo* treatment [149]. But, it has been reported that PPs are efficient promoters of certain genotoxic carcinogenic-induced lesions or other cells initiated spontaneously such as cells in the liver of untreated mice and rats [36] [148]. In rats pretreated with diethylnitrosamine, nafenopin was found to promote preneoplastic cells [211]. Age is another determinant of PP-induced tumour progression in rodents. Several studies have demonstrated the presence of numerous foci in the

livers of untreated old rats and mice [210]. These lesions act as spontaneous initiation to the cells in the older animals, which increase the incidence of liver tumor after treating the animals with peroxisome proliferators [150]. It has been posited that peroxisome proliferators have greater ability to produce liver tumor in old rats than in young rats. This view is based on the observation that in two studies with nafenopin [210] and WY -14,643 [151] using two groups of rats with different ages, old rats (57 to 60 weeks) and young rats (8 to 13 weeks). These studies revealed that development of liver tumors was higher in old rats than in young rats. The increase of liver tumor in old rats can be attributed to the promotion of a greater number of pre-existing spontaneously developed lesions, whereas the development of tumour in young can be attributed promotion of fewer pre-existing lesion [152] [209]. Figure 1.4 summarize the hypothetical mechanism of peroxisome proliferators as promotion agents in the tumour development.



(Modified from Suga, 2004 [42])

Figure 1.4 Hypothetical mechanism of peroxisome proliferator-induced hepatocarcinogenesis.

Section 1.10 Assessment of human hazard

Human are frequently exposed to peroxisome proliferators due to the number of PPs in the environment. This alerted many governments and regulatory agencies to assess whether these agents were safe for humans or not [43]. Since it was first observed that hypolipidemic drugs such as nafenopin induce carcinogenesis [153], many peroxisome proliferators that have been adequately tested for carcinogenicity in chronic dose caused tumors in rodent [51]. In contrast to the hepatocarcinogenesis in rodents, there is no published evidence that PPs are carcinogenic in humans. Several studies have asserted that PPs do not pose a carcinogenic risk to human [103]. An epidemiological study by the World Health Organization on clofibrate involving 208,000 men showed no excess cancer mortality [154] [155]. Furthermore, a volunteer study on humans exposed to therapeutic doses of peroxisome proliferators (clofibrate, fenofibrate, ciprofibrate, and gemfibrozil) showed no convincing evidence for peroxisome proliferation [103]. Moreover, the hypolipidemic drugs, that lower abnormally high plasma concentrations of cholesterol or triglycerides in human, are not accompanied by peroxisome proliferation nor by induction of peroxisomal β-oxidation or other activities induced by PP in rodents [51]. Tugwood reported that cloned human PPARα functions in a manner similar to its rodent counterpart but the PPARα expression is lower in human cells [75]. Other findings support the same concept that human hepatocytes express PPARα at 5-10% of the level found in rodent hepatocytes [79] [76]. Interestingly, there is interindividual variation in human PPARα cDNA obtained from different individuals [75]. Thus, existence of variability in PPAR sequences among human suggest that some individuals may be at risk to exposure to these potentially carcinogenic agents [208]. Finally, although the molecular mechanism of the carcinogenic action of PPs in the rodent liver is not fully understood, the phenomena associated with the action of PPs on rodent hepatocytes such as peroxisome proliferation and induction of cell proliferation are absent in human hepatocytes. These data provide substantial weight of evidence to support that the PP class of

nongenotoxic rodent hepatocarcinogens does not pose a cancer risk for human liver.

Section 1.11 Methodologies to measure DNA synthesis

Several techniques and methods are available to determine the rate of DNA synthesis whether *in vivo* or *in vitro*. DNA synthesis is determined by administering isotopically-labelled precursor ([^{3}H]thymidine (TdR)) or non-isotopically labelled precursor 5-Bromo-2-deoxyuridine (BrdU), a synthetic analog of thymidine, and these are incorporated into DNA during S-phase in hepatocytes [46]. These labelling agents can be administered via two different methods. First, the pulse labeling method, where a single dose of BrdU or TdR is administered 1-2 hours before an animal's sacrifice. Pulse labelling is the method of choice for evaluating rapid induction of DNA synthesis or in tissues with high division rates such as small intestine. Second, the continuous labelling method is another method for introducing the labeling agent. With this technique, labelling agent can be administered to the animal for several days by either using an osmotic pump placed under the skin of the animal or by delivery of the labelling agent in the drinking water of the test animals [46] [156]. The advantage of this method over pulse labelling method is that all cells going into S-phase during the period of labelling will be labelled and this will increase the sensitivity of the assay [46]. Once the labelling agent is incorporated into DNA, BrdU-immunohistochemistry (BrdU-IHC) or Flow Cytometry (FC) techniques can be used to determine the labelling index. In the immunohistochemistry method, incorporated BrdU in newly synthesized DNA strands of actively proliferating cells is detected by using Anti-BrdU antibodies and developed by e.g. the immunoperoxidase system, and visualised under the microscope. Flow cytometry provides a method for sorting different cell types based on size, shape and granularity by using specific light-scatter characteristics and detection of fluorescent probes. In FC, a laser beam is used as the source of scatter and fluorescence light. As a cell passes through the laser beam, it will scatter the light in all directions. Consequently, forward scatter can be used to determine the size of the cell, whereas side scatter can be used to measure gran-

ularity and structural complexity inside the cell [13]. The total cellular DNA content can be measured by staining a cell with DNA-binding dye such as Hoechst 33342, propidium iodide or 7-aminoactinomycin D. Incorporated BrdU can be determined either by using a monoclonal antibody against BrdU or using DNA-binding dye, one that shows altered fluorescence with BrdU substitution [157].

Section 1.12 Aims of the thesis

This thesis aimed to characterise the induction of liver growth by peroxisome proliferators. The specific objective were:

-To determine the true time and dose-dependency of induction of PP-induced DNA synthesis in liver of mouse

- To determine whether mouse strains respond to peroxisome proliferators with different time- and dose-dependency for induction of hepatic DNA synthesis

- To determine whether these effects (induction of liver DNA synthesis and liver growth) are mediated by the PPARα

- To determine if rats and mice have a different time-course for induction of hepatic DNA synthesis.

This information is an essential prerequisite for investigating the mechanisms of liver growth, as it is essential to be able to relate the timing of changes in regulating genes to the timing of DNA synthesis itself.

Chapter 2 Materials and Methods

Section 2.1 Materials

Section 2.1.1 Animals

Male and female 129S4/SvJae mice wild type or PPARα-null background (129S4/SvJae-Ppara$^{tm1Gonz/tm1Gonz}$) were bred locally in the breeding colony, Biomedical Services Unit, University of Nottingham. Inbred Male C57BL/6J, DBA/2J and BALB/c mice were obtained from Charles River Laboratories, UK. Outbred male Alderley Park (AP) mice were a gift from AstraZeneca Pharmaceuticals. Male Fischer 344 (F344/NHsd) rats were purchased from Harlan UK limited (Bicester, UK). Animal husbandry and handling were performed in accordance with the animals (Scientific Procedures) Act 1986. All Animals were housed in groups in plastic cages and were maintained on a 12-hr light/dark cycle.

Section 2.1.2 Chemicals

Section 2.1.2.1 Immunohistochemistry materials

All chemical were obtained at highest grade possible. 5-bromo-2'-deoxyuridine (BrdU) and Tris base ultrapure were obtained from Melford. 3,3' Diaminobenzidine tetrahydrochloride (DAB), Polyoxyethylene sorbitane monolaureate (Tween-20), bovine serum albumin (BSA) and fomalin solution, 10% formalin (approx. 4% formaldehyde) were obtained from Sigma. Acetic acid glacial, xylene, ethanol, methanol, sodium phosphate and haematoxylin stain were purchased from BDH. DPX (Distyrene, plasticiser, and xylene), hydrochloric acid and 30% hydrogen peroxide were obtained from Fischer scientific, Ammonium hydroxide from Aldrich and cobaltous chloride from AnalaR. Amersham cell proliferation kit were purchased from GE healthcare (UK). Poly-L-lysine-coated slides (Polysine™) were obtained from Fischer, pure paraffin wax (MP 56 deg C) from RA lamb, Peel-A-Way® disposable histology molds from Polysciences Inc and TAAB embedding stub (25mm) from TAAB. Pure water was produced in this laboratory at

a quality of < 0.2 μS.

Section 2.1.2.2 Peroxisome proliferators

2-4-(2,2-dichlorocyclopropyl)phenoxy)2-methylpropanoic acid (Ciprofibrate) was a kind gift from Dr. T.J.B Gray, Sanofi-Aventis (Alnwick, UK), methylclofenapate (MCP) from Dr. C.R. Elcombe, CXR Bioscience (Dundee, UK). 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid (Wy-14,643) was obtained from Chemsyn, Lenexa, a division of Aptuit. All these chemical were dissolved in corn oil directly except Wy-14,643 which was dissolved in absolute ethanol (70mg ml^{-1}) and then dissolved in corn oil to bring up concentration 7mg ml^{-1} (dosing volume, 20ml kg^{-1} b.wt).

Section 2.1.2.3 Other chemicals

Dimethyl sulphoxide (DMSO) 99.9% and 1,4-Bis[2-(3,5-Dichloropyridyloxy)] Benzene (TCPOBOP) were purchased from Sigma. TCPOBOP was dissolved in DMSO (7.5mg ml^{-1}) and then dissolved in corn oil to bring up concentration 0.2mg ml^{-1} (dosing volume, 20ml kg^{-1} b.wt).

Section 2.2 Experimental design

Animal experiments were designed on the basis of using power analysis to estimate the least number of animals required for meaningful statistical analysis of DNA synthesis. To exemplify power analysis, we have typical control mean values for hepatic labelling index of ~0.26% with a variance of 0.07%, and treated values of ~3% with a variance of 1.5%. To obtain a significance test with $P<0.05$ for a treated mean of 1.8%, with a power of 90%, the number of animals per group should be 6 [158]. Moreover, the least stressful procedure, suffering, distress of lasting harm are employed, given that the experiment will be sufficient to produce satisfactory results. In addition, all experiment were designed to study the liver growth and DNA synthesis in animal as a result of peroxisome proliferators action without toxic or adverse effects where possible.

The animals were matched for age within range of 9-10 weeks. The animals were matched for sex, strain, and supplier. In addition, when animal arrived, they were randomised on the basis of body weight into appropriate number of groups to minimise random variation. Positive and negative controls were introduced and optimized to standardise the experiments and appropriate method for administration of chemical and quantification of labelling index were optimized as well so that scheduled DNA synthesis due to exposure to a PP can be measured. Pilot study was introduced to the project to gather useful information prior to the start of larger scale studies. A small feasibility experiment, can reveal deficiencies in the design of a experiment that can be addressed before the start of larger experiments.

Section 2.2.1 Dosing of Animal

Section 2.2.1.1 BrdU-labelling protocol

In order to administer the DNA precursor, BrdU, without adverse effects, the BrdU-dosing regime was optimised. The animals were kept in a temperature-controlled room with alternating 12hrs dark/light cycle. The animals were given free access to food and tap water until the beginning of the experiments. At time zero (10a.m), the animals were randomly housed into groups and then acclimatized to 10% orange juice as a sole source of drinking water for one week. Then orange juice supplemented with 0.8mg ml^{-1}BrdU was given continuously in drinking water from one day prior to dosing with PP. All bottles-containing BrdU were protected from light by aluminum foil and were changed every 3 days.

Section 2.2.1.2 Single dose protocol without acclimatisation

Mice were dosed with 0.8mg ml^{-1} BrdU in drinking water for 3 days; after one day, the animals were dosed by gavage with appropriate dose of peroxisome proliferators and the animals were

killed after two days (Figure 2.1).

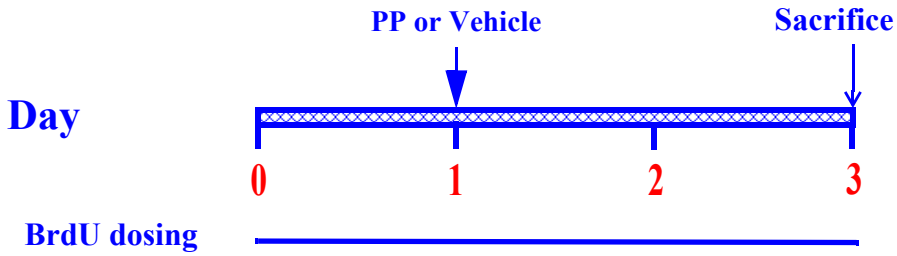


Figure 2.1 Schematic representation of single dose protocol 1. The schematic diagram represents the single dose protocol without acclimatisation to 10% orange juice. The hash bar shows the number of days on the experiment. The closed arrow indicates the time of dosing with peroxisome proliferator (PP) or vehicle, whereas the open arrow indicates the sacrifice day of the animals. The solid horizontal line shows the period of time where the animals were given BrdU continuously in drinking water (labelled BrdU dosing).

Section 2.2.1.3 Single dose protocol after acclimatisation to 10% orange juice

After using the labelling protocol (Section 2.2.1.1), treated animals were gavaged with a single dose of a peroxisome proliferator (PP) dissolved in corn oil (20ml kg^{-1}). Control group was kept under the same conditions, with the exception that gavage dose did not contain a peroxisome proliferator. Animal were killed after three days or as stated (Figure 2.2).

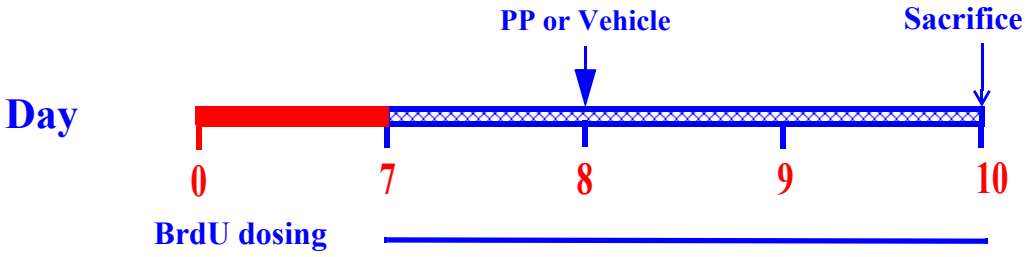


Figure 2.2 Schematic representation of single dose protocol 2. The schematic diagram describes the single dose protocol of induction of DNA synthesis by peroxisome proliferator after acclimatisation with 10% orange juice. The solid red bar shows the 7-day period where the animals were given 10% orange juice as sole source of drinking water and the hash bar shows the number of days on the experiment. The closed arrow indicates the time of dosing with peroxisome proliferator (PP) or vehicle, whereas the open arrow indicates the day of killing the animals. The solid horizontal line shows the period of time where the animals were given BrdU continuously in 10% orange juice, their sole source of drinking water (labelled BrdU dosing).

Section 2.2.1.4 Time-course protocol

After using the labelling protocol (Section 2.2.1.1), groups of six animals were gavaged daily with a peroxisome proliferator in corn oil (e.g. 100 mg kg^{-1} day^{-1}ciprofibrate with 129S4/SvJae mice) and killed on appropriate time points (e.g days 2, 3, 4, 5 and 6), and the control vehicle groups were killed on first and last time points (e.g days 2 and 6) after dosing with vehicle control. No control animals were used (on days 3-5) because we assume that control hepatic DNA synthesis rates are at steady state (which is tested by killing control animals at days 2 and 6) and we have a legal obligation to reduce the number of animal used by home office legislation. Animal body weight was measured daily throughout the experiment as part of adverse effects assessment (Figure 2.3).

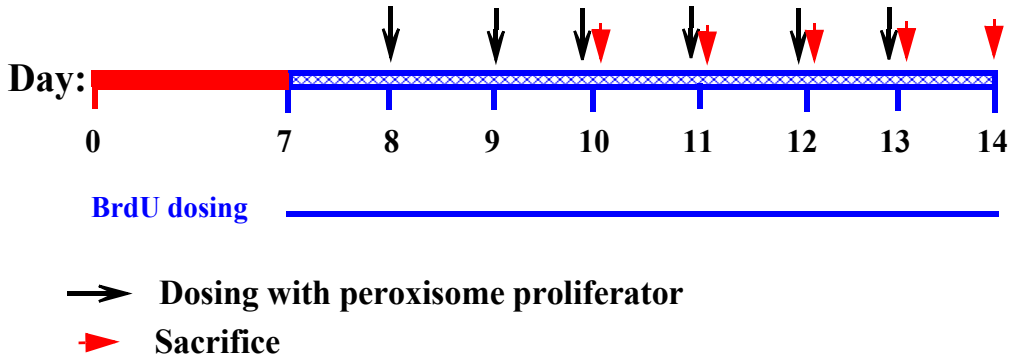


Figure 2.3 Schematic representation of multiple dose protocol 1. The schematic diagram shows a protocol for studying the time course of induction of DNA synthesis, with multiple doses of peroxisome proliferator or vehicle. The solid red bar shows the 7-day period where the animals were given 10% orange juice as sole source of drinking water and the hash bar shows the number of days on the experiment The black arrows represent the day of dosing with peroxisome proliferator or vehicle, whereas the red arrowheads represent the day of killing the animals. The control groups of animals were killed in appropriate days (e.g on days 2 and 6 after dosing with vehicle control). The solid horizontal line shows the period of time where the animals were given BrdU continuously in their sole source of drinking water (labelled BrdU dosing).

Section 2.2.1.5 Dose dependency protocol

After using the labelling protocol (Section 2.2.1.1), groups of six male 129S4/SvJae mice (9-10 wks old) were gavaged with 0,1,3,10,30,100 or 200 mg kg^{-1} $day^{-1}$of ciprofibrate in corn oil for 3 days (Figure 2.4), and other set were gavaged with 0,10,30,100 mg kg^{-1} $day^{-1}$of ciprofibrate for 4 days (Figure 2.5). Control groups were dosed with corn oil vehicle. The animals were humane by killed at the end of experiment by an overdose of pentobarbital (Dolethal)

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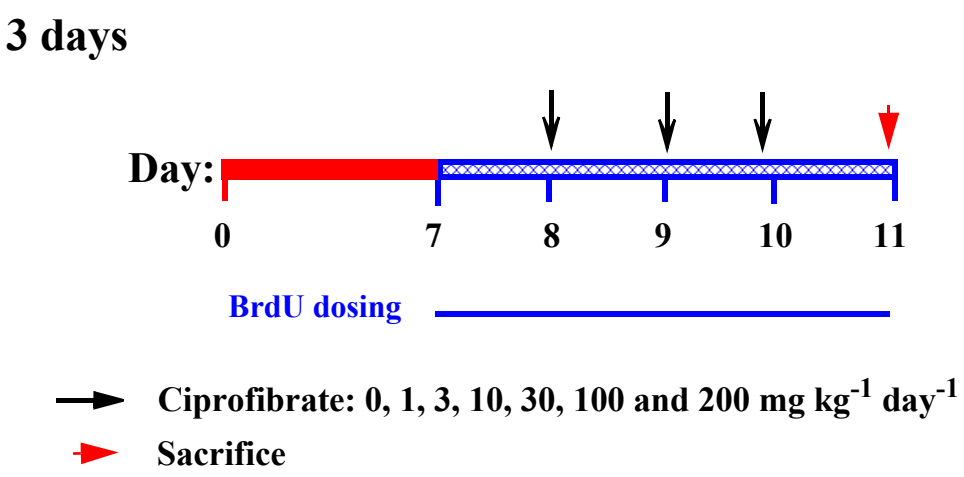

Figure 2.4 Schematic representation of multiple dose protocol 2 . The schematic diagram shows a protocol for studying the dose dependency of induction of hepatic DNA synthesis, with multiple doses of ciprofibrate for three days. The solid red bar shows the 7-day period where the animals were given 10% orange juice as sole source of drinking water and the hash blue bar shows the number of days on the experiment. The black arrows represent the day of dosing with ciprofibrate or vehicle, whereas the red arrowhead represents the day of killing the animals. The solid horizontal line shows the period of time where the animals were given BrdU continuously in their sole source of drinking water (labelled BrdU dosing).

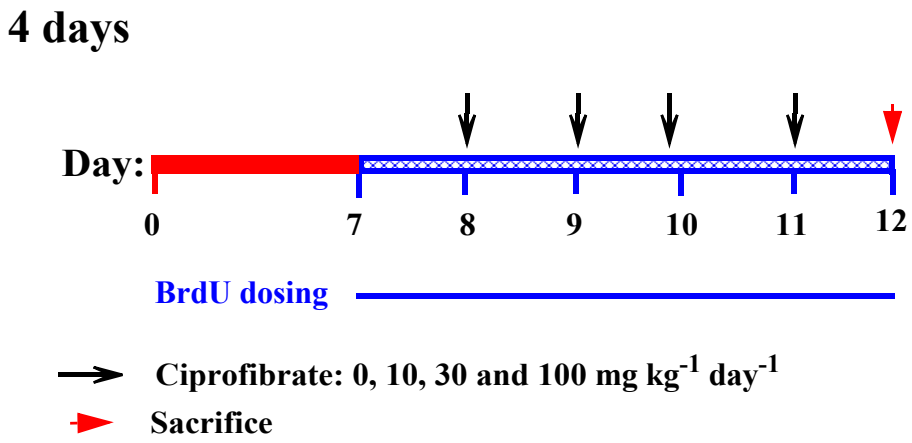

Figure 2.5 Schematic representation of multiple dose protocol 3. The schematic diagram shows a protocol for studying the dose dependency of induction of hepatic DNA synthesis, with multiple doses of ciprofibrate for four days. The solid red bar shows the 7-day period where the animals were given 10% orange juice as sole source of drinking water and the hash blue bar shows the number of days on the experiment. The black arrows represent the days of dosing with ciprofibrate, whereas the red arrowhead represents the day of killing. The solid horizontal line shows the period of time where the animals were given BrdU continuously in their sole source of drinking water (labelled BrdU dosing).

Section 2.2.2 Tissue processing

Following treatment, the animals were exsanguinated under terminal anaesthesia with pentobarbital. Immediately after death, a blood sample was collected by using cardiac-puncture. The whole liver was removed and weighed, and a section of left lobe of liver and small intestine (as positive control) were fixed in 10% neutral buffered formalin overnight. The remaining liver was snap-frozen in liquid nitrogen and kept at -80^{o}C until use. Blood samples were centrifuged and serum was frozen at -80^{o}C until subsequent analysis.

Fixed tissues were dehydrated, cleared and infiltrated with paraffin by using Shandon Citadel 2000 Automated Tissue Processor with the following protocol:

Paraffin-infiltered tissues were manually embedded with paraffin and kept overnight at 4^{o}C to cool down before sectioning. Sections (4.5µm) were mounted on adhesive coated slide, poly-

sine™ slide, and dried at 37°C overnight.

Table 2.1 Tissue processing protocol.

70% Ethanol	6.5 hrs
80%, 90%, 95%, and 3X 100% Ethanol	1 hr each
3X Xylene	1 hr each
2X Paraffin wax	1 hr each

Section 2.2.3 BrdU immunohistochemistry

Immunostaining was performed by dewaxing the sections in xylene for 30 minutes.The sections were then rehydrated through a graded series of ethanol solutions (100%, 70%, 50%) and finally washed in distilled water for 2 minutes. Endogenous peroxidase was quenched by treating the slides with 3% hydrogen peroxide in methanol for 15 minutes. The sections were then rinsed with phosphate buffer saline (PBS 100mM, pH 7.4/100 mM sodium chloride) twice for 5 minutes each. Antigen was retrieved by using heat induced epitope retrieval (HIER) technique using 700 watt-microwave. Sodium citrate buffer (10mM, pH 6) was microwaved at highest power until boiling. The slides were then plunged into the boiling buffer, then heated again at same power until boiling, followed by an additional 50 min at lowest power. The sections were washed with PBS solution for 5 min, followed by a 15-min incubation with 0.5% BSA, 0.5% Tween-20 in PBS for 5 minutes to reduce nonspecific binding. The slides were incubated with Amersham mouse monoclonal anti-BrdU antibody (1:1000 dilution in TBS) for 1 hour and the sections were then rinsed in distilled water for 5 minutes. Amersham HRP-conjugated goat anti-mouse antibody (1:50 dilution in TBS) were added for 30 minutes and again the slides rinsed with PBS twice for 5 minutes each. BrdU incorporation was detected by incubating the slide with developing reagent (0.05% DAB, 0.025% Cobaltous chloride in 10mM Tris-HCl, pH 7.6) for 10 minutes. The slides were then rinsed with distilled water for 3 minutes then counter-stained, with Harris haematoxylin for 5 sec, dehydrated, and then mounted with DPX (Disty-

rene, plasticiser, and xylene). All incubations were done in a humidity chamber at room temperature.

Section 2.2.4 Detection and quantification of DNA synthesis

Hepatocyte DNA synthesis was quantified under Leitz Wetzlar™ light microscope by scoring at least 2000 nuclei in random fields at 400X magnification per animal. The labelling index (LI) in hepatocytes was generated by dividing the number of BrdU-labelled nuclei by the total number of nuclei counted and expressing the results as a percentage. Hepatocytes were identified morphologically by using Leitz Wetzlar™ light microscope at 400x.

Section 2.2.5 Alanine aminotransferase (ALT) measurement

Serum ALT activity was measured as a biomarker for liver injury since the ALT is highly specific to the liver [159]. The measurement was performed by using Vitros ALT slides (Ortho-Clinical Diagnostics). Serum ALT test was performed in the clinical chemistry section, pathology department in Queen Medical Centre (QMC), Nottingham.

Section 2.2.6 Statistics

Statistical evaluation of data was performed by using Excel, Prism, and Sigmaplot programs. Students T-test was used for comparison of two groups; Paired t-test was used to test significantly of relative body weight loss. The comparison was tested between the day of BrdU dosing and the last day unless stated otherwise. Unpaired t-test was used to analyse significance between two groups on other occasions. One-way ANOVA followed by a post-hoc test (e.g. Dunnett's, Newman-Keuls tests) was used for comparison of multiple treatment groups (e.g. during a time course, or a dose-response). The assumption that liver DNA synthesis is parametric data is described in [160]. Relative body weight was estimated by measuring the actual body weight on the daily basis and calculated as relative to body weight on 0-day for each individual animal.

Chapter 3 Results

Section 3.1 Method validation

Section 3.1.1 Fixation and detection

Section 3.1.1.1 Assessment of immunohistochemistry protocol with positive control

It was necessary to ensure this protocol was able to localise incorporated BrdU in the cell nuclei with a good signal-to-noise ratio. A positive control tissue which was known to have high levels of BrdU labelling was used: this was xenograft tumour tissue (a kind gift from Dr. Phil Clarke, from the cancer studies unit). Substantial efforts were made to test three different techniques to restore antigenicity of incorporated BrdU, trypsin digestion, 0.5 M sodium hydroxide (denaturing solution), and heat induced epitope retrieval (HIER) microwave method. Microwave treatment was chosen as optimal (data not shown). A set of slides of liver section were stained by immunohistochemical protocol as follow: (1) without primary antibodies to demonstrate non specific binding; (2) stained without secondary antibodies to act as negative staining control; (3) slide was stained in presence of both antibodies. As shown in Figure 3.1, there were no immunoreactivity observed in the slides A and B due to absence of primary and secondary antibodies respectively. In contrast, the slide C which was stained in presence of both primary and secondary antibodies showed dark brown labelled nuclei. This demonstrates that the staining in (c) was dependent upon the presence of both the primary and the secondary antibodies, and shows that the staining was specific. These results confirm that the detection system works with good signal-to-noise ratio.

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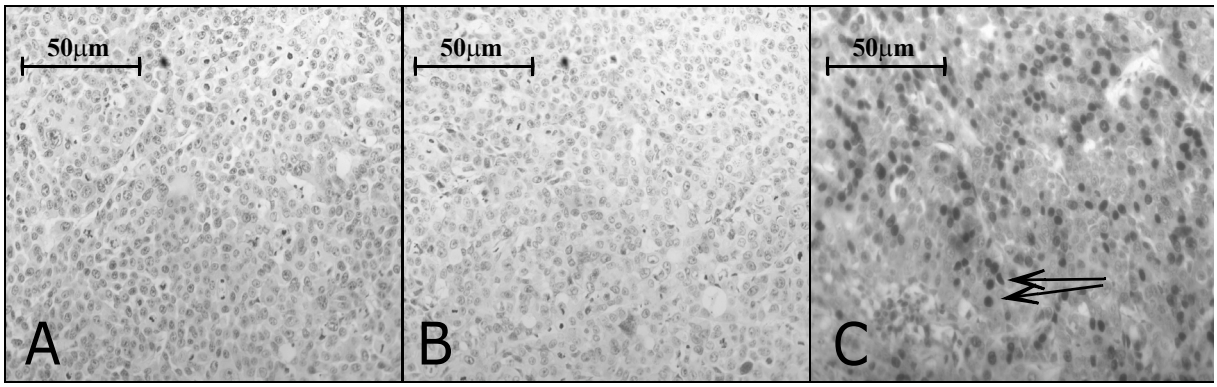


Figure 3.1 Evaluation of immunodetection protocol on formalin-fixed, paraffin embedded tissue.
BrdU-labelled Xenograft was stained with the immunohistochemical procedure and counterstained using Harris haematoxylin as described in materials and methods Section 2.2.3. (A) Shows staining after the immunohistochemical procedure without primary monoclonal anti-BrdU antibody. (B) Shows staining after the immunohistochemical procedure without secondary goat anti-mouse antibody conjugated with HRP. (C) Stained in the presence of primary antibody for BrdU and secondary antibodies. Arrows show BrdU-labelled nuclei**.** The scale bar = 50μm.

In order to assess the ability of detection system to detect the BrdU-labelled nuclei after *in vivo* treatment of mice with BrdU, groups of two 129S4/SvJae mice were treated with 0 and 0.8mg ml^{-1}BrdU in tap water (over three days), which was the sole source of drinking water. In the absence of BrdU administration, there was no immunostaining of nuclei in the liver or gut sections (Figure 3.2 A and B). The BrdU-labelled nuclei in the liver and gut sections of mice dosed with 0.08% BrdU was stained with a high signal-to-noise ratio (Figure 3.2C). The crypts and half of the villi section of the gut labelled with the BrdU, because cell division of epithelial cells occurs in the crypts and gradually move toward the tips of the villi (Figure 3.2D). This results confirm that labelling agent BrdU at this level was incorporated into the replicating DNA and can be detected by the immunostaining protocol.

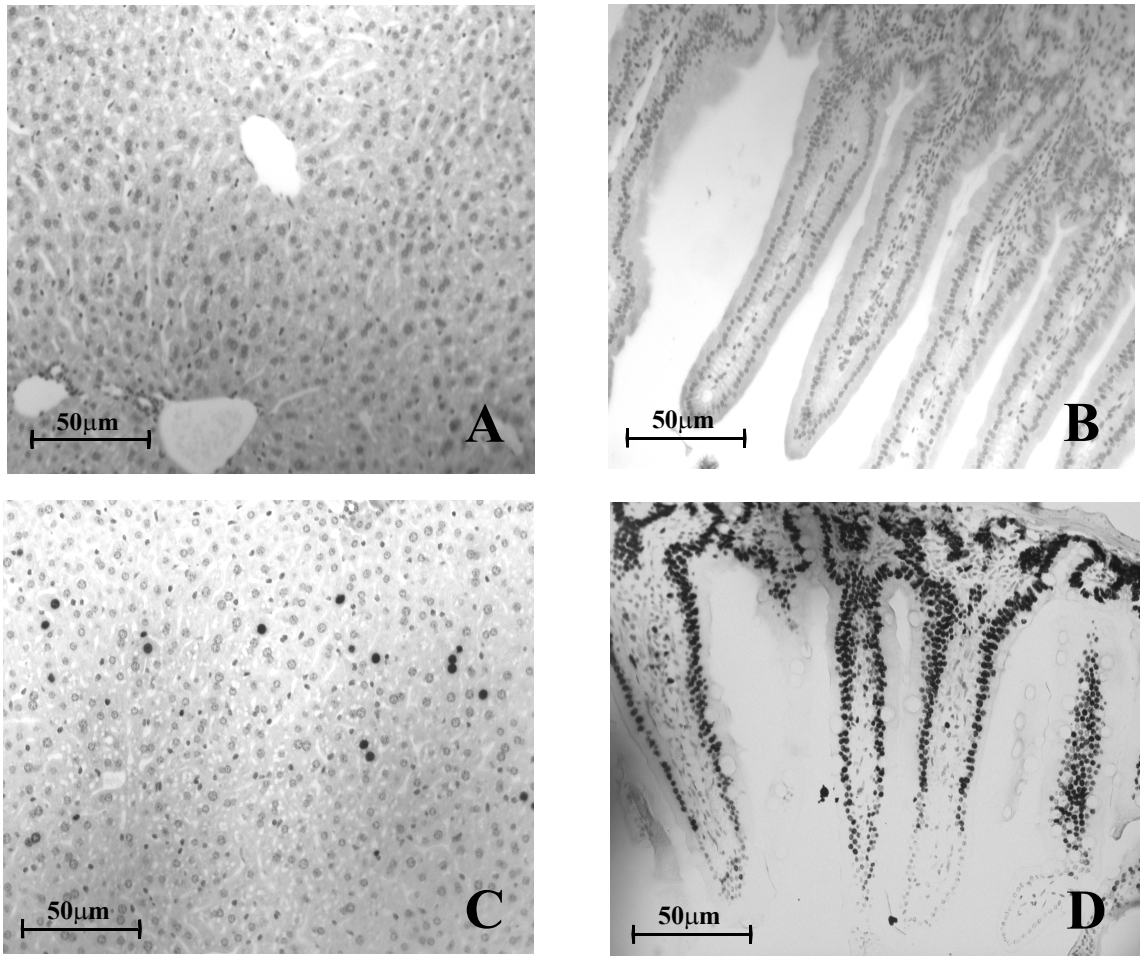


Figure 3.2 Assessment of anti-BrdU immunoreactivity in paraffin sections. Groups of two 129S4/SvJae mice were dosed with water and 0.8 mg ml^{-1} BrdU in drinking water for 3 days. Liver section (A) and gut section (B) from water-treated mice and liver (C) and gut section (D) from BrdU-treated mice were harvested, fixed and processed for paraffin sections as described in materials and methods, Section 2.2.2. The sections were stained with immunohistochemical protocol using Amersham cell proliferation kit and counterstained with Harris haematoxylin as described in Figure 3.1. Gut section was used throughout all project as positive control tissue to make sure BrdU was taken up by the animal. The scale bar = 50μm.

Section 3.1.1.2 Effects of fixation time and temperature on immunohistochemistry

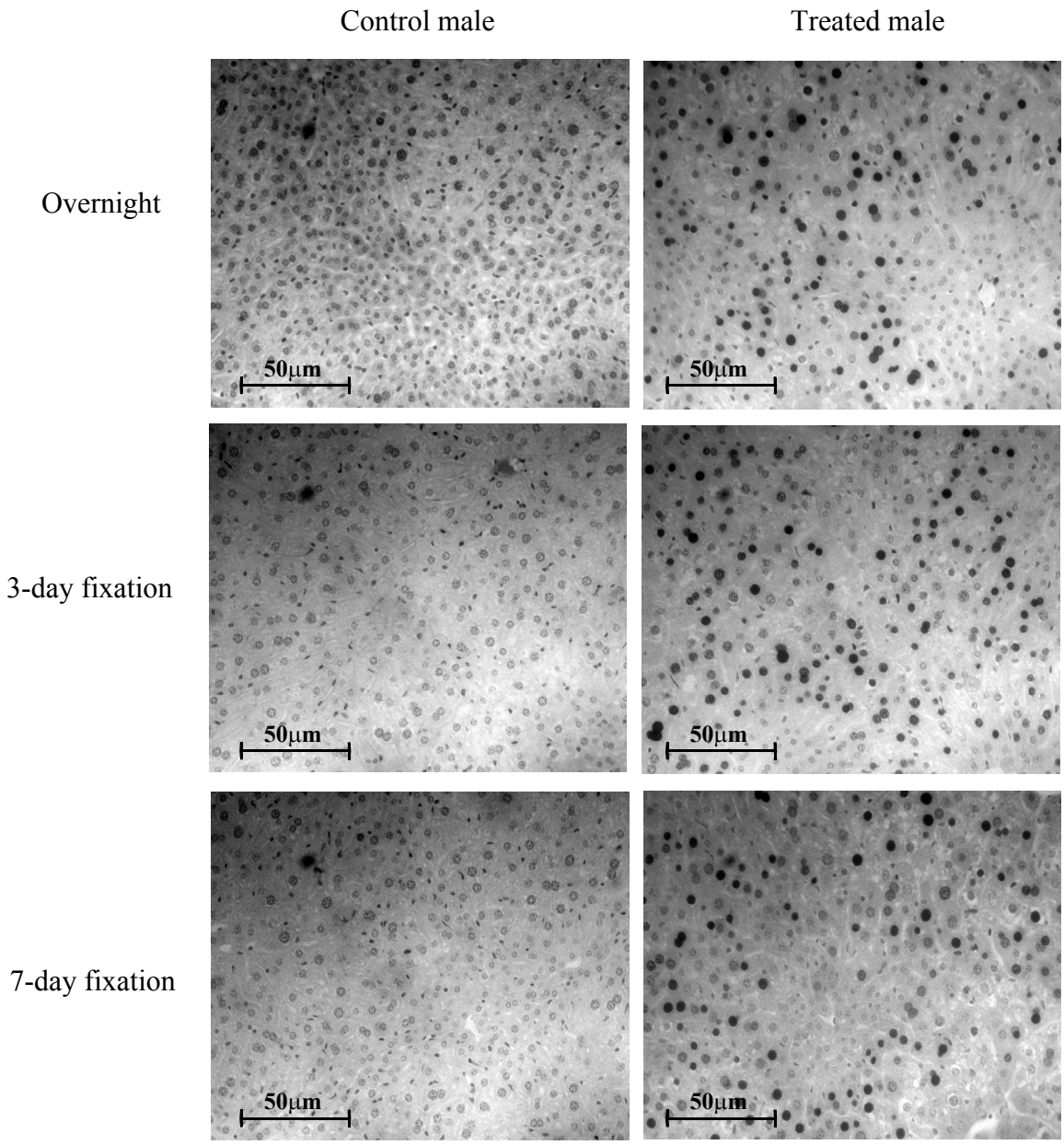
The aim of this experiment was to evaluate the effects of fixation time and temperature on the incorporated BrdU immunoreactivity in mouse gut and liver. Liver and gut tissues of groups of two 129S4/svJae mice were fixed in 10% neutral buffered-formalin (cross-linking agent) overnight, 3 or 7 days either at 4^{o}C or room temperature (RT) and then evaluated for BrdU immunohistochemistry. There was strong staining of nuclei with the immunohistochemical protocol (not shown). Table 3.1 shows the length of fixation time has no significant effect on immunoreactivity of incorporated BrdU in liver sections but some effect on the gut sections. The liver and gut sections fixed at room temperature had better BrdU-staining than those fixed at 4^{o}C, which was consistent with published data that the process of formalin fixation was temperature dependent, i.e. higher temperatures produce more rapid fixation [161] [162]. Thus, this finding confirms that fixing tissues at room temperature was better than at 4^{o}C and immunohistochemical staining of BrdU does not change after seven days fixation at room temperature.

Formalin fixation time	**Gut section**		**Liver section**	
Days	*4^{o}C*	*RT*	*4^{o}C*	*RT*
Overnight	+ + +	+ + +	+ +	+ + +
3	+	+ +	+ + +	+ + +
7	+	+ +	+ +	+ + +

Table 3.1 Effects of length of formalin fixation and temperature on immunohistochemistry. Liver and small intestine from groups of two 129S4/SvJae mice were fixed in 10 neutral buffered-formalin overnight, 3 or 7 days and stored either at 4^{O}C or room temperature. The section from each tissues were stained by immunoperoxidase staining as described in Figure 3.1. Intensity of staining was graded from + to + + +.

Section 3.1.1.3 Effects of fixation time on BrdU immunohistochemistry from mouse liver induced with TCPOBOP

Formalin a cross-linking agent seems to mask the reaction site of BrdU in DNA,. which may make it difficult to identify BrdU-labelled nuclei [162]. It was necessary to evaluate the effects of length of fixation duration on the BrdU-signal intensity in liver of mice with a high labelling index. Liver and gut tissues from groups of five male or female 129S4/SvJae mice treated with 0 or 3 mg kg^{-1} TCPOBOP (Section 3.3) were fixed into 10% neutral buffered formalin for 1, 3 and 7 days at room temperature. The formalin-fixed paraffin-embedded sections were examined microscopically for the strength and signal-to-noise (S/N) ratio of BrdU-labelled nuclei. As shown in Figure 3.3, BrdU-labeled nuclei in the different fixation time show a similar strength of signal intensity of BrdU-labelled nuclei in both male and female mice. Moreover, the S/N ratio was similar after different fixation times in both male and female mice. These findings verify that fixation length up to 7 days did not affect the strength of BrdU-labelled nuclei intensity nor the signal-to-noise ratio.



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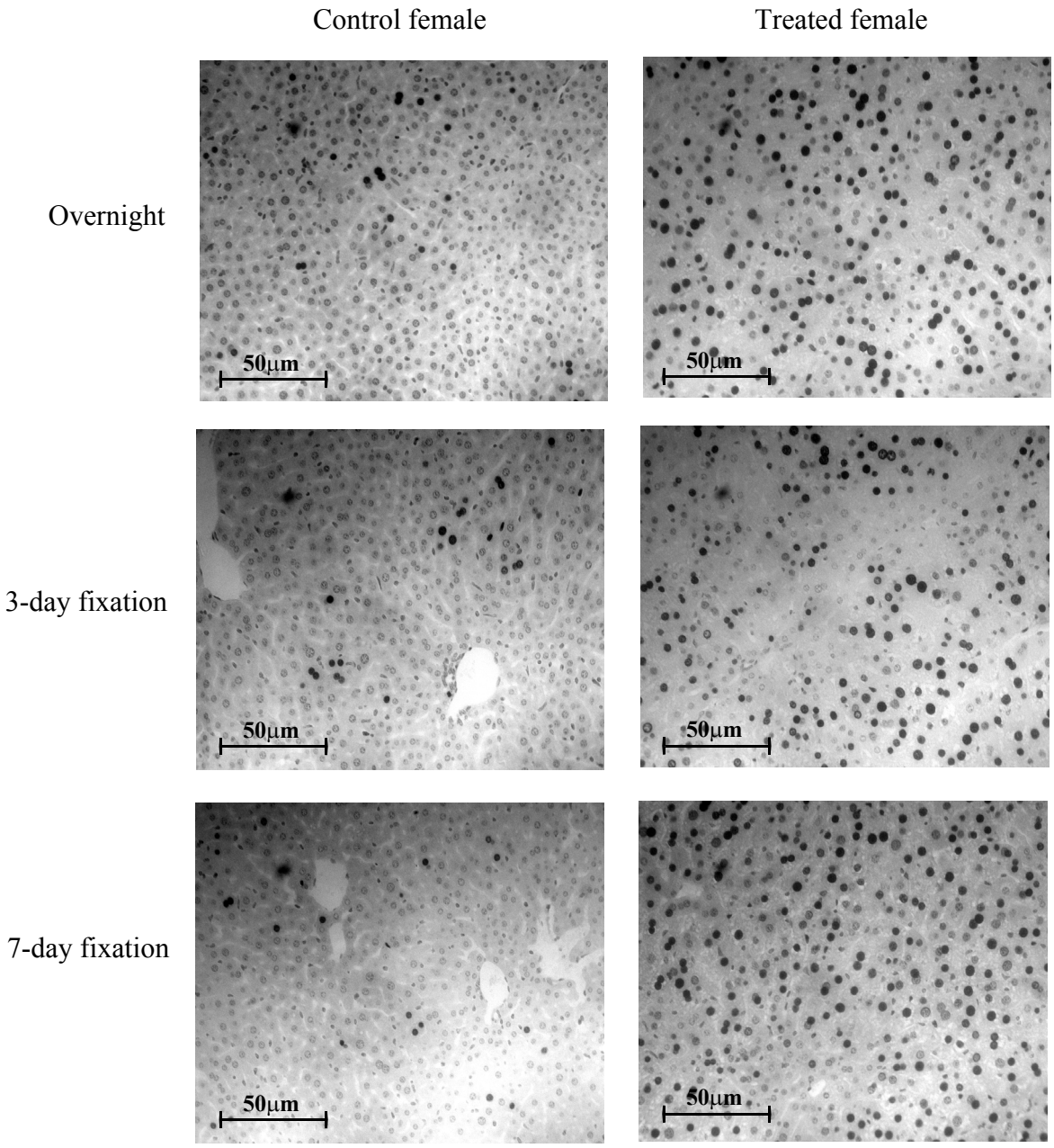

Figure 3.3 Effects of fixation duration on the TCPOBOP-treated tissues. Liver and gut segments from control and treated of male and female 129S4/SvJae mice were fixed into 10%NBF for overnights, 3 and 7 days at room temperature. Gut was used as positive control to ensure BrdU solution get into animal body as shown in Figure 3.2. The tissues were processed and stained by routine protocol as described in Section 2.2.3. The sections were examined under light microscope at 400x and positively labelled hepatocytes as dark brown in color against a light blue color counter-stain.

Section 3.1.2 Optimisation of *in vivo* BrdU dosing

Section 3.1.2.1 Induction of hepatic DNA synthesis by ciprofibrate: Pilot study

The aim of this experiment was to determine the dose of ciprofibrate that gives good DNA induction without any adverse effects, and also to reveal a better idea of inter-animal variability. 129S4/SvJae mouse strain was chosen in this project for several reasons: a) capability of breeding and supply the 129S4/SvJae strain in our location; b) this strain has a targeted knock-out of the PPARα gene, which can be used to examine if the action of PPARα agonist, peroxisome proliferators was mediated by PPARα receptor; c) since there was no published data in strain differences of the induction hepatic DNA synthesis, we anticipated that this strain would be valuable to study liver growth.

Two male 129S4/SvJae mice per group (of different age) were treated with a single dose of 0, 12.5, 25, 75mg kg^{-1} ciprofibrate, and killed after two days. The liver:body weight ratio in ciprofibrate-treated groups did not differ obviously from the control group. (Figure 3.4A). Labelling index was not obviously increased in the treated group at all exposure levels of ciprofibrate over the control (Figure 3.4B). In fact, inter-individual variation between the animals was large. It was impossible to draw reliable conclusions from using two animals per group. Therefore, it was essential in the future to use a larger group size to do statistical analysis.

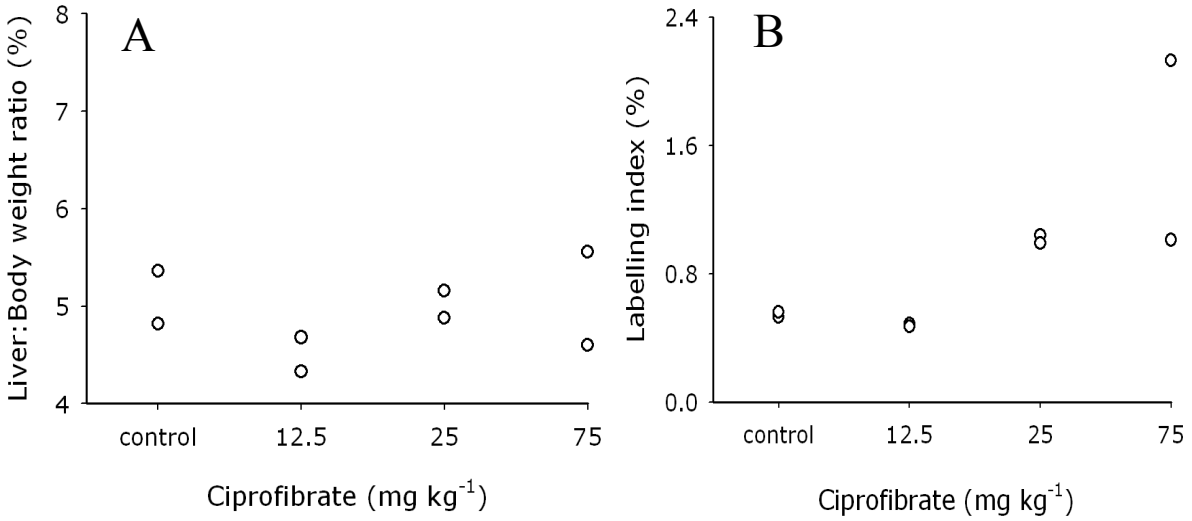

Figure 3.4 Effect of dose of ciprofibrate on liver growth. Groups of two male129S4/SvJae mice of varying age (within the range 8-14 weeks) were dosed with 0.8mg ml^{-1} BrdU in drinking water for 3 days; after one day, the animals were dosed by gavage with ciprofibrate (12.5, 25, or 75 mg kg^{-1}) and corn oil for control group. The animals were killed after 2 days of ciprofibrate treatment as described in single dose protocol 1 (Section 2.2.1.2). (A) Liver-to-body weight ratios are expressed as liver/body weight ratio percent for each individual mouse; (B) Labelling index was determined by immunocytochemical localization of BrdU and counterstaining with Harris haematoxylin as described in the Figure 3.1, and calculated by counting at least 2000 hepatocyte nuclei per liver under microscope. The LI was expressed as number of (labelled nuclei / total nuclei)x100 for each mouse as described in the materials and methods, Section 2.2.4. n=2.

Section 3.1.2.2 Induction of hepatic DNA synthesis in BALB/c mice

In order to demonstrate the induction of hepatic DNA synthesis in mice, the strain mice BALB/c was used to induce hepatic DNA synthesis by ciprofibrate. Budroe et al. reported that BALB/c mice demonstrated 24-fold increase in the labelling index after being exposed to 250ppm ciprofibrate for four days [11]. Groups of three Balb/c male mice (9-10 weeks old) were dosed with BrdU (0.8mg ml^{-1}) in drinking water, dosed once with ciprofibrate (0-300 mg kg^{-1}), Wy 14,643 (100 mg kg^{-1}) or methylclofenapate (25 mg kg^{-1}) by gavage after one day of BrdU treatment, and killed after two days. Body weight was measured daily. There was a dose dependent increase in relative liver: body weight ratio in mice treated with ciprofibrate up to 157% of the control value. In addition, the mice treated with a single dose of 25mg kg^{-1} methylclofenapate showed a significant increases in liver: body weight ratio (37% increase) compared to the con-

trol, whereas the group of mice treated with 100 mg kg^{-1} Wy 14,643 did not display any significant difference as compared to the control (Figure 3.5A). Unexpectedly, the control group, 300mg kg^{-1}ciprofibrate-treated group and Wy 14,643-treated group showed a significant decrease in body weight after three days of BrdU dosing, which was ~20% in the control group (Figure 3.5B). This decrease in body weight was a confounding issue, since the effect on body weight per se could have an effect on liver growth so the experiment provides no reliable information about control of liver growth and it was difficult to interpret the results. It was therefore necessary to alter the methodology to maintain the body weight of the mice before continuing.

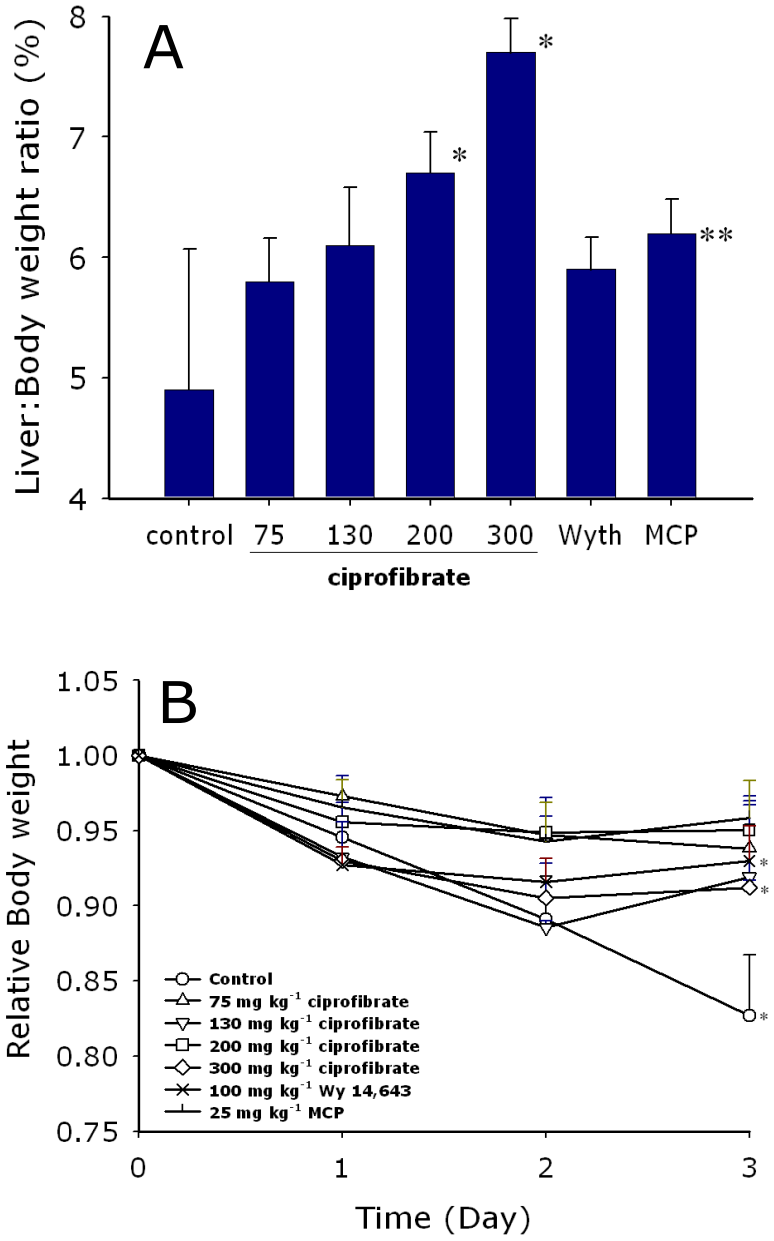

Figure 3.5 Effect of peroxisome proliferators on liver growth in Balb/c mice . Group of three male BALB/c mice were dosed with 0.8mg ml^{-1} BrdU in drinking water for 3 days; after one day, the animals were dosed by gavage with increasing concentrations of ciprofibrate (75,130,200 and 300 mg kg^{-1}), Wyeth-14,643 (100 mg kg^{-1}) or methylclofenapate (MCP; 25mg kg^{-1}), and the animals were killed after 2 days as described in single dose protocol 1 (Section 2.2.1.2). (A) Liver-to-body-weight (LBW) ratios were calculated as liver weight to the mouse body weight on the day of killing. (B) Body weight was measured on daily basis for each individual mouse and relative body weight (RBW) was calculated relative to the body weight for each individual mouse on day one. All data are expressed as mean±SD, n=3. One-way ANOVA and Dunnett's test were used in statistical analysis of A (LBW), whereas paired t-test was used for statistical analysis of RBW between the day of BrdU dosing and the day of killing as described in materials and methods, Section 2.2.6. $* = p<0.05$ and $** = p<0.01$.

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Section 3.1.2.3 Optimisation of BrdU uptake in drinking water

The hypothesis was the unpleasant taste of BrdU caused mice to reject BrdU-containing water. One report showed that mice given BrdU in drinking water had slight body weight loss compared to the control [163]. The labelling protocol was optimised whereby groups of three 129S4/SvJae mice (-/-) were given various concentrations of BrdU (0.08, 0.05%) in 16% orange juice, as taste enhancer, in tap water, which was the sole source of drinking water. The control group was given tap water only and the other control group was given only diluted orange juice (16%). The idea here was to enhance the amount of BrdU-containing water imbibed either by decreasing level of BrdU (with the idea that the taste of BrdU will be reduced so the animal will drink it) or hiding the BrdU tasting by using taste enhancer (orange juice). Figure 3.6 shows there was no statistical difference in relative body weight of mice given orange juice, BrdU or BrdU supplemented with orange juice as compared to the controls. However, while the mice in the control group gained 4% of body weight after three days, the mice dosed with 0.08% BrdU + orange juice or 0.05% BrdU lost 4% and 1% of body weight after three days of dosing respectively. The mice dosed with 0.08% BrdU only was showed no change in relative body weight after three days of dosing. The groups given 0.05% BrdU + orange juice or orange juice alone were the only two groups which had gained 1% of body weight after three days of dosing. This study shows 0.05% BrdU in 16% orange juice regime was the best regime to maintain the body weight throughout the dosing period for this experiment only.

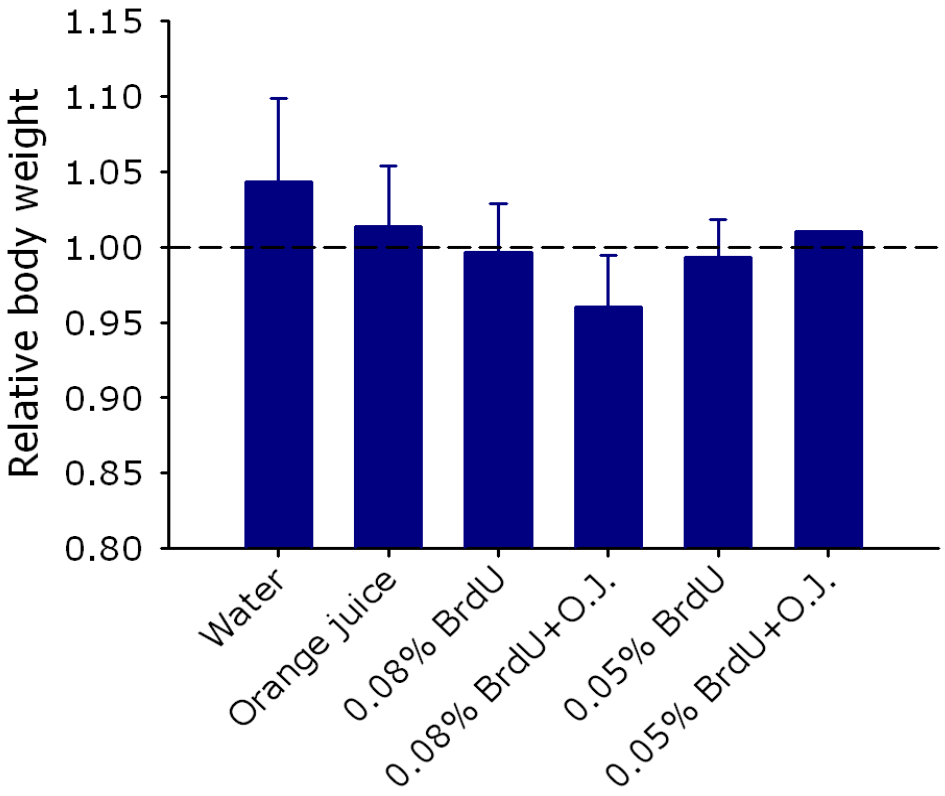

Figure 3.6 Effect of orange juice and BrdU on body weight. Groups of three 129S4/SvJae mice -/- were dosed with 0.5 or 0.8 mg ml^{-1} BrdU in drinking water with and without 16% orange juice for 3 days. Body weight was measured on daily basis for each individual mouse and relative body weight was determined relative to the body weight for each individual mouse on day one. RBW was calculated as body weight in day 3 to body weight in day 0 for each individual mouse. No significant difference was observed between treated groups and control (water) group. Data are the mean±SD of the mice in each group, n=3. Statistical analysis was calculated by one-way ANOVA with Dunnett's posttest.

Section 3.1.2.4 Induction of hepatocellular DNA synthesis by ciprofibrate

The objective of this study was to induce DNA synthesis by ciprofibrate in mice treated with 0.05% BrdU supplemented with 16% orange juice regime. In this study, the labelling regime optimised in Section 3.1.2.3 was used to prevent body weight loss during the BrdU-dosing protocol. Groups of five male 129S4/SvJae mice (9-10 weeks old) were administered BrdU (0.5mg ml^{-1}) in 16% orange juice in drinking water, dosed once with ciprofibrate by gavage (0-300 mg kg^{-1}), and killed after two days. There was a dose-dependent increase in the liver: body weight

ratio, rising from 4.5± 0.3% in the control group (i.e. 16% orange juice plus BrdU) to 6.2±0.2% in the 300 mg kg^{-1} ciprofibrate-treated group, which was significant at $P<0.05$ (Figure 3.7A). Labelling index was not significantly increased in treated groups as compared to the control (Figure 3.7B). However, the control and treated groups showed a ~ 4.5% loss in body weight, which was statistically significant at day 3 (Figure 3.7C). ALT level, as a toxicity marker, in the treated groups did not differ significantly from the control group (Figure 3.7D). This finding shows that mice still lose some body weight after BrdU dosing, which necessitates further measures to reduce body weight loss. Figure 3.7C shows that the orange juice + BrdU regime results in a significant decrease in body weight, whereas Figure 3.6 failed to detect any decrease: this may be explained by the larger number of animals per group (5 vs. 3 respectively), thereby giving greater power for the detection of statistically significant difference.

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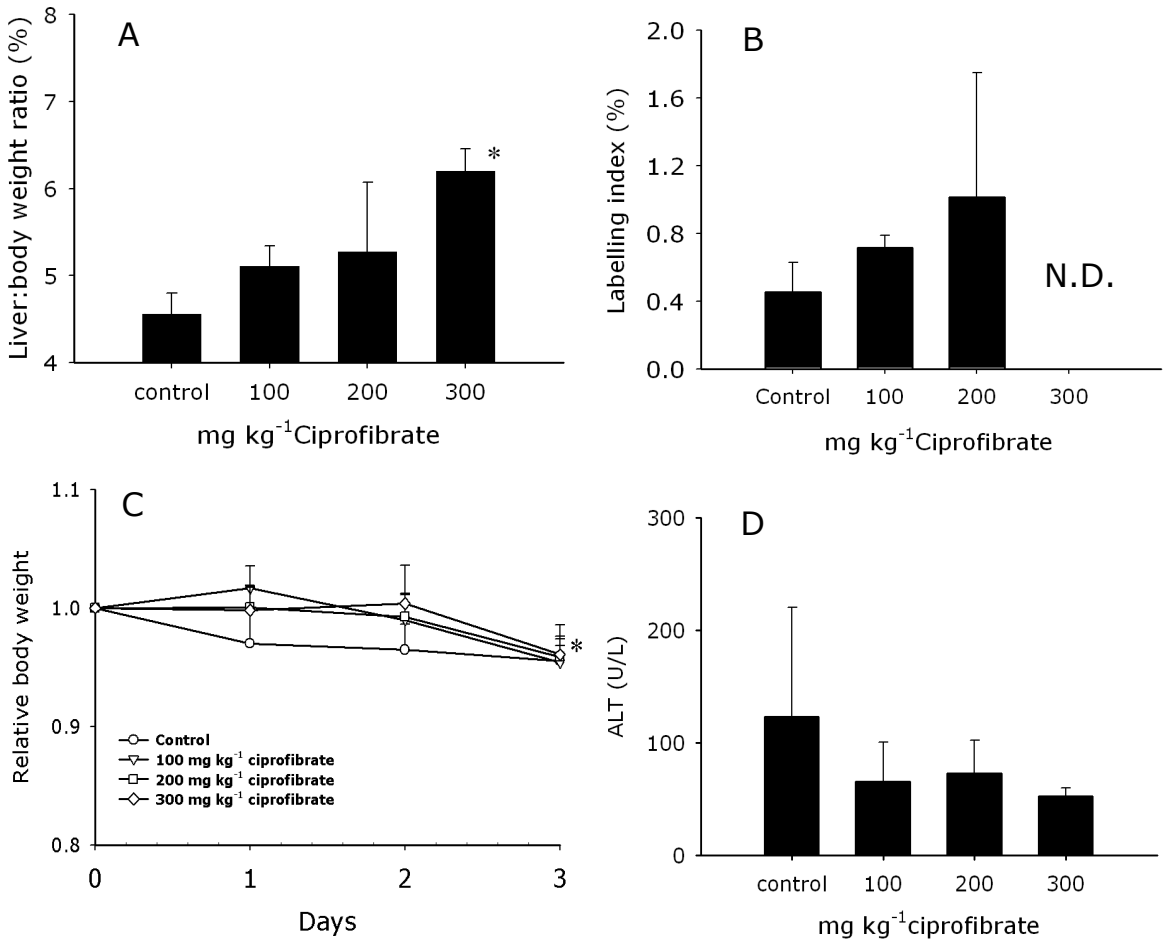

Figure 3.7 Effect of ciprofibrate on liver growth in 129S4/SvJae mice. Group of five male129S4/SvJae mice were dosed 0.5mg ml^{-1} BrdU in 16% orange juice for three days; after one day of BrdU dosing, the animals were dosed once by gavage with 0,100,200 and 300 mg kg^{-1} ciprofibrate, and the animals were killed after 2 days as described in single dose protocol 1 (Section 2.2.1.2). (A) Liver-to-body-weight ratios are expressed as a percentage of mean±SD; (B) Labelling index was calculated as described in Figure 3.4; (C) Relative body weight was calculated and statistically tested using paired student t-test as described in Figure 3.5; (D) Serum alanine aminotransferase (ALT) activity was determined using Vitros ALT slides (Ortho-Clinical Diagnostics). The procedure was performed in the clinical chemistry department, Queen medical center (QMC) at Nottingham. One-way ANOVA and Dunnett's test were used for statistical analysis of A, B and D, while paired t-test was used for figure C as described in Figure 3.5. ∗ = p<0.05. N.D.: data not analysed. n=5.

Section 3.1.2.5 Effect of acclimatisation and juice on body weight gain

The mice exposed to 0.05% BrdU with 16% orange juice showed a significant decrease in body weight (Section 3.1.2.4). We set out other protocols to maintain body weight during the BrdU-dosing procedure by adapting the mice for one week to flavoured juice. This step was important to acclimatise the mice to the juice taste before starting dosing. 10% juice was used instead of 16% dilution to reduce juice acidity. The adaptation protocol was used in different mice strains,

129S4/SvJae, DBA/2J, C57BL/6J and Alderley park (AP), and rats (F-344/NHsd) to assess the effect on body weight.

Section 3.1.2.5.1 129S4/SvJae mice

Groups of three 129S4/SvJae PPARα -/- mice were acclimatised with different diluted juices (orange, apple and blackcurrant) without BrdU for one week, then the animals were given 0.8mg ml^{-1} BrdU + diluted juices (10% v/v) as a sole source for drinking water for 3 days. The relative body weight in mice given juice were similar to the mice in control group. After BrdU dosing, the mice treated with BrdU + juice did not show any reduction in body weight and there were no significant differences in RBW increases between the treated group. These finding show that BrdU solution has no negative effect on body weight, if mice were previously acclimatised to drinking diluted juices. Thus, this acclimatisation regime will be used in labelling protocol prior treating the animal with peroxisome proliferator (Figure 3.8).

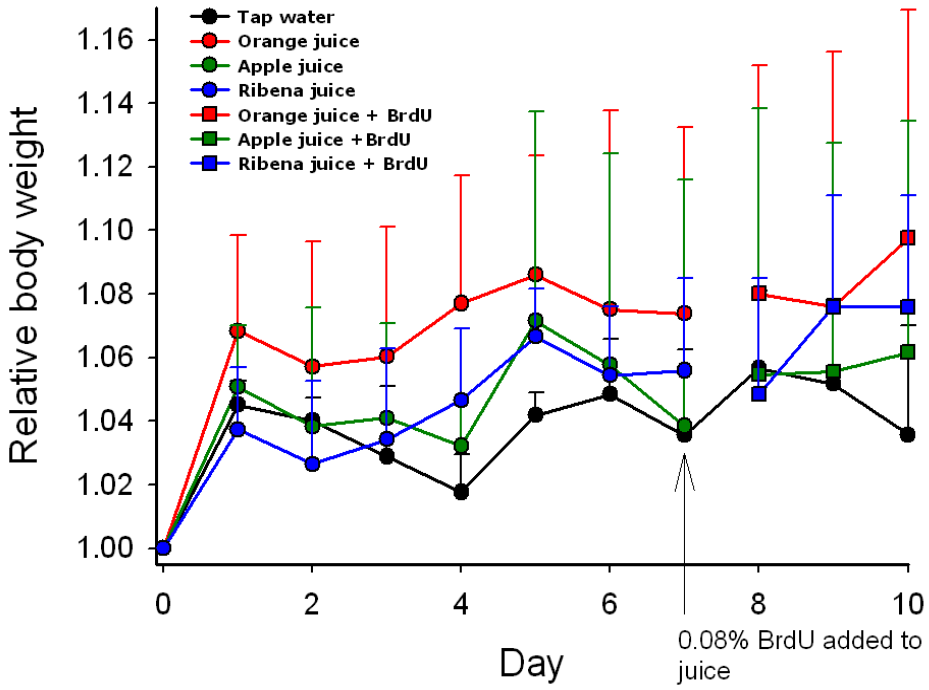

Figure 3.8 Effect of acclimatisation and juice on 0.08% BrdU on relative body weight. Four groups of three 129S4/SvJae PPARα -/- mice were acclimatised to different flavour juices (10% v/v in tap water) for a week as their sole water source, then to 0.08% BrdU in 10% juice for 3 days. Mice body weights were measured on daily basis for each individual mouse and relative body weight was calculated relative to the body weight for each individual mouse on day 0. The arrow shows the start of administration of BrdU. Data are the mean±SD.

Section 3.1.2.5.2 DBA/2J mice

Groups of two DBA/2J mice were acclimatised in 10% orange juice for a week, then were dosed 0.8mg ml^{-1} BrdU supplemented with 10% orange juice as a sole source of drinking water for six days. Control group was dosed with tap water. No body weight reduction was observed in the mice after administration of BrdU (Figure 3.9). This finding shows that the labelling protocol can be used to administer BrdU in this strain (DBA/2J) of mice without causing any reduction in the body weight.

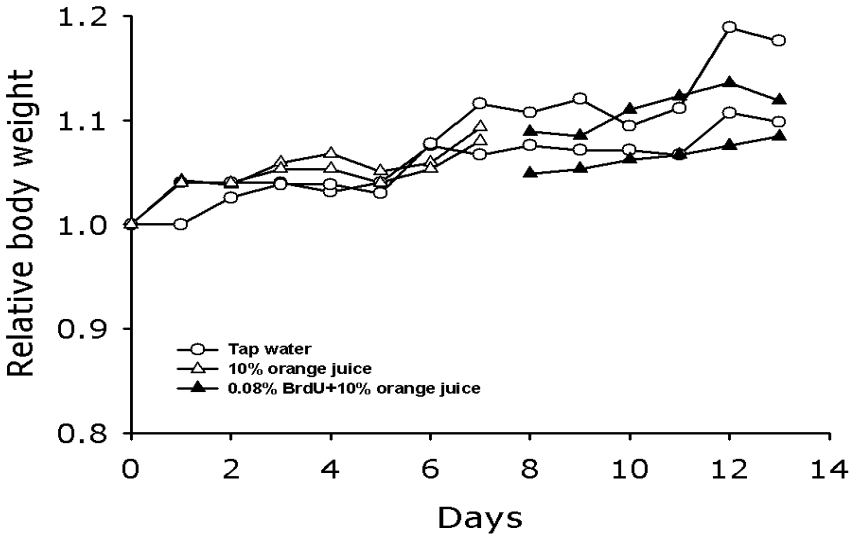

Figure 3.9 Effect of acclimatisation and orange juice on body weight of DBA/2J mice. Group of two DBA/2J mice were acclimatised in 10% orange juice for a week, then were dosed with 0.08% BrdU+orange juice for six days. Control group was given a tap water. Relative body weight was measured as described in Figure 3.8. Data was expressed for each individual mouse

Section 3.1.2.5.3 C57BL/6J mice

In order to assess the adaptation of C57BL/6J strain onto the labelling protocol, groups of two male C57BL/6J mice were acclimatised to 10% orange juice for a week, then were dosed 0.8mg ml^{-1} BrdU supplemented with 10% orange juice as a sole source of drinking water. Control group was dosed with tap water. No body weight reduction was noticed in the BrdU-treated mice after BrdU treatment. Thus, the labelling protocol with BrdU does not have an adverse effect on body weight.

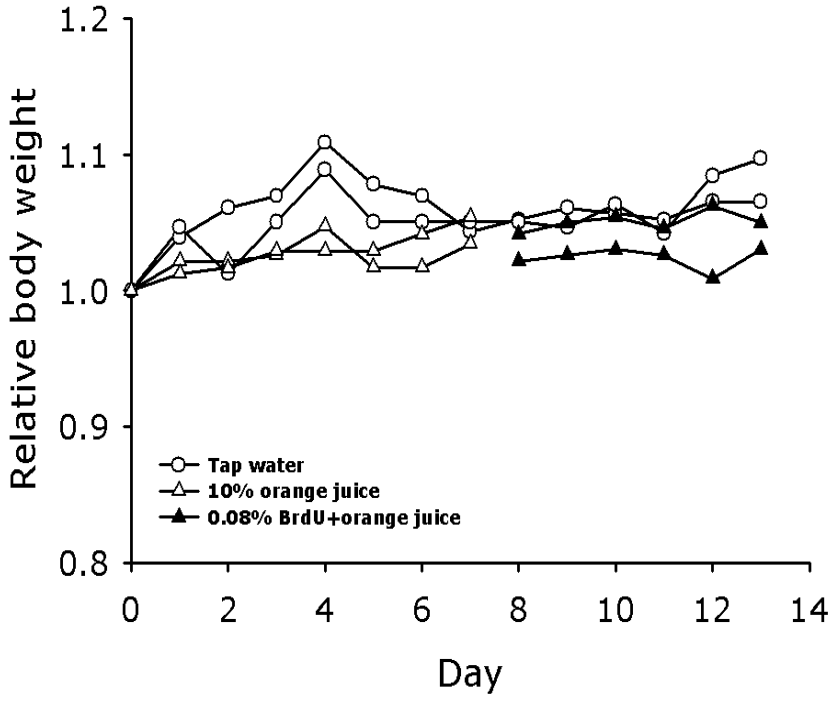

Figure 3.10 Effect of acclimatisation and orange juice on body weight of C57BL/6J mice. Group of two C57BL/6J mice were acclimatised in 10% orange juice for a week, then were dosed with 0.08% BrdU+orange juice for six days. Control group was given a tap water. The relative body weight was measured as described in Figure 3.8. Data was expressed for each individual mouse.

Section 3.1.2.5.4 Alderley park (AP) mice

Group of four mice were dosed with 10% orange juice for a week, then dosed with 0.8mg ml^{-1} BrdU + orange juice for four days. The orange juice period was used as a control due to the limited availability of this strain. No body weight loss was observed after dosing the mice with 0.08% BrdU (Figure 3.11). The labelling protocol was good for administration of BrdU in drinking water.

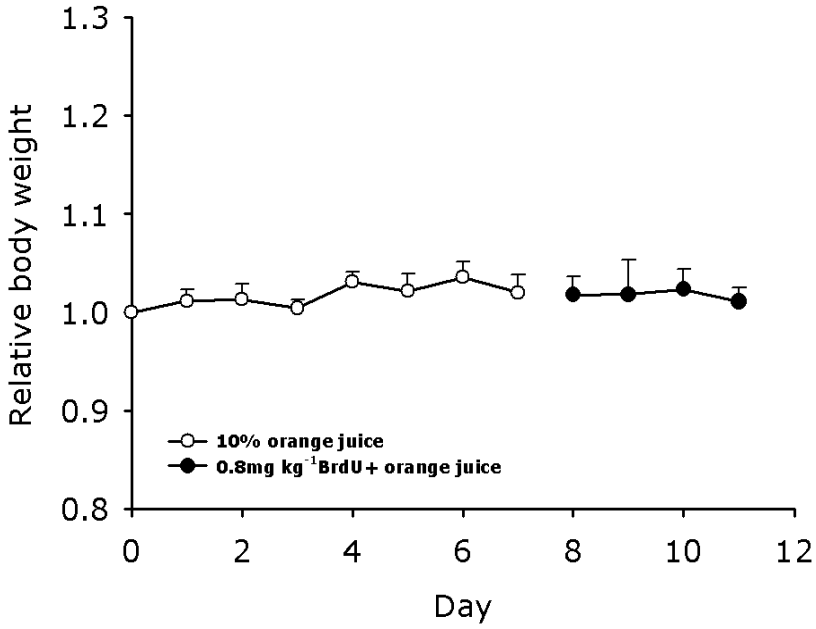

Figure 3.11 Effect of acclimatisation and orange juice on body weight gain of Alderley park (AP) mice. Group of four mice were dosed with 10% orange juice for a week then 0.8mg ml^{-1} BrdU + orange juice for four days. Relative body weight was measured as described in Figure 3.8. The time of orange juice dosing was used as control for BrdU+orange juice dosing. Student paired t-test was used in the analysis. Data was expressed as mean±SD.

Section 3.1.2.5.5 F344/NHsd rats

The BrdU-labelling protocol (Section 2.2.1.1) was assessed in male F-344/NHsd rats before designing the experiment of species differences. Groups of two male F-344/NHsd rats were acclimatised to 10% orange juice for a week, then were dosed with 0.8mg ml^{-1} BrdU supplemented with 10% orange juice as a sole source of drinking water. Control group was dosed with tap water. No body weight reduction was noticed in the BrdU-treated rat after BrdU treatment. Thus, the labelling protocol with BrdU does not show an adverse effect on rat body weight.

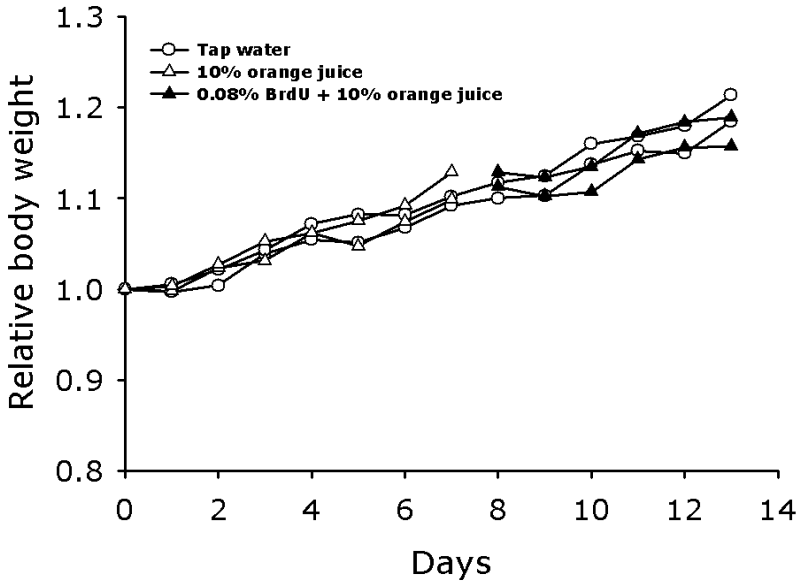

Figure 3.12 Effect of acclimatisation and orange juice on body weight gain of F-344/NHsd Rat.
Group of two F-344/NHsd rats were acclimatised in 10% orange juice for a week, then were dosed with 0.08% BrdU+orange juice for six days. Control group was given a tap water. The body weight was measured daily and relative body weight is relative to body weight on day one. Data was expressed as mean±SD.

Section 3.2 Induction of DNA synthesis by ciprofibrate in 129S4/SvJae mice; dose-dependency

Maintaining body weight during BrdU exposure prompted a repeat of dosing the mice with ciprofibrate to characterize the induction of hepatic DNA synthesis. In this study, the acclimatising regime (Section 2.2.1.1) was introduced in the experiment to maintain body weight. Five groups of five male129S4/SvJae mice were dosed with 100-400 mg kg^{-1}ciprofibrate once, and killed after two days. This range was chosen to find out the optimal dose of ciprofibrate that produce a significant induction of liver growth without toxicity since up to 75mg kg^{-1} ciprofibrate does not show any induction in the liver growth. Under these conditions, the relative body weights do not decrease over the 10 days of the study period (Figure 3.13A). Liver to body weight ratios increase substantially and significantly at the 100 mg kg^{-1}dose level, and hit a plateau at 200 mg kg^{-1} ciprofibrate, with a 44% increase over control (Figure 3.13B), validating that the protocol can be used successfully for chronic administration of doses of BrdU sufficient to measure DNA synthesis.

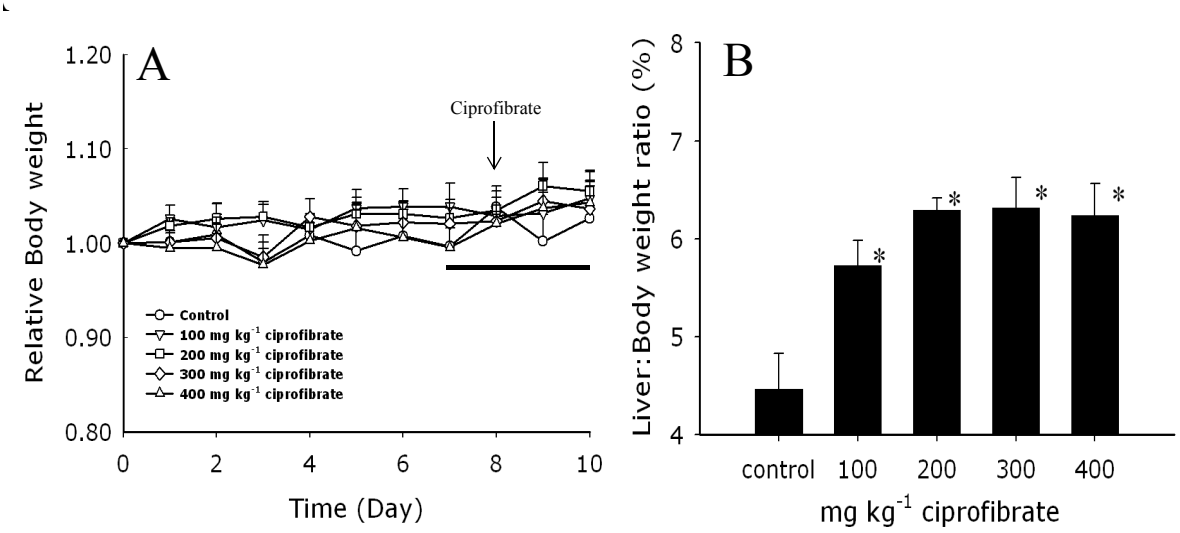

Figure 3.13 Effect of single dose of ciprofibrate over two days on liver growth. Group of five 129S4/SvJae mice were acclimatised on labelling protocol as described on material and method, Section 2.2.1.1. All groups were administered once with ciprofibrate by gavage (0-400 mg kg^{-1}), and vehicle for control group as described in single dose protocol 2 (Section 2.2.1.3). The mice were killed after two days. (A) Relative body weight was calculated as described in Figure 3.5. Paired student t-test was used for statistical analysis. (B) Liver-to-body weight ratio (as a percentage) was calculated as shown in Figure 3.5. One-way ANOVA with Dunnett's test as post hoc was used in statistical analysis. All data expressed as mean ± SD. Solid horizontal line represents a time when animal were exposed to BrdU in 10% orange juice.

However, although there was a non significant increase in labelling index (LI) at 300 mg kg^{-1} over the control, the mice exposed to a single dose of ciprofibrate (up to 400 mg kg^{-1}) did not show a significant induction of hepatic DNA synthesis within two days (Figure 3.14A). Alanine aminotransferase (ALT) was measured in serum as a test for any hepatic damage. ALT level in treated mice did not differ significantly from the control mice (Figure 3.14B). Thus, my results reveal: (1) the labelling protocol was good; (2) the ciprofibrate causes substantial induction of liver weight by two days; (3) even in the presence of a ~50% increase in liver weight, there was no increase in DNA synthesis; (4) the lack of induction was not due to gross liver toxicity, as there was no increase in ALT activity. Therefore, ciprofibrate does not induce LI within two days. This result was in contrast to the work of Styles, who showed that peroxisome proliferators strongly induce DNA synthesis in mouse liver by 24 hours [113] [164]. This finding requires further investigation to characterise the early proliferative response of hepatic DNA synthesis by ciprofibrate.

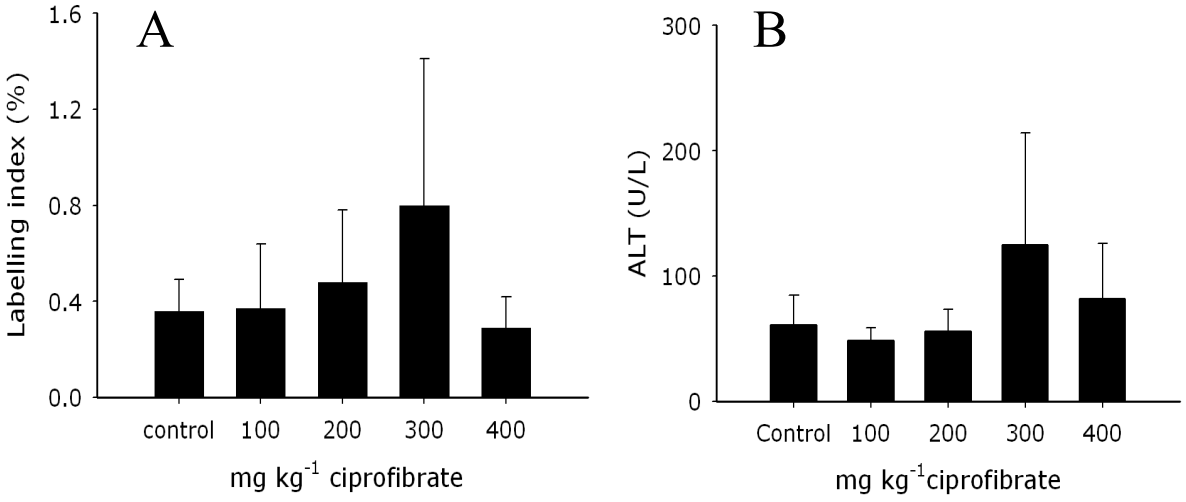

Figure 3.14 Effect of single dose of ciprofibrate on liver growth in 129S4/SvJae mice. Group of five male 129S4/SvJae mice were acclimatised to labelling protocol as described in Figure 3.13, then dosed once with ciprofibrate by gavage (0-400 mg kg^{-1}), and vehicle for control group. The mice were killed after two days. (A) Labelling index was determined as described in Figure 3.4. (B) Serum ALT activity was determined as described in Figure 3.7. One-way ANOVA with Dunnett's test as post hoc was used in statistical analysis. All data expressed as mean ± SD.

Section 3.3 Effect of TCPOBOP on DNA synthesis in 129S4/SvJae mouse

Section 3.2 had shown that there was no proliferative response to ciprofibrate within two days. In order to confirm whether failure of ciprofibrate to increase the LI was due to defective methodology used for measuring DNA synthesis in the liver, replicative DNA synthesis was examined with a positive control compound which acts via activation of the CAR receptor [207]. TCPOBOP was used to induce liver growth in male and female mice within 48 hours, as it was reported to cause induction of DNA synthesis within 48 hours [207]. Groups of five male or female 129S4/SvJae mice were acclimatised to the labelling protocol as described in Section 2.2.1.1 before treatment with a single dose of 3mg kg^{-1} TCPOBOP by gavage over two days. The control groups were treated with vehicle (dimethyl sulphoxide-corn oil solution). No body weight reduction was observed following BrdU administration among the mice in all groups (Figure 3.15A).

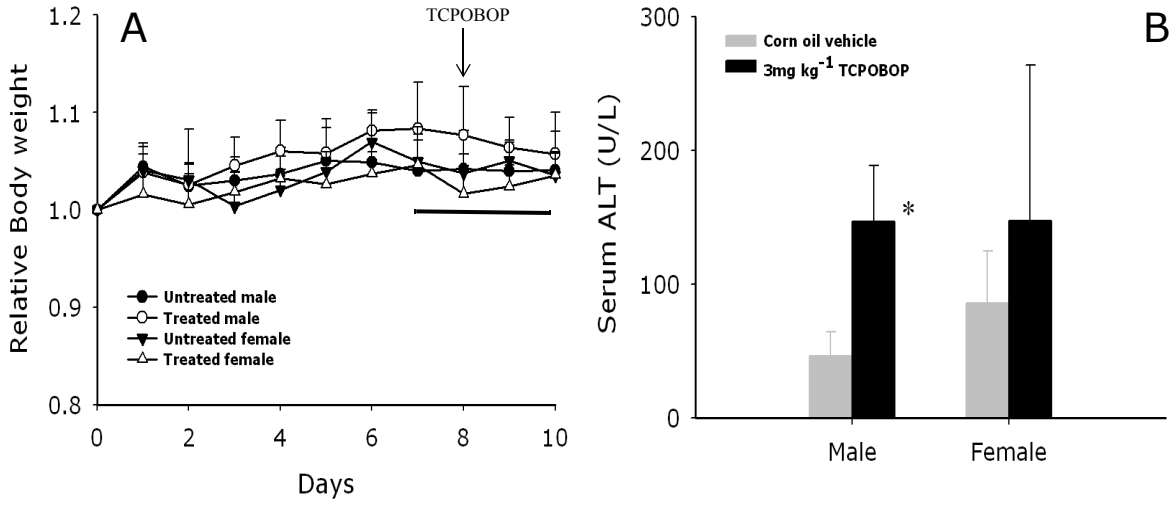

Figure 3.15 Effect of TCPOBOP on mice liver. Groups of five male and female 129S4/svJae mice treated with labelling protocol as described in Figure 3.13 were given a single dose of 3 mg kg^{-1} TCPOBOP dissolved in dimethyl sulphoxide as described in single dose protocol 2 (Section 2.2.1.3). Control groups received vehicle only. All animal were humanely killed after 48 hours of treatment. Blood was drawn by cardiopuncture technique to determine serum ALT. (A) Relative body weight was determined and calculated as described in Figure 3.5. (B) Serum alanine aminotransferase (ALT) activity was measured as described in Figure 3.7. Unpaired student t-test was used for statistical analysis of two groups (ALT data), whereas paired student t-test was used to analyse relative body weight as described in Figure 3.5. All data expressed as mean ± SD, *= $p<0.01$. Solid line represents a time when animal were exposed to BrdU in 10% orange juice.

However, treatment with TCPOBOP results in a statistically significant increase in serum ALT, an index of hepatocyte injury, in male treated mice, but not in the female-treated mice compared to corresponding control (Figure 3.15B). Administration of TCPOBOP significantly increased liver-to-body ratio by to 65 and 96% of control in male and female treated groups respectively (Figure 3.16A). TCPOBOP caused a significant increase in hepatic labelling index compared to corresponding control, rising from 0.2±0.1% and 1.4±1.0% in the control groups to 27.5±6.1% (~ 145-fold increase) and 48± 9.8% (35-fold increase) in the treated male and female groups respectively (Figure 3.16B). Histologically, Figure 3.16C showed liver sections from treated male and female mice with high magnitude of BrdU-labelled nuclei compared to the corresponding control. This amount of increase was greater in female than male mice. These findings confirm that the method used in this study detected hepatocyte labelling within 48 hours. This shows that the failure of ciprofibrate to increase the LI was not a consequence of defective methodology.

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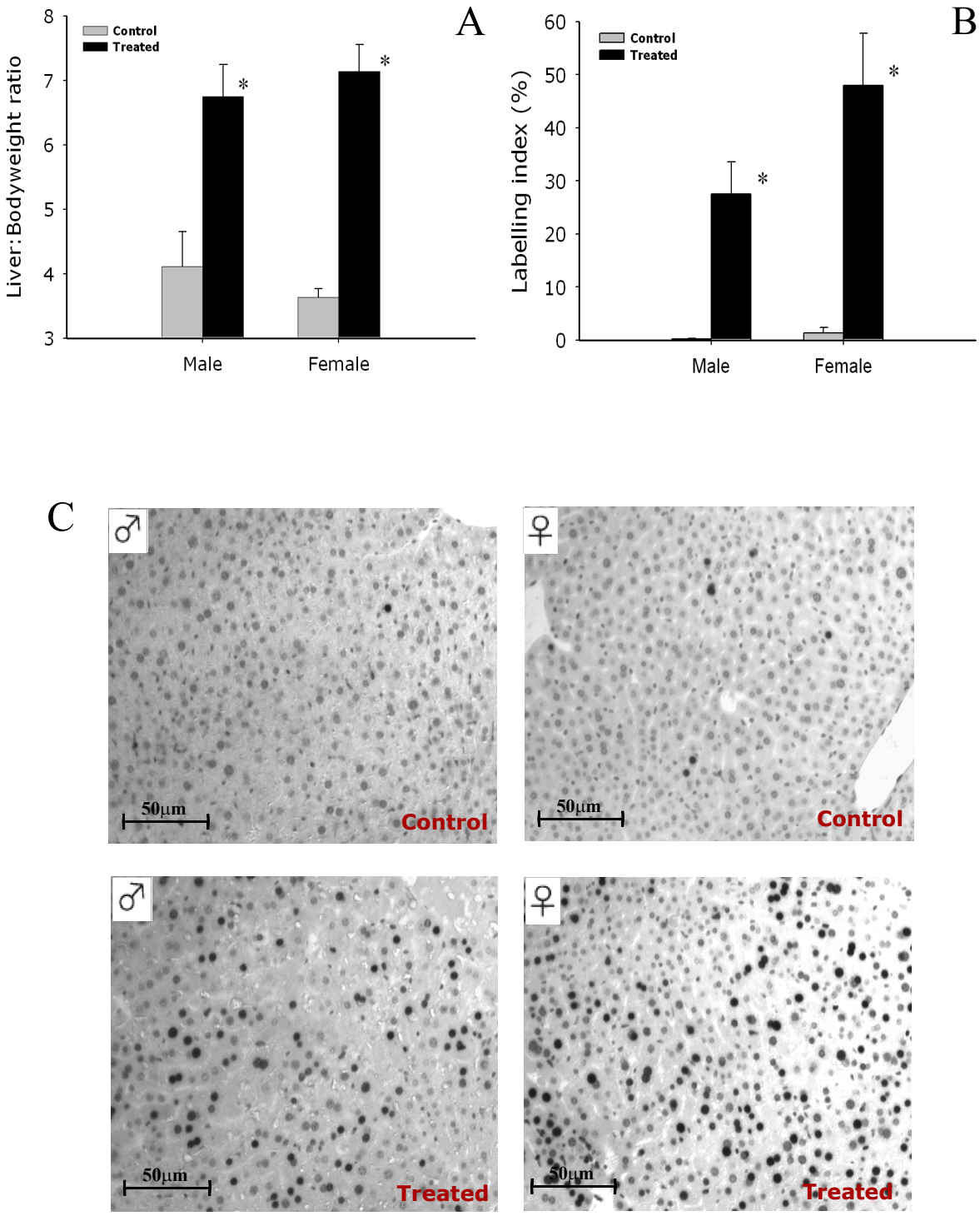

Figure 3.16 Effects of TCPOBOP on hepatic DNA synthesis in 129S4/SvJae mice. Groups of male and female mice were dosed with TCPOBOP as described in Figure 3.15. (A) Liver:body weight ratio (as a percentage) was calculated as shown in Figure 3.5. (B) Labelling index percent was determined as described in Figure 3.4. (C) Representative liver sections labelled with anti-BrdU antibody (Black nuclei) and counterstained with haematoxylin of TCPOBOP-treated (Treated) male (♂) and female (♀) mouse and untreated (Control) mouse. Liver sections from control and treated male and female mice were fixed overnight and stained with immunohistochemical protocol as described in Figure 3.1. Gut section was used as positive control as described in Figure 3.2. Results are expressed as means± SD of five mice per group. Significant difference was determined by Student's t-test; * =p<0.00001.

Section 3.4 Induction of hepatic DNA synthesis by Methylclofenapate (MCP)

An alternative hypothesis to explain the failure to detect early induction of DNA synthesis by ciprofibrate, was that this was an idiosyncratic response to ciprofibrate, which was not typical of PPARα agonists in general. Methylclofenapate (MCP) was a potent peroxisome proliferator and it has been reported to increase DNA synthesis (>10%) in mice at 24 hours after dosing with 25mg kg^{-1} [164]. To examine this hypothesis, a group of seven 129S4/SvJae mice were treated with a single dose of 25mg kg^{-1} MCP and killed after two days (as described in Section 2.2.1.3); a control group was dosed with corn oil vehicle. No body weight reduction occurred in the mice following administration of BrdU in drinking water (Figure 3.17A). Serum ALT in the treated group did not significantly differ from the control group (Figure 3.17B).

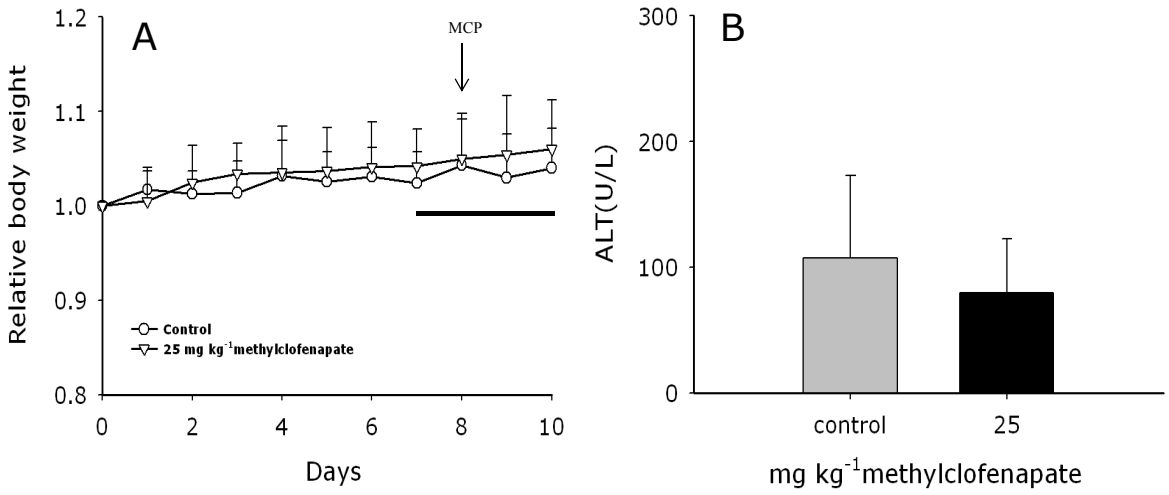

Figure 3.17 Effect of a single dose of methylclofenapate on relative body weight and ALT in mice.
Groups of seven male129S4/SvJae mice were acclimatised to the labelling protocol as described in Figure 3.13, and were dosed with 25mg kg^{-1} MCP-corn oil solution as described in single dose protocol 2 (Section 2.2.1.3). Control group was dosed with corn oil. (A) Relative body weight was determined as described in Figure 3.5. (B) Serum alanine aminotransferase (ALT) as liver toxicity marker was measured as described in Figure 3.7. Data was expressed as mean ± SD for groups of seven mice (n = 7). Paired and unpaired student t-test were used for statistical analysis of A and B respectively. The arrow represents the day of dosing with MCP or vehicle, whereas solid line represents a time when animal were exposed to BrdU in 10% orange juice.

Liver-to-body weight ratio was significantly increased in the MCP-treated group compared to the control group (Figure 3.18A), whereas the labelling index in the MCP-treated group did not

differ significantly from the control group (Figure 3.18B). This result shows that the failure of induction of DNA synthesis within two days was not unique to ciprofibrate, and suggests it was common to PPARα agonists in general.

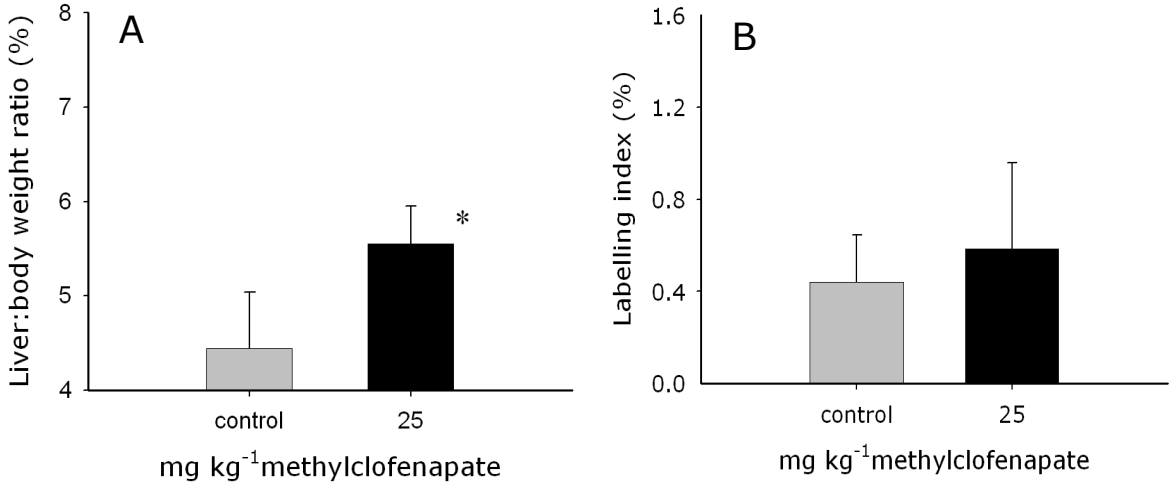

Figure 3.18 Induction of hepatic DNA synthesis after a single dose of methylclofenapate. Group of seven mice treated with labelling protocol as described in Figure 3.13 were given a single dose of 25mg kg^{-1} MCP or corn oil vehicle and killed after two days. (A) liver:body weight ratio was calculated as described in Figure 3.5. (B) hepatocyte labelling index was calculated as described in Figure 3.4. Significantly difference was determined by unpaired Student's t-test, $*=p<0.01$.

Section 3.5 Effect of ciprofibrate on liver growth over five days

Administration of a single dose of ciprofibrate over two days does not cause induction of DNA synthesis (Section 3.2). In this study, induction of DNA synthesis after a single dose of ciprofibrate over five days was tested to determine if ciprofibrate (300mg kg^{-1}) was capable of inducing DNA synthesis over five days. Groups of five 129S4/SvJae mice were treated with a single dose of 300 mg kg^{-1} ciprofibrate or vehicle and killed after five days. No body weight loss was observed in either group, but the treated group showed a significant body weight gain on the day of killing as compared to the day of BrdU dosing (Figure 3.19A). Relative liver weight was significantly increased from 4.2±0.39% in the control group to 5.3±0.22% in the group treated with 300 mg kg^{-1} ciprofibrate (Figure 3.19B). The labelling index in the treated group was significantly increased from 0.2±0.1% in the control to 3±1.3% (15-fold increase) in the treated group

(Figure 3.19C). ALT activity in treated mice did not significantly differ from the control group (Figure 3.19D). This result shows that induction of DNA synthesis occurs within 5 days of a single dose of ciprofibrate without liver toxicity. Together with Figure 3.14, it shows that ciprofibrate induces DNA synthesis more than two days after treatment with ciprofibrate.

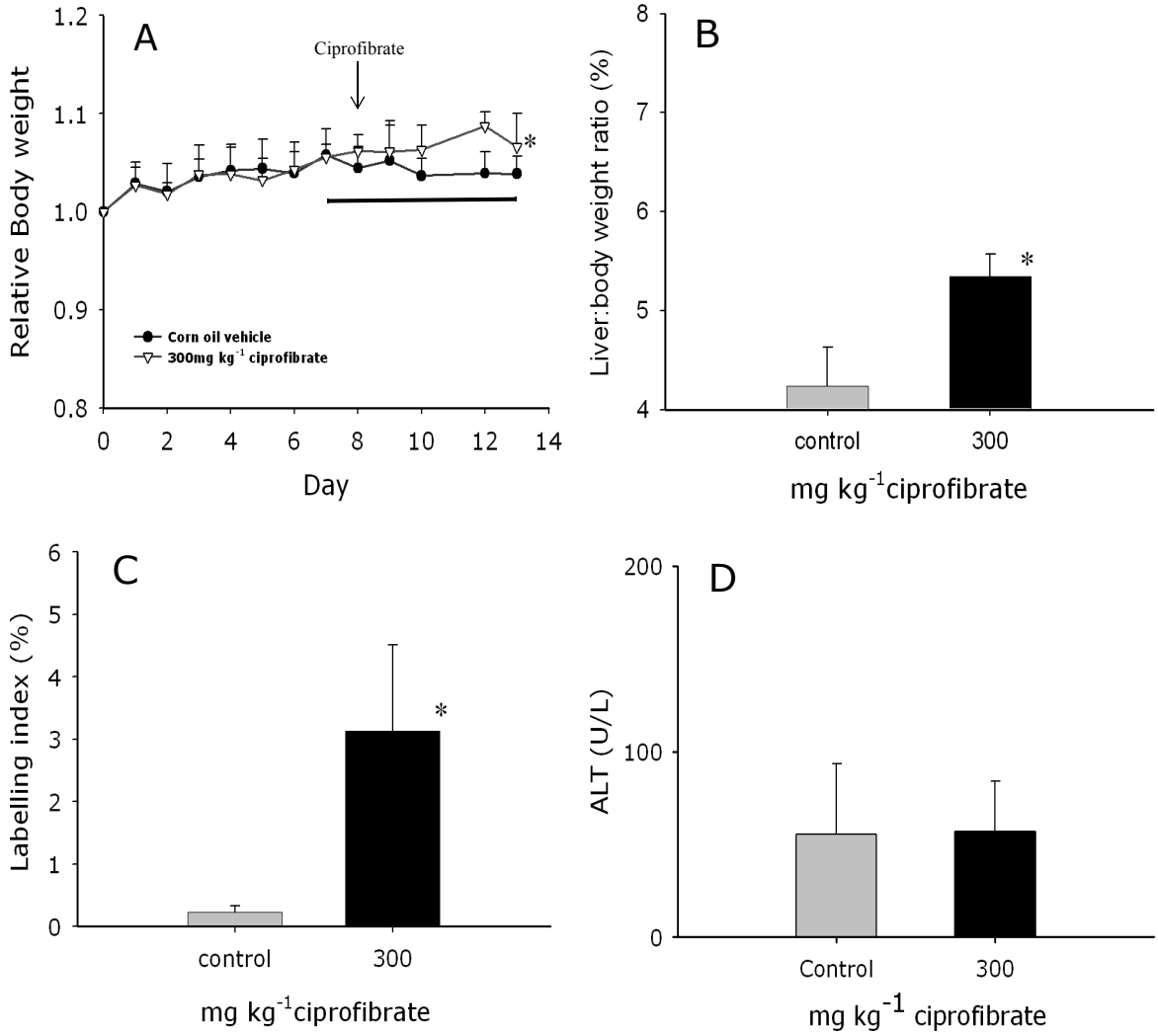

Figure 3.19 Induction of DNA synthesis in hepatocytes by ciprofibrate over five days. Single dose of 300 mg kg^{-1} ciprofibrate was administered to five 129S4/SvJae male mice by gavage for five days as described in single protocol 2 (Section 2.2.1.3). Control group was dosed corn oil as vehicle. All mice were acclimatised in labelling protocol as described in Figure 3.13. A: relative body weight and B: Liver-to-body weight percent were calculated as described in Figure 3.5; C: Labelling index as percentage was calculated as described in Figure 3.4; D: serum ALT level as marker for hepatic damage was determined as described in Figure 3.7. The bars show the mean ± SD and significant difference was determined by unpaired Student's t-test for B, C and D and paired student t-test for A as described in Figure 3.5, $*= p<0.05$. The arrow represents the day of dosing with ciprofibrate or vehicle, whereas solid line represents a time when animal were exposed to BrdU in 10% orange juice.

Section 3.6 Kinetic studies on induction of liver growth in 129S4/SvJae mice

Previous sections had shown that peroxisome proliferators (ciprofibrate and methylclofenapate) do not induce hepatic DNA synthesis within 48 hours (Section 3.2 and Section 3.4). We set out experiments to characterise the time- and dose dependency of hepatic DNA synthesis, and whether these effects were mediated by the PPARα receptor.

Section 3.6.1 Time-course effect of ciprofibrate in wild type 129S4/SvJae mice

The aim of the time-course experiment was to find out the earliest time point at which the increase in hepatic DNA synthesis was significantly greater than control levels. Groups of five male129S4/SvJae mice were acclimatised as described in Section 2.2.1.1, and were treated with 100mg $kg^{-1}day^{-1}$ ciprofibrate and killed on days 2, 3, 4, 5 and 6. Control groups were killed on days 2 and 6 after dosing with the vehicle control. No significant body weight loss was observed in the mice over 14 days (Figure 3.20A). Serum alanine aminotransferase (ALT) levels in treated groups at all time points did not differ significantly from the control groups (Figure 3.20B)

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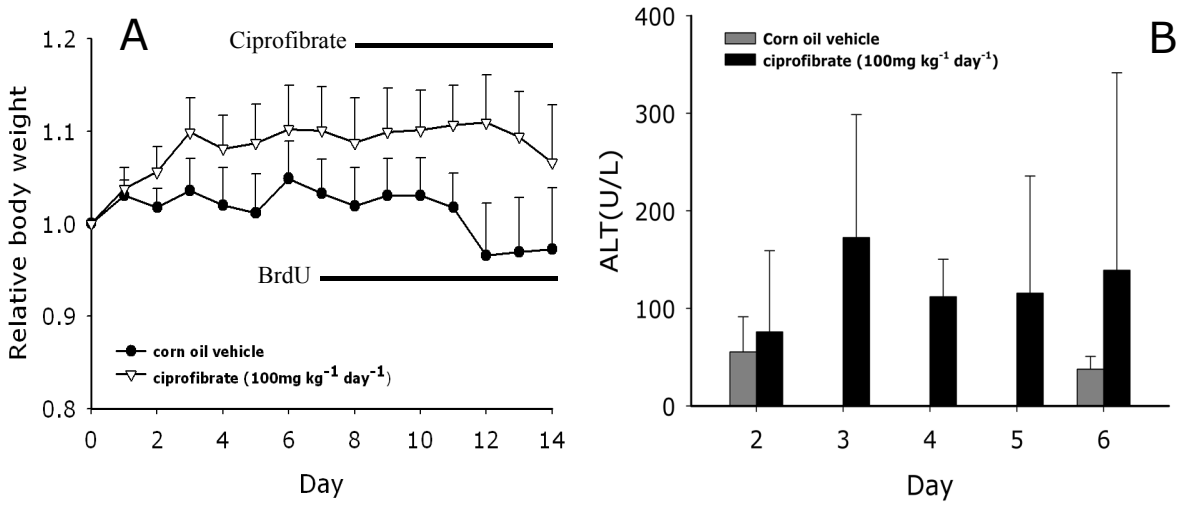

Figure 3.20 Time course of effect of ciprofibrate on relative body weight and ALT level on wild-type 129S4/SvJae mice. Groups of five mice were dosed with ciprofibrate (100mg $kg^{-1}day^{-1}$) for 2, 3, 4, 5 and 6 days as described in multiple dose protocol 1 (Section 2.2.1.4). Control groups were dosed with corn oil on days 2 and 6. (A) Relative body weight (RBW) of the control and the treated groups pooled into two groups were determined as described in the Figure 3.5. Statistical analysis was performed between the day of BrdU dosing and the last day by using paired student t-test. Although the result shows no significant body weight loss in the groups, 4-days and 6-days (wild type) treated groups showed a significant increased in RBW (B) Serum ALT level was measured as stated in Figure 3.7. Statistical analysis was performed using ANOVA described in Figure 3.5. Results are expressed as mean± SD of five mice per group. 5- and 6-day groups were given BrdU on day 7. The remaining groups were given BrdU in day 8. Closed circles are control and open triangles are treated. Solid line labelled BrdU represents the time when animals were exposed to BrdU in the 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Liver-to-body weight (LBW) ratio was significantly increased in treated groups by 48% at 2 days up to 113% at 6 days, as compared to the control (Figure 3.21A). Ciprofibrate failed to induce DNA synthesis in the liver in the first two days, but days 3, 4 and 6 days showed significant induction (11, 20 and 14-fold, respectively) compared to the control group (Figure 3.21B). Additionally, even though the inter-animal variation in the treated groups at days 3 and 4 was high, each individual animal has a labelling index that was higher than any of the animals treated with corn oil vehicle. These results revealed that day 3 was the earliest time point when DNA synthesis was significantly greater than control level. Treatment with 100mg $kg^{-1}day^{-1}$ for 6 days ciprofibrate does not cause liver damage in 129S4/SvJae mice.

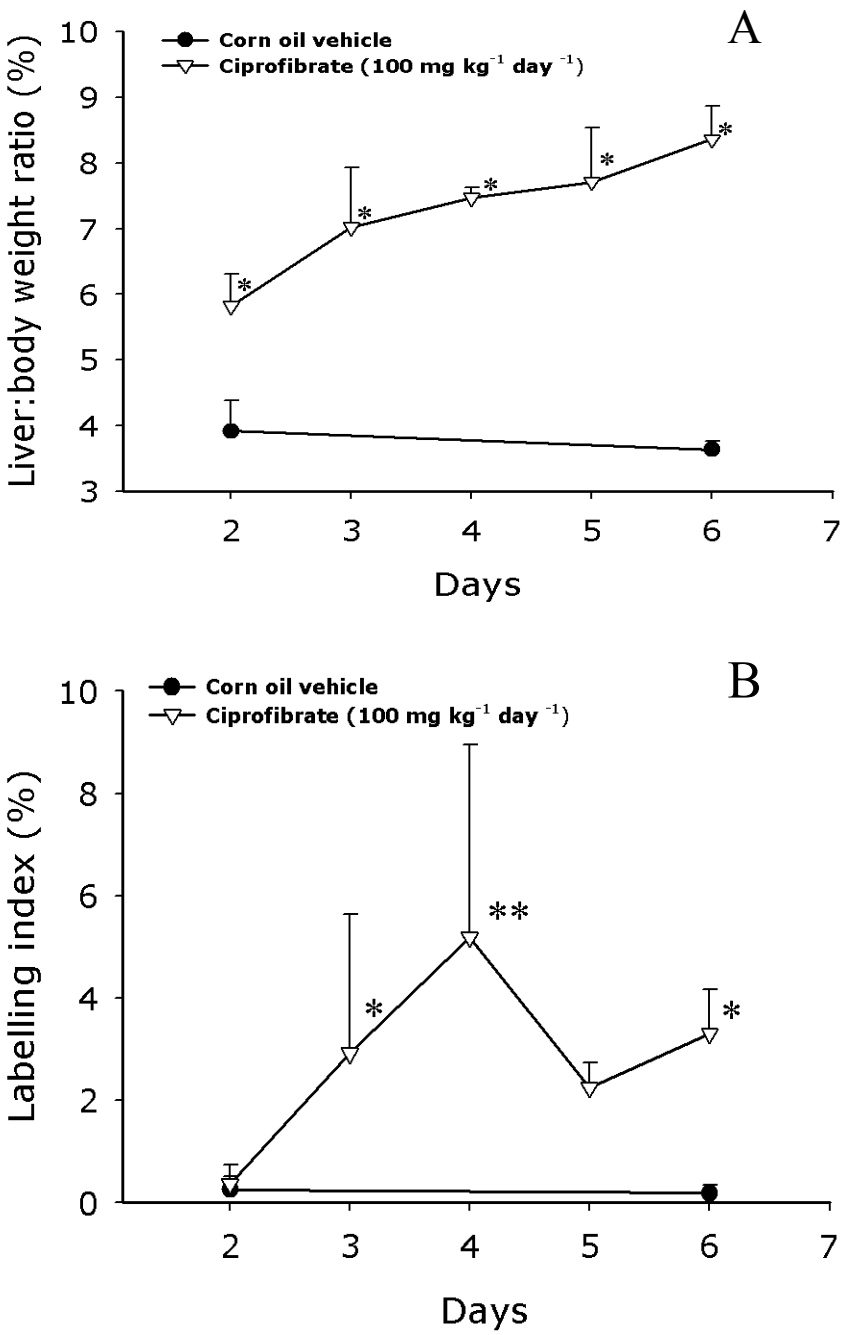

Figure 3.21 Time course of effects of ciprofibrate on liver-to-body weight ratio and labelling index in male 129S4/SvJae mice. Groups of five mice were treated as described in Figure 3.20. (A) Liver:body weight (LBW) ratio as a percentage was determined as described in Figure 3.5. (B) Labelling index was calculated as described in Figure 3.4. Data shown are mean ± SD. Significantly different from the control was determined by one-way ANOVA and Dunnett's test. * = $p<0.01$; ** = $p<0.001$. Closed circles are control groups and triangle downs are ciprofibrate-treated groups.

Section 3.6.2 Effect of ciprofibrate in PPARα knockout mice

The aim of this study was to test whether the effects of ciprofibrate seen in the Section 3.6.1 were mediated by the PPARα. The experiment was performed by using PPARα -/- mice (129S4/SvJae background) [81]. Group of five null mice were treated with 0 and 100mg kg^{-1} day^{-1} ciprofibrate then killed on days 2 and 6. No significant body weight reduction was observed in treated and control groups (Figure 3.22A). Liver enzyme alanine aminotransferase level in treated groups did not differ from vehicle control after two or 6 days of treatment with ciprofibrate.

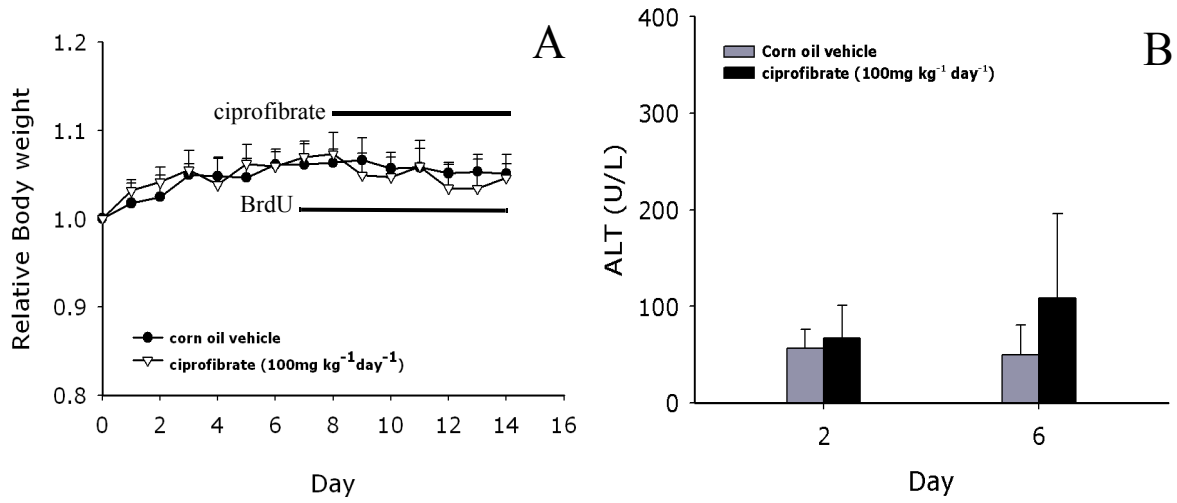

Figure 3.22 Assessment of body weight and liver status on 129S4/SvJae null mice treated with ciprofibrate. Groups of five null mice were dosed with ciprofibrate (100mg $kg^{-1}day^{-1}$) for 2 and 6 days. Control groups were dosed with corn oil.(A) Relative body weight (RBW) of the control and the treated groups pooled into two groups were determined as described in the Figure 3.5. Statistical analysis was performed between the day of BrdU dosing and the last day by using paired student t-test. Relative body weight; (B) ALT level on null mice were measured as described in Figure 3.7. Statistical analysis was performed using ANOVA described in Figure 3.5. Results are expressed as means± SD of five mice per group. Closed circles are control groups and open triangle are ciprofibrate-treated groups. Solid line labelled BrdU represents the time when animals were exposed to BrdU in the 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Administration of ciprofibrate (100mg kg^{-1} day^{-1}) did not affect the liver weight of PPARα KO mice. The LBW ratio in ciprofibrate-treated mice was not significantly different compared to control (Figure 3.23A). Moreover, labelling index in treated groups also did not differ from the

control group on both days (Figure 3.23B). This finding demonstrate that the induction of DNA synthesis by ciprofibrate was dependent on the PPARα receptor

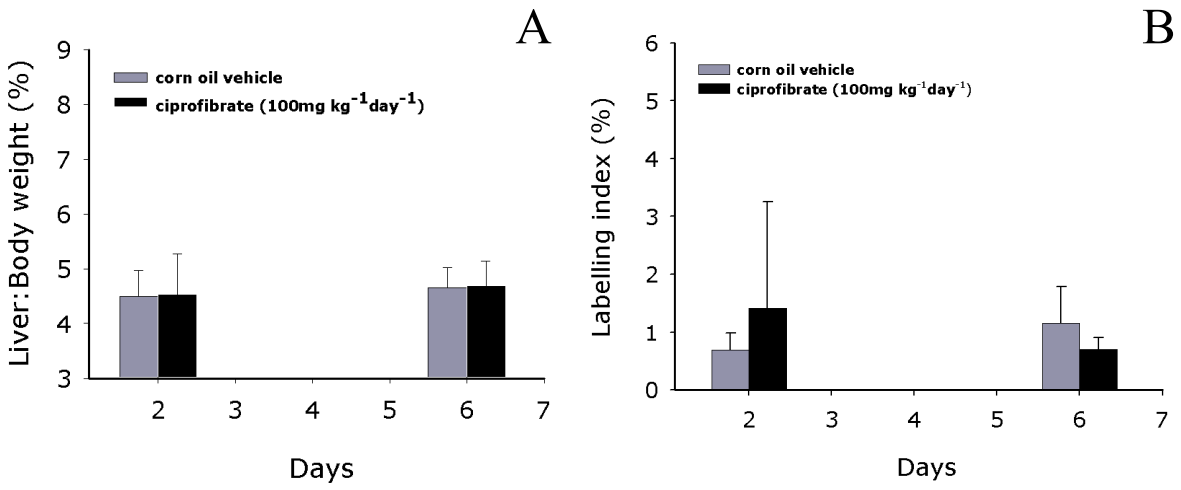

Figure 3.23 Effect of ciprofibrate on induction of DNA synthesis in 129S4/SvJae null mice. Group of five null mice acclimatized as described in Section 2.2.1.1 were dosed with 100mg $kg^{-1}day^{-1}$ciprofibrate for 2 and 6 days. (A) Liver/body weight ratio was calculated as described in Figure 3.5; (B) Labelling index percent was calculated as described in Figure 3.4. Unpaired t-test was used for statistical analysis. Data was expressed as mean±SD.

Section 3.6.3 Dose-related effects of ciprofibrate on hepatic DNA synthesis in 129S4/SvJae mice

To demonstrate whether there was a dose dependency in the response of mouse liver to induction of DNA synthesis by the peroxisome proliferator ciprofibrate, groups of six mice were gavaged with 0, 1, 3, 10, 30, 100 or 200 mg kg^{-1} day^{-1} ciprofibrate in corn oil for 3 days, or were gavaged with 0,10,30,100 mg kg^{-1} day^{-1} ciprofibrate for 4 days. Control animals were given vehicle (corn oil). 3 and 4 days of treatment were chosen in this study because they showed a maximal induction of DNA synthesis in the time-course study (Section 3.6.1). Dosing the animals with ciprofibrate and BrdU did not cause any body weight reduction during the study (Figure 3.24A). However, RBW at the day of killing in 3-day control showed a statistical decrease,

whereas in the 4-day treated group there was a statistical increase in RBW as compared to the corresponding group in the day of BrdU dosing. Both these effects were small, and it was not clear if they were of biological significance. Moreover, serum ALT levels in treated groups of mice treated with ciprofibrate for 3 and 4 days did not display any significant difference from the corresponding control group (Figure 3.24B).

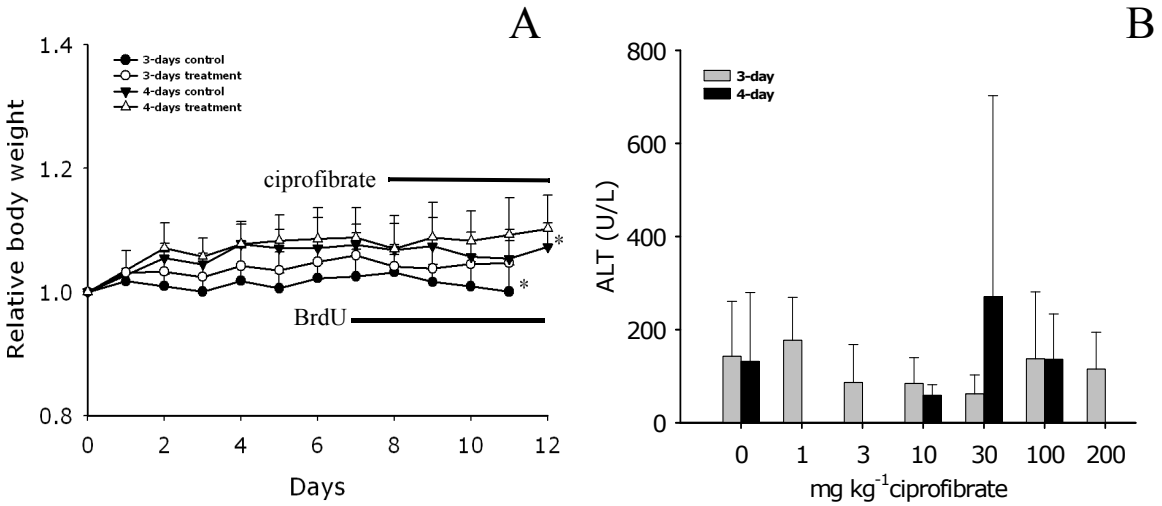

Figure 3.24 Assessment of body weight and liver status in dose-related effects of ciprofibrate. Groups of six male 129S4/SvJae mice were dosed with 0, 1, 3, 10, 30, 100 or 200 mg kg^{-1} day^{-1} ciprofibrate in corn oil for 3 days (Figure 2.4), or were gavaged with 0,10,30,100 mg kg^{-1} day^{-1} ciprofibrate for 4 days (Figure 2.5). Control animals were given vehicle (corn oil). (A) Relative body weight (RBW) was calculated and statistical tested as described in Figure 3.5; (B) Serum ALT level were measured and statistically tested as described in Figure 3.7. Results are expressed as means±SD of six mice per group. Closed circles are 3-day control, open circles are 3-days treatment, closed triangle downs are 4-days control and open triangle up are 4-day treatment. Solid line labelled BrdU represents the time when animals were exposed to BrdU in the 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Figure 3.25A showed that dosing of ciprofibrate for 3-day period at concentration up to 3mg $kg^{-1}day^{-1}$ produced no change in the liver-to-body weight (LBW) ratio. However, the dose-dependency study revealed that liver to body weight ratios were statistically increased in mice treated with 10mg $kg^{-1}day^{-1}$ and greater ciprofibrate at 3- and 4-days. The LBW ratio was 5.2% (1.2-fold over control) at 10mg kg^{-1} day^{-1} for both exposure times. The LBW ratio increased in a dose-dependent manner with maximum increase to 6.7% (1.5-fold over control) at 200mg $kg^{-1}day^{-1}$ for 3-day dosing, and 7% (1.7-fold over control) at 100mg $kg^{-1}day^{-1}$ for 4-day dosing. Induction of hepatic DNA synthesis was measured in these animals. Figure 3.25B showed that

after 4 days of ciprofibrate treatment, the animals demonstrated progressively increased BrdU-labelled DNA of 2.7±0.97% (8-fold over control) and 3.7±1.49% (11-fold over control) at dose levels of 30 and 100mg $kg^{-1}day^{-1}$ ciprofibrate respectively as compared to control, while animals treated with ciprofibrate over 3 days showed a significant increase in labelling index by 7-fold increase (1.0±0.59%) over the control only at an exposure level of 100mg $kg^{-1}day^{-1}$ ciprofibrate. The magnitude of induction of DNA synthesis at dose levels 30 and 100mg $kg^{-1}day^{-1}$ in 4 days treatment were 7- and 4-fold higher than those in 3 days treatment. These results confirm that ciprofibrate at dose 30 mg $kg^{-1}day^{-1}$ for four days or 100mg $kg^{-1}day^{-1}$for three days were doses that induce hepatic DNA synthesis significantly over the control male 129S4/SVJae mice without any liver toxicity.

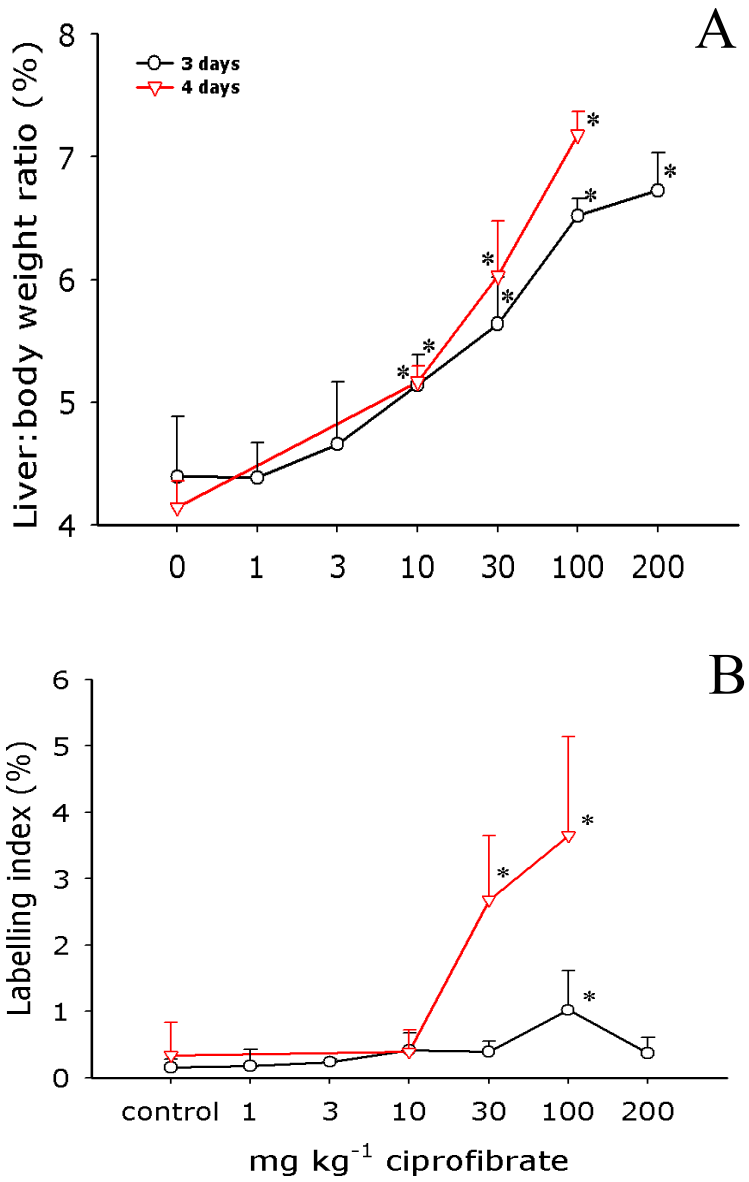

Figure 3.25 Dose-related effects of ciprofibrate on liver growth in 129S4/SvJae mice. Group of six mice were acclimatized as described in Section 2.2.1.1. The groups were gavaged with 0, 1, 3, 10, 30, 100 or 200 mg kg^{-1} day^{-1} ciprofibrate, or with 0,10,30,100 mg kg^{-1} day^{-1} ciprofibrate and killed after three or four days respectively. Control groups were dosed with corn oil as vehicle. (A) liver-to-body weight ratio was calculated as described in Figure 3.5; (B) hepatic labelling index was determined as described in Figure 3.4. Statistical analysis was performed as described in Figure 3.21. Data shown are mean ±SD. Circles are 3 days treatment and triangles down are 4 days treatment.

Section 3.7 Induction of liver growth by ciprofibrate in female 129S4/ SvJae mice

In order to characterise the induction of DNA synthesis in both sexes of 129S4/SvJae mice, groups of female mice were dosed with 100mg $kg^{-1}day^{-1}$ ciprofibrate by gavage then killed after 3 or 4 days of treatment.

Section 3.7.1 Three days dosing

Group of seven female 129S4/SvJae mice (9-10 weeks old) were dosed with 100mg $kg^{-1}day^{-1}$ ciprofibrate by gavage for three days. control group was dosed with corn oil as vehicle. All mice were acclimatised as described in Section 2.2.1.1 before treatment with ciprofibrate. Relative body weight and serum ALT level were measured. As shown in Figure 3.26A, no body weight reduction was seen in the mice in either group. However, the treated group showed a significant increase in body weight at the day of killing when compared to the day of BrdU dosing. Serum ALT level of the ciprofibrate-treated group was not statistically different from the control group (Figure 3.26B).

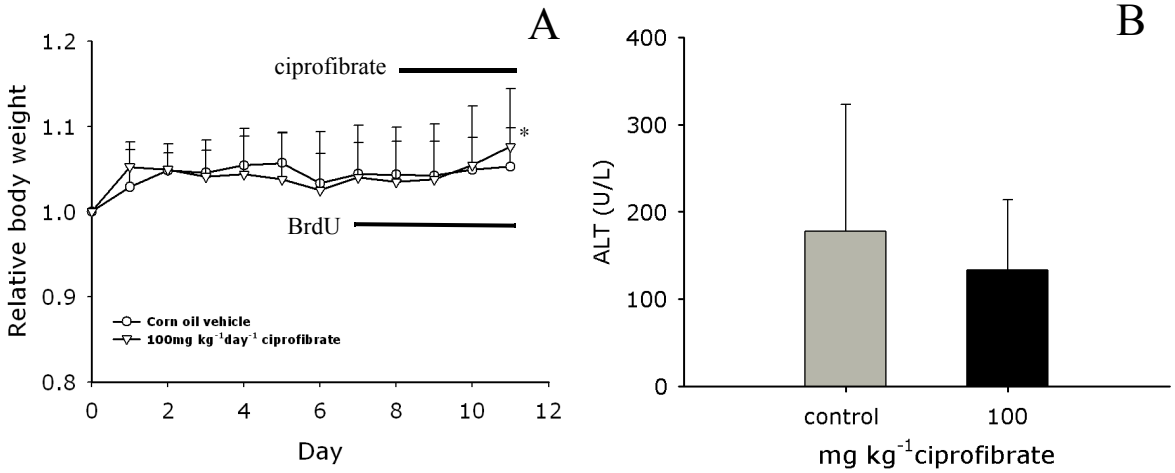

Figure 3.26 Body weight and ALT of female 129S4/SvJae mice treated with ciprofibrate for three days. Groups of seven female mice were dosed with 100mg kg^{-1} day^{-1} ciprofibrate then killed after three days as described in multiple protocol 2 (Figure 2.4). Control group was dosed with corn oil. All mice were acclimatised as described in Figure 3.13. (A) Relative body weight was calculated and statistically tested as described in Figure 3.5; (B) Serum ALT level was measured and statistically tested as described in Figure 3.7. Data was expressed as mean±SD, n=7. Solid line labelled BrdU represents the time when animals were exposed to BrdU in the 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Figure 3.27A shows liver:body weight ratio was significantly increased from 4±0.4% in the control group to 6±0.3% in the 100mg $kg^{-1}day^{-1}$ treated group. However, although labelling index was higher in the treated group as compared to the control, labelling index in the treated group did not differ significantly from the control (Figure 3.27B). These results shows that although three days of ciprofibrate (100mg $kg^{-1}day^{-1}$) dosing induced hypertrophy in liver, it did not significantly induce DNA synthesis over the control.

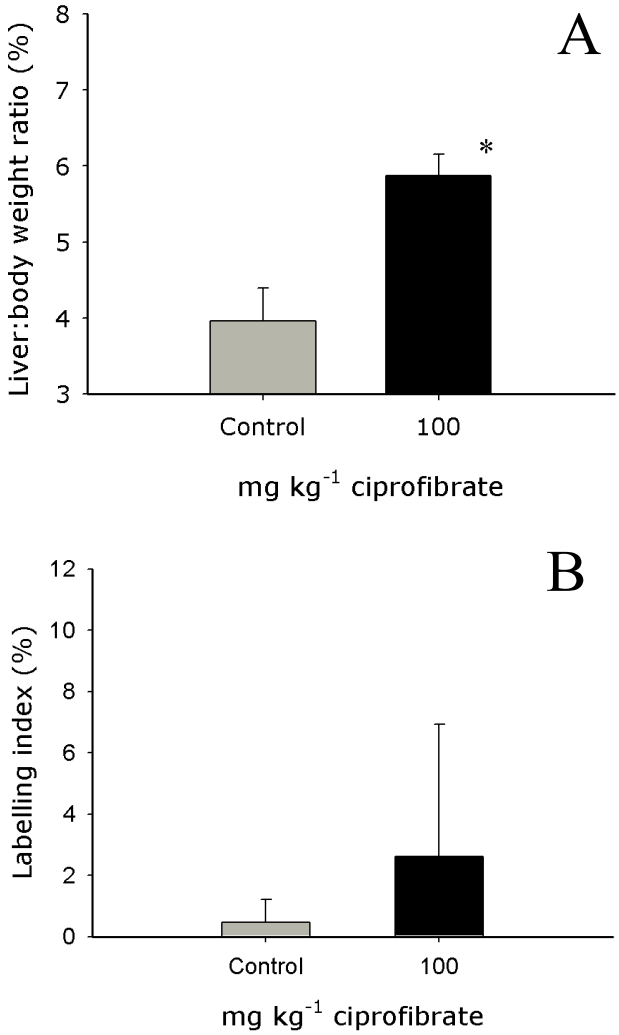

Figure 3.27 Induction of DNA synthesis in female 129S4/SvJae mice by ciprofibrate for three days.
Group of seven female 129S4/SvJae mice were dosed with 100mg $kg^{-1}day^{-1}$ ciprofibrate and killed after three days. The mice were treated as described in Figure 3.26. (A) Liver: body weight ratio (%) was calculated as in Figure 3.5 (B) Labelling index was calculated as described in Figure 3.4. Significantly difference was determined by Student's t-test, *= p<0.01. Data was expressed as mean±SD.

Section 3.7.2 Four days dosing

Groups of six female 129S4/SvJae mice was gavaged with 100mg $kg^{-1}day^{-1}$ ciprofibrate for four days. The control group was treated with corn oil vehicle. All animals were acclimatised using the protocol described in Section 2.2.1.1. before dosing. No body weight loss was observed in the mice after dosing with BrdU. In the treated group, there was a significant increase in RBW at day 12 as compared to the day of BrdU dosing (Figure 3.28A). There was no significant difference in ALT between treated and control mice after four days of ciprofibrate dosing (Figure 3.28B).

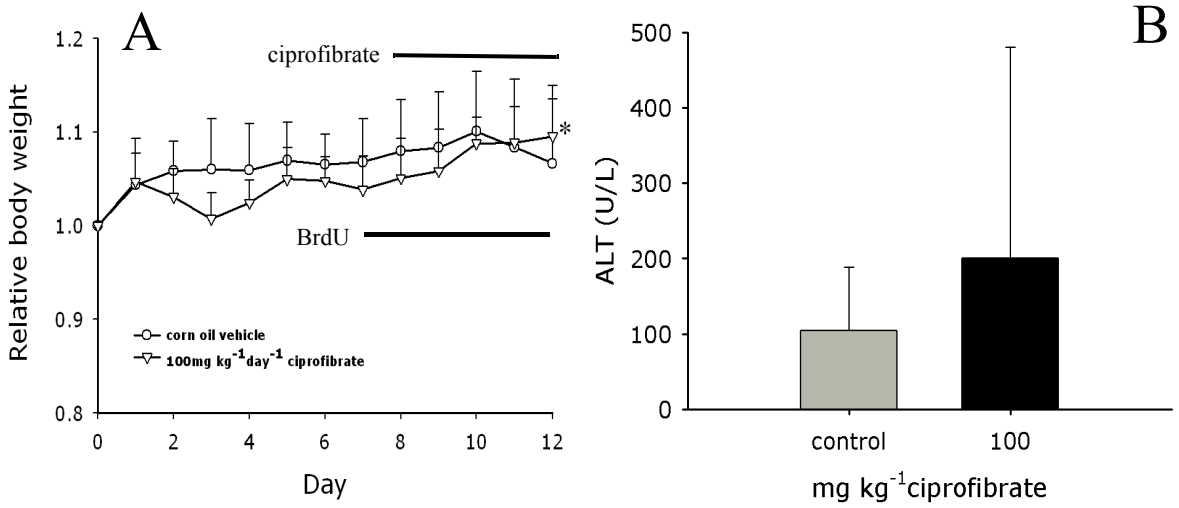

Figure 3.28 Body weight and ALT level of female 129S4/SvJae mice treated with ciprofibrate for four days. Groups of female mice were dosed with 100mg kg^{-1} day^{-1} ciprofibrate and killed after four days as described in multiple protocol 2 (Figure 2.5). Control group was dosed with corn oil. All mice were acclimatised as described in Figure 3.13. (A) Relative body weight was calculated and statistically tested as described in Figure 3.5; (B) Serum ALT level was measured and statistically tested as described in Figure 3.7. Data was expressed as mean±SD, n=6. Solid line labelled BrdU represents the time when animals were exposed to BrdU in 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Mice treated with ciprofibrate showed a significant increase in liver:body weight ratios, rising from 4±0.7% in the control group to 6.8±0.5% in the treated group (Figure 3.29A). In contrast to 3 days dosing, the labelling index was significantly increased from 0.7±0.5% in the control group to 4.4±3.9% in the treated group (6-fold increase) after 4 days dosing with ciprofibrate (Figure 3.29B). Thus, four days dosing with ciprofibrate (100mg $kg^{-1}day^{-1}$) can induce DNA synthesis (hyperplasia) as well as liver growth (hypertrophy) in female mice.

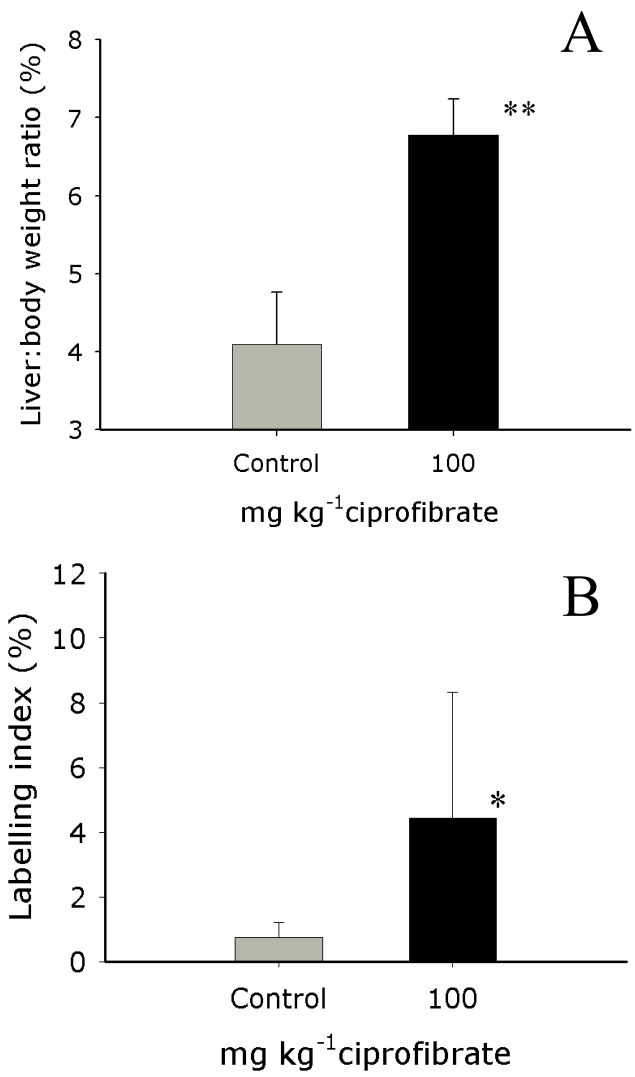

Figure 3.29 Induction of DNA synthesis in female 129S4/SvJae mice by ciprofibrate for four days.
Group of six female 129S4/SvJae mice were treated as mentioned in Figure 3.28 (A) Liver: body weight ratio (%) was calculated as in Figure 3.5 (B) Labelling index was calculated as described in Figure 3.4. All data was expressed as mean±SD.Significant difference was determined by Student's unpaired t-test, *= $p<0.05$ and **= $p<0.0001$.

Section 3.8 Sex differences of liver growth induction by ciprofibrate in 129S4/SvJae mice

The aim of this study was to examine sex differences in the induction of liver growth by ciprofibrate. The effect of ciprofibrate (100mg $kg^{-1}day^{-1}$) treatment on hepatic DNA synthesis was compared in male and female mice by comparing the results of female mice in the Section 3.7 with the results of male mice in the Section 3.6.1 and Section 3.6.3. As shown in Figure 3.30, liver:body weight (LBW) ratio in treated-males was significantly greater than in treated-females receiving ciprofibrate for three days. Moreover, LBW of treated male mice (experiment 1) was significantly higher than those in treated female mice over four days.

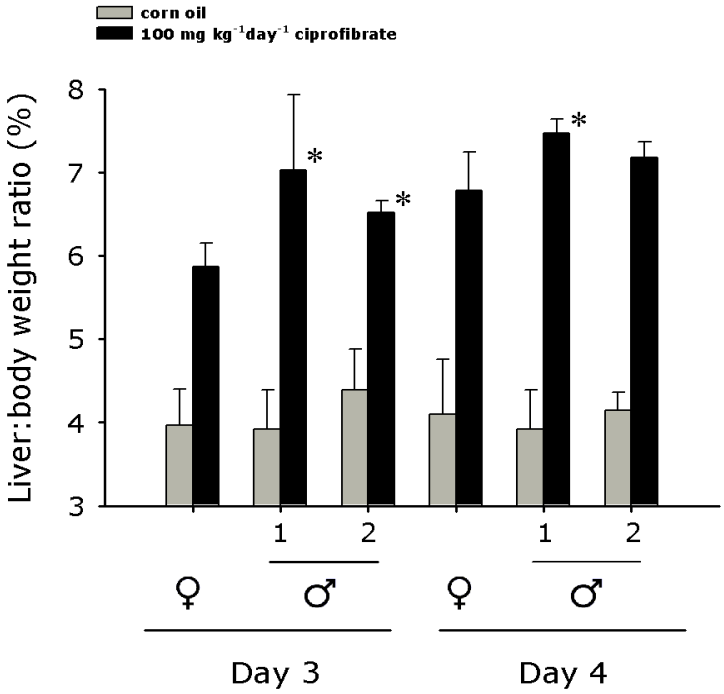

Figure 3.30 Sex differences in relative liver growth on 129S4/SvJae mice induced by ciprofibrate. Liver:body weight (LBW) ratios of treated female group dosed with 100mg $kg^{-1}day^{-1}$ciprofibrate were compared with those of treated-male groups over three and four days. Liver:body weight (LBW) ratio from two experiments, time-course (1) and dose dependency studies (2) of male mice were used for comparison with LBW of female mice. All data was expressed as mean±SD. Significant difference was determined by one-way ANOVA and posthoc by Newman-Keuls. *= significant difference was compared between treated female and treated male of same day.

No sex differences in the labelling index induction of DNA synthesis by ciprofibrate was observed as the labelling index in treated males did not significantly differ from those in the treated

females (Figure 3.31).

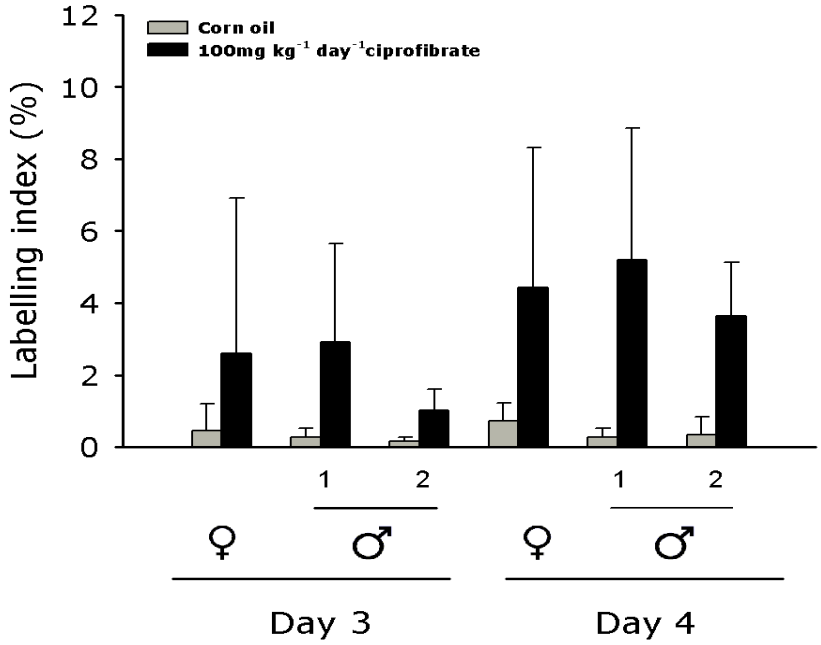

Figure 3.31 Sex differences in labelling index on 129S4/SvJae mice following treatment with ciprofibrate. Labelling index of treated female group dosed with 100mg $kg^{-1}day^{-1}$ciprofibrate were compared with those of treated-male group over three and four days. The male mice had the same dose of ciprofibrate. Labelling index from two experiments, time-course (1) and dose dependency studies (2) of male mice were used for comparison with LI% of female mice. All data was expressed as mean±SD. Significant analysis was determined by one-way ANOVA and Newman-Keuls. *= significant difference as compared to treated female in the same day.

Section 3.9 Time-course effect of methylclofenapate on 129S4/SvJae mice

Section 3.6.1 had shown that the induction of DNA synthesis occur after three-to-four days of ciprofibrate dosing (Section 3.6.1). To investigate if this time-course of DNA induction was an idiosyncratic response to ciprofibrate, methylclofenapate was used to study the time-course of induction of DNA synthesis. Groups of six male 129S4/SvJae mice (9-10 weeks old) were dosed with 25mg $kg^{-1}day^{-1}$ methylclofenapate and killed 2,3 and 4 days later. The control groups were killed on days 2 and 4 after dosing with corn oil as vehicle. Administration of BrdU or MCP did not cause any statistically significant body weight loss in the mice, as determined using a paired t-test on the pooled control or MCP-treated relative body weights, comparing between the initial day of dosing and subsequent days. However, there was a statistically significant increase in relative body weight in the animals killed after 2 or 3 days treatment with MCP (but not vehicle), as compared with day 0 (paired t-test, $p<0.05$) (Figure 3.32A). No liver toxicity was noticed among the mice treated with MCP as ALT activities in treated mice were not significantly different from those in the control groups (Figure 3.32B).

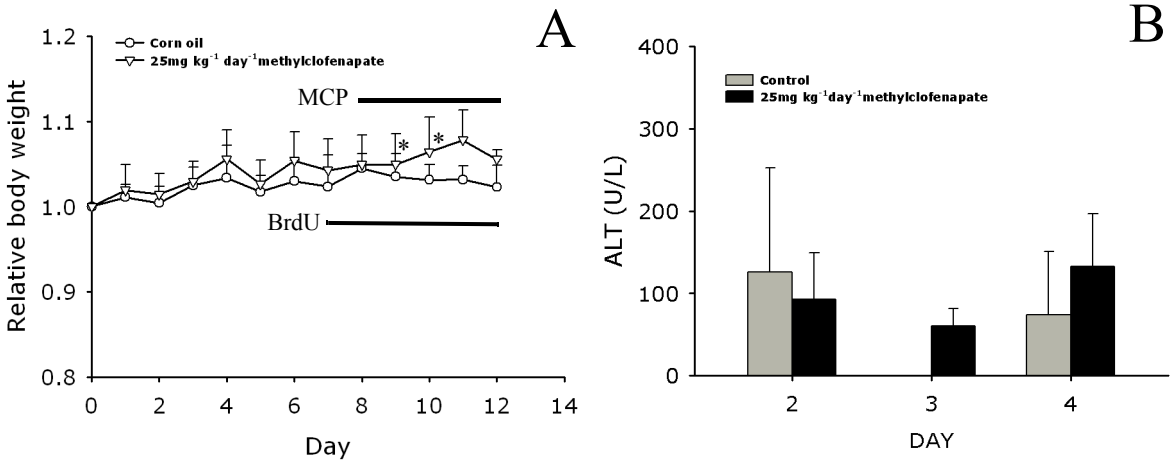

Figure 3.32 Time course effect of methylclofenapate on relative body weight and ALT activity. Groups of six 129S4/SvJae mice were dosed with 25mg kg^{-1} day^{-1} MCP and killed on days 2, 3 and 4 as described in multiple protocol 1 (Figure 2.3). Control groups were dosed with corn oil as vehicle and killed on days 2 and 4 after dosing. All mice were acclimatised as described Figure 3.13. (A) Relative body weight was calculated and statistically tested as described in Figure 3.5; (B) Serum ALT level a liver toxicity marker was measured and statistically tested as described in Figure 3.7. All data was expressed as mean±SD, n=6. Solid line labelled BrdU represents the time when animals were exposed to BrdU in 10% orange juice, whereas the solid line labelled MCP represents the period of MCP dosing at daily intervals.

As shown in Figure 3.33A, treatment of the mice with 25mg $kg^{-1}day^{-1}$ MCP for four days caused enlargement of the liver by up to ~66% of control. Liver-to-body weight (LBW) ratio was significantly increased from 4.0±0.27% in control group up to 7.3±0.19% in treated groups at day 4. The LBW increased in a time dependent manner (Figure 3.33B). Similar to ciprofibrate action, methylclofenapate (25mg $kg^{-1}day^{-1}$) failed to induce hepatic DNA synthesis within 2 days, but a significant increase in labelling index was observed by day 3 and maintained on day 4 (Figure 4.32C). Thus, the delayed time course of induction of hepatic LI was not a consequence of the particular PP (ciprofibrate), since another peroxisome proliferator methylclofenapate (MCP) gives similar results.

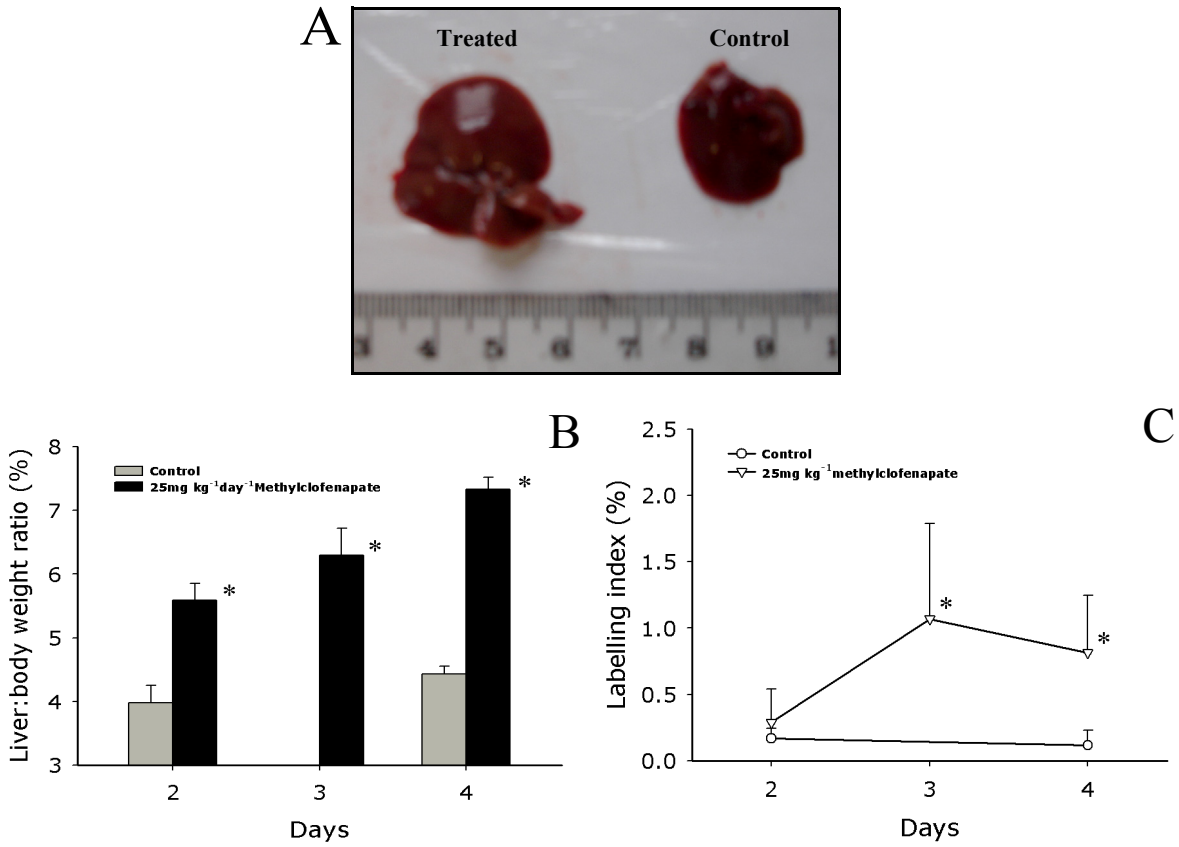

Figure 3.33 Time course of effects of methylclofenapate on DNA synthesis in 129S4/SvJae mice. Groups of six male mice were acclimatized as described Figure 3.13 before gavage with 25 $mg.kg^{-1}.day^{-1}$ methylclofenapate (MCP) in corn oil. The mice were killed after 2, 3 and 4 days of treatment with MCP. The control groups were killed on first and last time points. (A) Enlargement the liver after treated with MCP for 4 days. (B) Liver:body weight ratio (%) was determined and statistically tested as described in Figure 3.5. (C) Labelling index (%) was calculated and statistically tested as described in Figure 3.4. Data shown are mean ± SD. * = $p<0.01$.

Section 3.10 Strain differences in induction of DNA synthesis by methylclofenapate in mice

A time course was performed in a different mouse strain, Alderley Park mice, to find out if there was a strain-dependent difference in DNA synthesis using the peroxisome proliferator MCP. Styles had reported that treatment of AP mice with 25mg $kg^{-1}day^{-1}$ methylclofenapate (MCP) induced hepatic DNA synthesis by ~ 24 hours [113]. Groups of six AP mice were treated with 25mg $kg^{-1}day^{-1}$ methylclofenapate and killed on days 1, 2, 3 and 4. Control groups were killed on days 1 and 4 after dosing with a vehicle control. Body weight was not significantly decreased by treatment with MCP or corn oil (Figure 3.34A). However, 3 days-treated group shows significant body weight gain at the day of killing compared to the day of BrdU dosing. No liver injury occurred since the ALT activities in the treated groups did not significantly differ from those in the control group (Figure 3.34B).

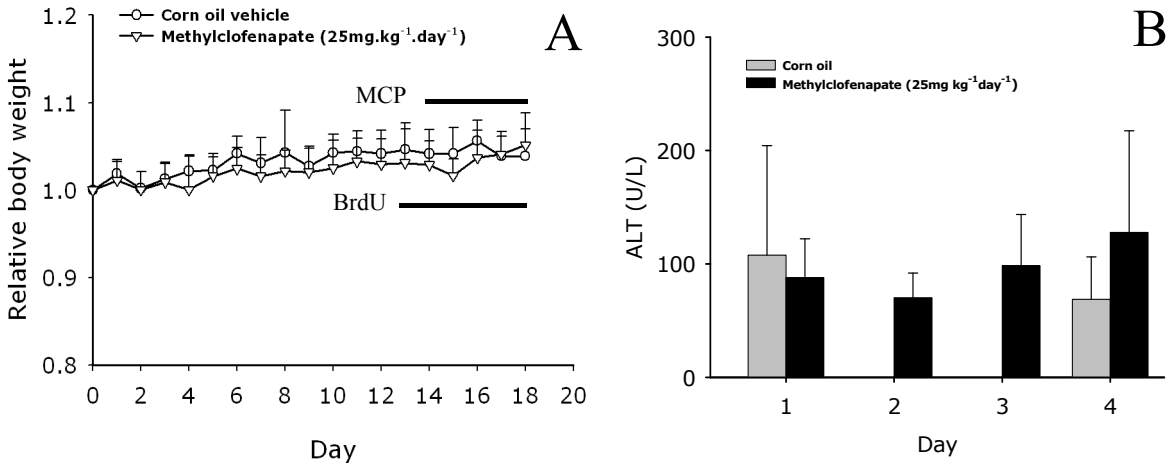

Figure 3.34 Time course of effects of methylclofenapate (MCP) on relative body weight and ALT activities in Alderley park (AP) mice. :Groups of six Alderley Park mice were dosed with 25mg kg^{-1} day^{-1} MCP and killed on days 1,2, 3 and 4 as described in multiple protocol 1 (Figure 2.3). Control groups were dosed with corn oil as vehicle and killed on days1 and 4 after dosing. All mice were acclimatised as described Figure 3.13 (A) Relative body weight was calculated and statistically tested as described in Figure 3.5; (B) Serum ALT level a liver toxicity marker was measured and statistically tested as described in Figure 3.7. All data was expressed as mean±SD, n=6. Solid line labelled BrdU represents the time when animals were exposed to BrdU in 10% orange juice, whereas the solid line labelled MCP represents the period of MCP dosing at daily intervals.

The liver:body weight ratio was significantly increased from 5±0.55% in the control group to 5.7±0.34% (14% increase), 6.8±0.49% (36% increase), 7.9±0.5% (58% increase) and 8.3±0.7%

(66% increase) in treated groups at 1, 2, 3,and 4 days respectively (Figure 3.35A). However, MCP induced DNA synthesis in AP mice after 4 days of dosing. This increase was statistically significant (584% increase) at day 4, but not significant (183% increase) at day 3 compared to the control group (Figure 3.35B). These results show that treatment of AP mice with 25mg $kg^{-1}day^{-1}$ methylclofenapate (MCP) failed to induce DNA synthesis within 48 hours as Styles reported, and confirm my data with a different peroxisome proliferator (ciprofibrate) and different strain (129S4/svJae), and show that the difference between our results and those of Styles were not due to mouse strain or peroxisome proliferator.

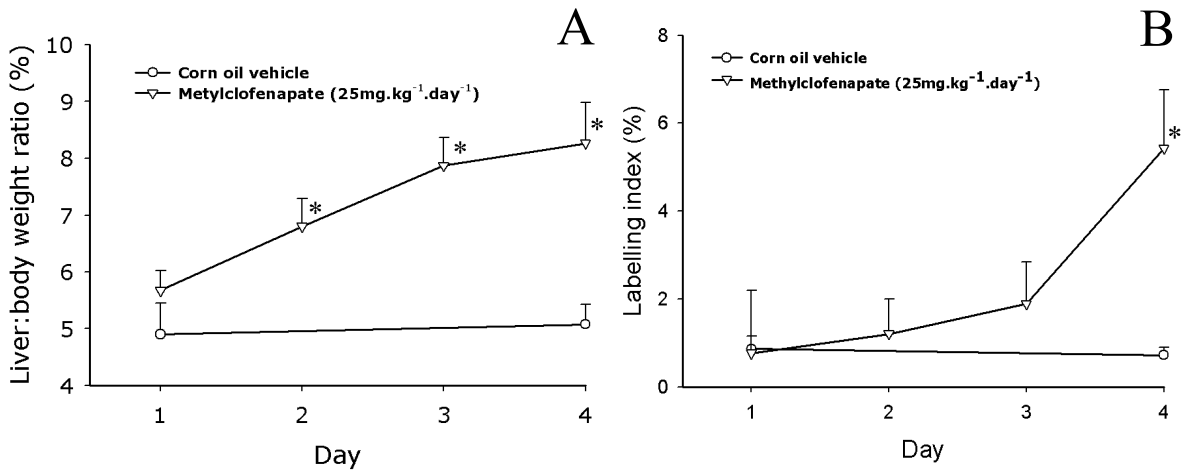

Figure 3.35 Time course of effects of methylclofenapate (MCP) on DNA synthesis in Alderley park (AP) mice. Groups of six male AP mice were treated as described in Figure 3.34. (A) Liver:body weight ratio (%) was determined and statistically tested as described in Figure 3.5; (B) Labelling index ratio (%) was calculated and statistically tested as described in Figure 3.4. Data shown are expressed as mean±SD, n=6.

For comparison purposes, liver:growth ratio and labelling index data from 129S4 and AP mice treated with 25mg $kg^{-1}day^{-1}$ methylclofenapate were plotted together to examine strain differences. As shown in Figure 3.36, data for the 129S4 mice day one was not available as this group had shown previously no induction on DNA synthesis within 48 hours (Section 3.4). The basal levels of Liver:body weight ratio in AP mice were greater than those in 129S4 mice. Also, the liver:body weight (LBW) ratio in MCP-treated AP mice was significantly greater than at in

MCP-treated 129S4 mice at all time points. However, the magnitude of induction of LBW in 129S4 (84% increase) was greater than that in AP mice (65% increase) at day 4 (Figure 3.36A). On the other hand, the basal levels of labelling index in AP mice (0.8±0.9) were greater than those in the 129S4 mice (0.15±0.1). Labelling index was significantly increased in 129S4 mice on days 3 and 4, but it increased significantly at day 4 in AP mice compared to the corresponding control.,

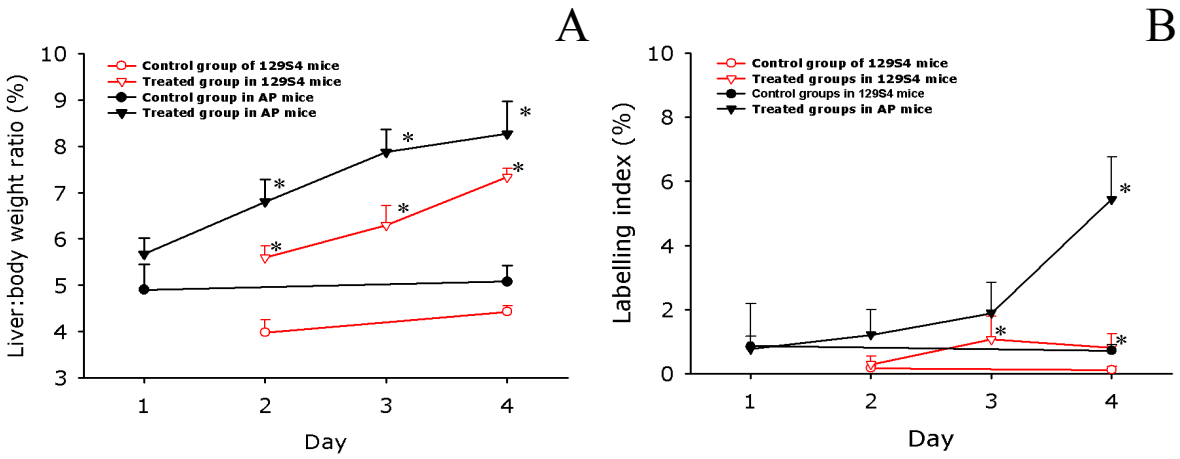

Figure 3.36 Strain differences in induction of liver growth by methylclofenapate (MCP). Groups of six male 129S4/SvJae and AP mice were dosed with 25mg $kg^{-1}day^{-1}$MCP for 2, 3, 4 or 1, 2, 3 and 4 days respectively. Control groups were dosed corn oil on the first and last days of dosing. (A) Liver: body weight ratio (%) was determined and statistically tested as described in Figure 3.5; (B) Labelling index ratio (%) was calculated and statistically tested as described in Figure 3.4. Data was expressed as mean±SD, n=7.

Section 3.11 Time course of ciprofibrate on liver growth in F-344/NHsd Rat

The hypothesis that ciprofibrate induces hepatic DNA synthesis during the process of augmentative growth in rats with a different time-course to mice was examined. The species differences was examined by dosing groups of male F344/NHsd rats with 50mg $kg^{-1}day^{-1}$ ciprofibrate for 1, 2, 4 days. Control groups were dosed with corn oil as vehicle and killed on the first and last days of dosing.

Unexpectedly, in this experiment, dosing the rats with 0.08% BrdU resulted in a confounding issue. There was a significant (p<0.01) loss in the body weight which was 5% in the control

groups and 7% in the treated groups after BrdU-dosing. This confounding result limits the interpretation of information from this study of the liver growth in the rat (Figure 3.37A). However, treatment with MCP (50mg $kg^{-1}day^{-1}$) did not cause liver toxicity to the rat since the ALT level in the treated rat did not significantly differ from the control rats (Figure 3.37B).

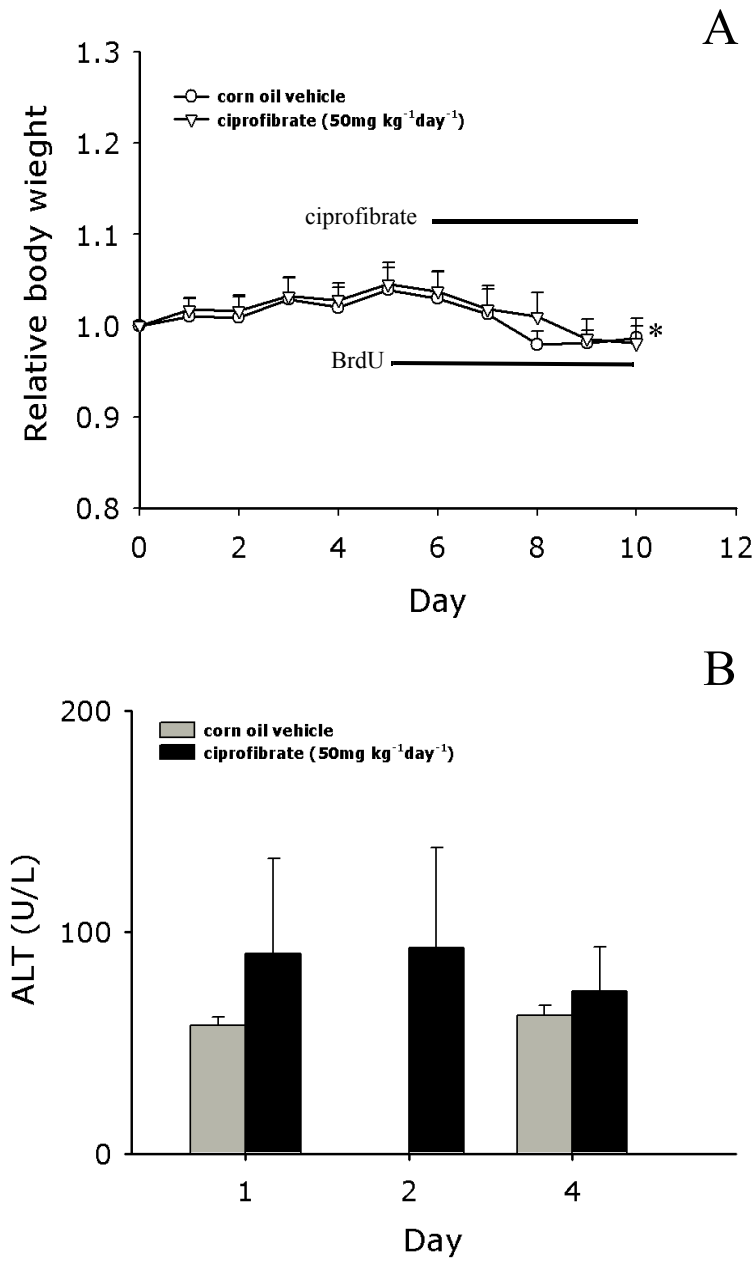

Figure 3.37 Time course of effects of ciprofibrate on relative body weight and ALT activities in F-344/ NHsd rat. Groups of six rats acclimatised as described in Figure 3.13 were dosed with 50mg kg^{-1} day^{-1} ciprofibrate and killed on days 1,2, and 4 as described in multiple protocol 1 (Figure 2.3). Control groups were dosed with corn oil as vehicle and killed on days1 and 4 after dosing. All mice were acclimatised as described Figure 3.13 (A) Relative body weight was calculated and statistically tested as described in Figure 3.5; (B) Serum ALT level a liver toxicity marker was measured and statistically tested as described in Figure 3.7. All data was expressed as mean±SD, n=5, *= $p<0.001$. Solid line labelled BrdU represents the time when animals were exposed to BrdU in 10% orange juice, whereas the solid line labelled ciprofibrate represents the period of ciprofibrate dosing at daily intervals.

Liver: body weight ratio was significantly increased in treated groups at all times in a time-dependent manner, rising from 3.7±0.23% in the control group to the 4.2±0.29% (14% increase), 4.65±0.25% (26% increase), and 5.66±0.3% (54% increase) in the treated groups at 1, 2, 4 days respectively (Figure 3.38A). Labelling index was significantly increased rising from 1.3±0.37 in the control to 24.3±10%, and 38.4±16% in the treated groups at 2 and 4 days respectively (Figure 3.38B). Although there was a significant body weight loss during the dosing, labelling index was significantly increased at day 2 of ciprofibrate dosing, which shows that rat has an earlier induction of DNA synthesis than mouse.

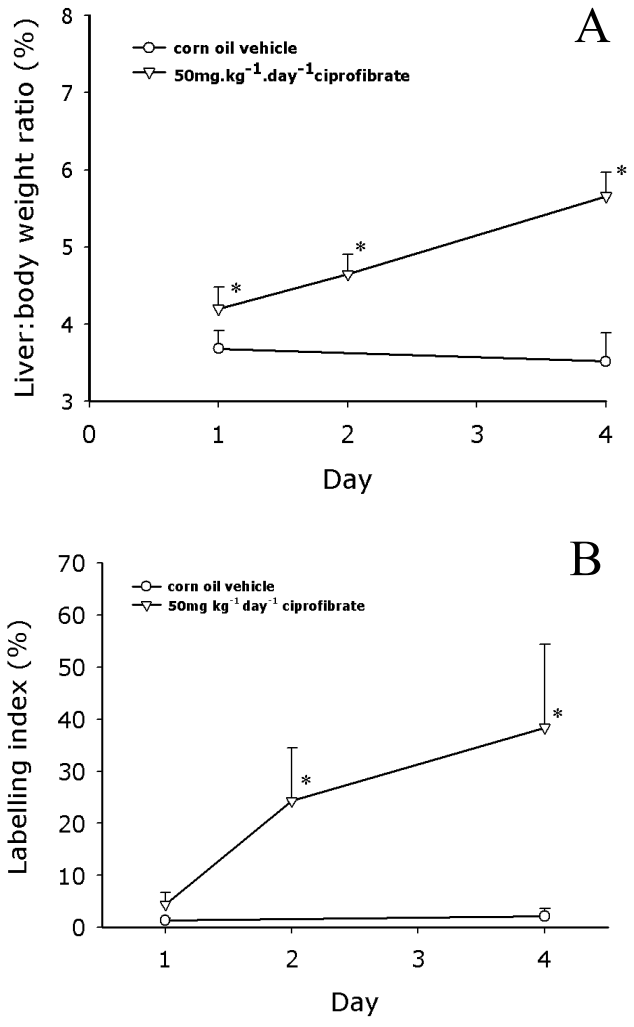

Figure 3.38 Time course effect of ciprofibrate on liver growth on F-344/NHsd rat. Groups of six rats were treated as described in Figure 3.37. (A): Liver-to-body weight percent was measured and statistically tested as described in Figure 3.5; (B): Labelling index (%) was calculated and statistically tested as described in Figure 3.4. The bars show the mean ± SD. *= $p<0.01$.

Chapter 4 Discussion

Section 4.1 Method validation

Section 4.1.1 Assessment of immunohistochemical detection

Immunohistochemistry is a common technique for detection of incorporated BrdU during replication of DNA in hepatocytes [165] [166] [207]. The procedure requires successful denaturation of DNA to facilitate the binding of Anti-BrdU antibodies to incorporated BrdU in DNA. Moreover, an optimized concentration of primary and secondary antibodies is also required to yield a good signal-to-noise ratio for BrdU-labelling. For these reasons, histologic estimation of S-phase labelling indices was employed to ensure the ability of the detection protocol to detect BrdU-labelled nuclei with a good signal-to-noise (S/N) ratio and to exclude any false positive or false negative. The detection system was examined by using different levels of positive control tissues: xenograft tumour tissue, which was known to have a high level of BrdU incorporation, to evaluate the immunohistochemical detection; liver and gut tissues from mice treated with BrdU (0.8mg ml^{-1}) to assess the labelling and detection of BrdU-labelled nuclei *in vivo*. The results showed that the detection system is specific and reliable for determination of BrdU in tissue (Figure 3.1 and Figure 3.2).

Section 4.1.2 Assessment of fixation time and temperature on immunohistochemistry

Fixation time and temperature play a role in successful immunohistochemistry. Formalin is the most commonly used fixative [167], and forms a cross-linking “methylene bridge” between basic amino acids. This cross-link can mask the antigenic sites of BrdU in DNA and longer fixation in formalin is associated with less intense staining [162]. For this reason, liver and gut tissues from 0.08% BrdU-treated mice were used to optimise the fixation time and temperatures. Furthermore, liver tissues from animals treated with TCPOBOP were also used to evaluate the affect of fixation time since the BrdU-labelled nuclei were higher in the TCPOBOP-

treated liver, which yielded easier microscopic characterisation to evaluate signal intensities of labeled nuclei (Figure 3.3). The data was consistent with the work of Hayashi *et al*. (1988) who showed that fixation length of up to 7 days does not affect the intensity of staining of BrdU-labelled rat gut tissues [162]. On the other hand, temperature effects on the fixation process were significant, with room temperature fixation yielding better staining than fixation at 4ºC. This may be due to higher temperature producing rapid fixation as proposed by [168].

Section 4.1.3 Optimisation of *in vivo* BrdU dosing

BrdU is rapidly degraded by dehalogenation in the liver [169] [170] [171], so the level of systemic BrdU will reduce rapidly after an acute bolus dose, *e.g*. by gavage or intra-peritoneal injection, leading to a limitation in availability of BrdU for incorporation into DNA [170]. Given the short half-life of BrdU, it was essential to validate that dosing with 0.8mg ml^{-1}BrdU in drinking water continuously for three days lead to effective labelling of DNA. In this *in vivo* model, gut sections were used as a positive control to evaluate BrdU delivery and immunohistochemical staining since this tissue has fast cell turnover (Figure 3.2B and D). After three days of treatment with BrdU, labelled nuclei in the gut covered all the intestinal crypts and most of the villi compartment; the proliferation and differentiation of epithelial cells occurs in the crypts, and the cells gradually move toward the tips of the villi [172] (Figure 3.2D).

Section 4.1.4 BrdU-labelling protocol

BrdU has been routinely used in study of induction of DNA synthesis for long time [156], frequently used as an alternative labelling agent in place of [^{3}H]-thymidine. [^{3}H]-thymidine and BrdU have been shown to give equivalent results under some circumstances [173], and BrdU allows faster studies without radioactive containment problems. The relationship between BrdU-labelling, as a function of time, and incorporation of BrdU during DNA synthesis in hepatocytes is not fully understood. In small intestine, as the duration of BrdU exposure increased,

the numbers of BrdU-labelled nuclei were increased [156]. This finding is consistent with my work; as the number of days of BrdU exposure increased, the percentage of labelled nuclei on the villi of gut section increase linearly. Moreover, Kiel *et al*. (2007) examined haematopoietic stem cells (HSCs) in C57BL/ka mice treated with BrdU, and found that BrdU incorporation over time was linear [174]. However, in liver, the linearity of BrdU labelling over time is controversial. Ton *et al*. (1997) have shown that the number of BrdU-labelled hepatocyte nuclei in mice increased over time when using a mini-pump, but not with drinking water, over three days of BrdU dosing [156]. However, this relationship between exposure time to BrdU, and labelling index, was not seen in rats in the same study. In present study, the labelling index (LI) after three days of BrdU in control group was similar to the LI of control group after 6 days of dosing, although the failure to discriminate between these two time-points might be due to a lack statistical power arising from the low labelling index in control animals. In general, potential explanations of non linearity include that the cells that undergo DNA synthesis subsequently die, or alternatively that the same individual cell undergoes DNA synthesis many times.

In this project, the continuous labelling method was chosen over single pulse injection based on published literature showing that continuous labelling increase the sensitivity of the assay, by measuring the cumulative number of BrdU-labelled cells in S-phase rather than labelling cells at a particular point in time [175] [46]. Additionally, continuous labelling avoids pitfalls associated with any diurnal variation in DNA synthesis [176]. Thus, continuous labelling is considered as a method of choice for studying cells characterised by low cell turnover *i.e.* hepatocytes [175]. The advantage of continuous labelling over a single dose was demonstrated by Marsman *et al*. (1988). Animals were treated with the peroxisome proliferators, Wy14,643 or DEHP, and the LI response evaluated with a single acute dose, or 7-day osmotic pumps, containing tritiated thymidine. The LI response detected with 7-day osmotic pumps increased 5- to 10-fold in the

rats receiving Wy14,643 as compared to control or DEHP-fed rat, whereas using a single acute dose of thymidine did not show any difference in labelling index between the three groups [145]. This is evidence that the chronic labelling protocol has greater sensitivity for detecting low levels of DNA synthesis.

BrdU can be continuously administered either by osmotic pump or in the drinking water. In this study, drinking water was used to administer BrdU since it involves easier application and avoidance of surgical stress to the animal [46] [156], by comparison to implanting mini-pumps. The dosing of BrdU at a concentration of 0.8mg ml^{-1} in drinking water was chosen based on previous literature [177] [46] [163] [156], and it has been reported that continuous administration of BrdU at up to 1mg ml^{-1} had no adverse effect on the animal [156].

In this study, dosing mice with BrdU in drinking water decreased body weight (Figure 3.5B). This issue might affect the induction of DNA synthesis and confound the interpretation. This phenomenon has been described previously in the literature. For example, Jecker *et al*. (1997) reported that water consumption was reduced when LEW rats were given 1mg ml^{-1} BrdU without 16% orange juice, and LEW rats refused to drink at a BrdU concentration of 1.5mg ml^{-1} [169]. Reome *et al*. (2000) also reported that C57BL/6 mice given 1 mg ml^{-1} BrdU in drinking water exhibited a body weight loss as compared to control [163]. However, the elementary control of measuring body weight is rarely described in the contemporary literature; thus many studies may have a confounding effect of body weight loss. It was therefore necessary to alter the experimental design to maintain the body weight of the mice before continuing; in all subsequent experiments, mouse body weight was measured.

An unpleasant taste of BrdU in water was postulated to be the factor causing the mice to stop drinking BrdU-containing water and then lose body weight. Several attempts were made to

overcome this problem. The unpleasant taste of BrdU was masked by adding orange juice (1:6) to 0.08% BrdU water as proposed by Jacker *et al*. (1997), decreasing the level of BrdU (up to 0.05%) or both. Although the 0.05% BrdU supplemented with 16% orange juice regime increased body weight about 2% in 3 days in the optimising experiment (Figure 3.6), this labelling regime (0.05%BrdU+16% orange juice) did not help to maintain body weight in control and treated mice as shown in Figure 3.7C. This might be because: a) the number of mice in the optimising experiment was insufficient to reveal the effect of 0.05%BrdU+16% orange juice regime; b) acidity of orange juice; or c) a lack of mouse acclimatisation.

The BrdU dosing protocol was varied in two parameters in an attempt to control body weight during experimental work. Firstly, review of experimental data revealed that mice undergoing experimental work lost approximately one gram in body weight in the first day of treatment. One possible explanation is that the mice were not used to handling, consequently urinated when handled, and so weight loss could arise partly from urination. Therefore, the animals were acclimatised to handling on a daily basis for seven days, prior to beginning dosing with xenobiotics. Secondly, the palatability of the BrdU remained as a possibility. So, animals were acclimatized to 10% juice, with the intention that this would mask the taste of the BrdU, whilst not making the orange juice too concentrated for consumption by the animals. This protocol was implemented in 129S4/Jae mice, and it successfully maintained body weight during dosing with 0.08% BrdU, and dosing with ciprofibrate (Figure 3.13A), whilst maintaining effective labelling of cells undergoing DNA synthesis (Figure 3.14A). The maintenance of mouse body weight during the experimental protocol removes a potentially confounding variable from the experiments.

Although Figure 3.13A validates the protocol for 129S4/SvJae mice, it did not immediately follow that the protocol could be simply applied to other mice strains. For example, there were

large strain differences in body weight, food intake, and water intake in 28 inbred mouse strains [178], and the variation in water intake between the mice strains would cause the mice to be exposed to different levels of BrdU on a mg/kg body weight basis. There were also the possibilities that different mouse strains could have different taste responses to BrdU, or differentially suffer from BrdU toxicity. For these reasons, it was necessary to test the BrdU dosing protocol on each strain of mouse (and F-344 rat) tested. DBA/2J (Figure 3.9), C57BL/6J (Figure 3.10) and AP (Figure 3.11) mice were acclimatized successfully to the protocol and administration of BrdU in 10% orange juice and did not show any body weight loss.

Section 4.2 Serum ALT as a marker for liver injury

PPARα ligands can cause focal necrosis [133], thereby leading to regenerative growth and cell proliferation. For example, Woods *et al*. (2007) have shown there was body weight loss in C57BL/6J and congenic $p47^{phox}$-/- mice after treatment with WY-14,643 (0.1%), ALT levels increased significantly after one week of dosing, and there was marked hepatic necrosis and inflammation [179]. Therefore, serum ALT (a marker of liver cell damage) was measured to ensure the doses used for the induction of liver growth were not cytotoxic, and consequently inducing regenerative regrowth, but were rather a PP-induced augmentative liver growth. ALT has been used extensively to assess liver injury in animals [180] [179]. ALT was preferred over AST because ALT is more specific for liver than AST; specifically, the ALT assay is not affected by hemolysis, whereas the AST assay is so affected [181] (Al Kholaifi *et al*., unpublished data). In this study, ALT activity in the serum was unaffected by the doses of PPARα ligands used either in rats or in mice. This finding was consistent with published data by Bondy *et al*., showing that dosing F-344 rat with 0.025% ciprofibrate up to 40 weeks did not elevate ALT level over the control [180]. Although administration of TCPOBOP, a CAR ligand, increased ALT levels three-fold in male mice, but not in females, the increased level of cell death associated with this increase in ALT would not be sufficient to induce large scale regenerative growth

in the liver, and consequently, the increase in ALT and cell death does not account for the potent mitogenicity of TCPOBOP (Figure 3.16). For example, an agent like carbon tetrachloride produces >10000-fold increase in serum ALT level in mice [182]; and so, TCPOBOP is only increasing hepatocyte death marginally.

Section 4.3 Induction of hepatic DNA synthesis *in vivo* by peroxisome proliferators

Section 4.3.1 Induction of DNA synthesis by single dose of peroxisome proliferators

It was reported that the induction of DNA synthesis in the mouse occurred as early as 24 hours after dosing with the potent PPARα ligand, MCP [113] [164]. Mouse was used as a model in the current study because the availability of mouse genetic models, such as knock-out mice, enables investigations that would not otherwise be possible. The 129S4/SvJae strain was characterized initially, as the PPARα null strain [81] is available on this genetic background, and the comparison of the effects of a chemical between PPARα wild-type and nullizygous mice enables an experimental determination of the role of PPARα in the response to the chemical.

Marked differences in potencies of PPARα ligands are known to exist [36] [92]. Reddy reported that treatment of rats with ciprofibrate (0.01%), DEHP (1%) or DEHA (1%) for 30 days increased the liver weight 100%, 75% or 12% respectively [92]. Treatment of rat hepatocytes with various PPs at 100-500μM for 72 hours induced peroxisome proliferation in the following sequence: ciprofibrate = nafenopin > benzafibrate > clofibric acid > di-(2-ethylhexyl)phthalate [183]. The available evidence supports the fact that ciprofibrate is a potent PPARα ligand; however, it is necessary to examine the possibility that the dose of ciprofibrate was insufficient to induce hepatic DNA synthesis. The range of doses of ciprofibrate (0-75mg kg^{-1}) used in the pilot study (Figure 3.4) did not provide any sign that induction of DNA synthesis occured over two days. This might be because the dose range used was not capable of inducing DNA synthe-

sis, and so a dose ranging study using 100-400 mg kg^{-1} ciprofibrate was tested in 129S4/SvJae mice acclimatised to 10% orange juice. This dose range of ciprofibrate caused a significant induction in the liver: body weight ratio in treated animals without any hepatic toxicity (*i.e.* no significant increase in ALT of treated mice, or grossly altered liver histology), but no significant induction in hepatic DNA synthesis was observed, and the incidence of mitotic figures was not measured. The fact that ciprofibrate caused an induction in the liver: body weight ratio is strong evidence that the ciprofibrate is being given at a dose that causes an effect; hence it was necessary to determine if other factors were responsible for the failure to detect or induce S-phase in the liver of ciprofibrate treated mice.

The first issue to be considered was whether the methodology was sensitive enough to measure an early induction of DNA synthesis in the liver within 48 hours. This issue was addressed by using a positive control compound, TCPOBOP; TCPOBOP does not activate PPARα, but instead activates a different nuclear receptor, CAR, to mediate its effects on liver [47]. TCPOBOP triggered a fast DNA synthesis response in mouse liver two days after dosing [207]. Figure 3.2 shows clear labelling of replicating cells in liver and intestine, and in mice treated with 3mg kg^{-1} TCPOBOP, there was a large and statistically significant induction of the hepatocyte labelling index compared to vehicle treated animals. In addition, because sex differences in induction of DNA synthesis by TCPOBOP exist, the current protocol was capable of measuring the difference in LI between male and female mice, which reflects the reliability of the protocol. This finding shows that the methodology was capable of detecting an induction of hepatocyte DNA synthesis within 48 hours after dosing with test compound, and hence, a defect in the ability of the methodology to measure hepatic DNA synthesis within this time frame is not the reason behind the failure to detect of induction of DNA synthesis by ciprofibrate within 48 hours. The consistency between the results in Figure 3.16B, and those of Columbano [207] provides addi-

tional support to the notion that the labelling protocol yields a reliable measure of hepatocyte labelling.

The possibility of an off-target effect is an aspect that needed to be considered in clarifying the failure of ciprofibrate to induce DNA synthesis over two days. It is known that one compound can have multiple modes of actions and be able to activate completely different signal pathways. For example, indomethacin is an antagonist of cyclooxygenase activity (COX-1 and COX-2), and it also binds and activates PPARs α and γ [184]. For this reason, the possibility that ciprofibrate had an off-target (*i.e.* non-PPARα mediated) effect that prevented induction of liver DNA synthesis (whilst leaving intact the liver growth function) was also considered. One way to address this issue is to use a different PPARα ligand that has previously been shown to induce hepatic DNA synthesis. MCP was chosen because Styles had shown an early induction in hepatic DNA synthesis after administration of methylclofenapate (MCP) [113] [164], and it is additionally known to be a potent PPARα ligand. In the current study methylclofenapate caused a hypertrophic response in treated mice, but that no induction in hepatic DNA synthesis following treatment for two days (Figure 3.18B). This result confirmed that the potent peroxisome proliferator MCP [96] [97] had similar effects to ciprofibrate, showing that the effects of ciprofibrate were not unique, and suggests that the absence of induction of hepatic DNA synthesis within two days of dosing is characteristic for PPARα ligands in mice.

The result from these control experiments, confirms that ciprofibrate fails to induce hepatocyte DNA synthesis within two days after dosing (Figure 3.14A), which was in contrast to our expectations from the work of Styles [113]. It was therefore essential to define the time frame wherein DNA synthesis is induced after treatment with ciprofibrate. Five days rather than two days exposure to a single dose of 300mg kg^{-1} ciprofibrate was investigated to characterise the early induction point of DNA synthesis in 129S4/SvJae mice. This dose was chosen because it

was the dose showing the highest LI (Figure 3.14A). Although there was a 15-fold increase in labelling index in treated mice five days after administration of 300mg kg^{-1} ciprofibrate, no hepatotoxicity was observed (Figure 3.19D). In conclusion, this result confirms that induction of hepatic DNA synthesis occurred later than 48 hours after dosing.

Section 4.3.2 Characterisation of induction of DNA synthesis by peroxisome proliferators

Analysis of the time-course of induction of liver DNA synthesis by ciprofibrate in 129S4/SvJae mice was evaluated in detail. 100mg $kg^{-1}day^{-1}$ciprofibrate was dosed to male129S4/SvJae mice for 6 days. This dose was chosen to avoid any hepatotoxicity that could result from the high dose of ciprofibrate. Although the previous experiment (Figure 3.14) had shown no obvious toxicity of a single dose of ciprofibrate up to 400 mg kg^{-1}, ciprofibrate has a hydrophobic (dichlorocyclopropyl) side chain, which gives the drug a longer half life (80 hour) [185] [186] and the LD_{50} for ciprofibrate is ~700mg kg^{-1}. If the mice were given 400mg kg^{-1} daily, there might be accumulation to frankly toxic concentrations as a result of the repeated dosing; the dose used did not show any toxicity to the mice. The present study revealed that treatment of mice with ciprofibrate caused enlargement of liver in time-dependent manner (Figure 3.21A). This indicate that there were significant changes occurring in the liver starting after two days of dosing, which saw the liver:body weight ratio increase 50% after 2 days of dosing with ciprofibrate. However, induction of hepatic replicative DNA synthesis does not synchronize with the liver growth (hypertrophy). This study shows the induction of DNA synthesis (hyperplasia) started at three days after treatment with ciprofibrate (Figure 3.21). The delayed response of the induction of DNA synthesis shows that there were different kinetics of liver growth and liver DNA synthesis in response to PPARα ligands, and begs the question of how these two responses are co-regulated. Additionally, the delay in response in DNA synthesis does not mean the mouse is not capable of having a rapid response because administration of TCPOBOP showed a fast response in

DNA synthesis in mouse. The delay in response by at least 48 hours after the start of dosing 100 mg kg^{-1} day^{-1} ciprofibrate is consistent with Ledda-Columbano's work, who reported that induction of labelling index was observed at 3 days of ciprofibrate feeding and was maximal at 4 days [207]. Notably, there was considerable animal to animal variability in LIs exhibited in treated mice on day 3 and 4 with a coefficient of variation (%CV) of 93% and 71% respectively. In control mice, hepatic DNA synthesis during the study period is at steady state.

The time course of induction of DNA synthesis was characterised using another peroxisome proliferator, methylclofenapate to find out if there was a discrepancy in the early time point of induction of DNA synthesis arising from an idiosyncrasy of ciprofibrate. The compound and the doses were selected because they were reported to show a high induction of hepatic DNA synthesis in treated mice [113] [164]. Studies reported by Styles, using 25 mg kg^{-1} MCP in AP mice, showed marked induction of DNA synthesis in the liver by 24 hours after dosing, using flow cytometry analysis to quantify replicative DNA synthesis [113]. In the current study, although the liver growth was increased by two days, significant induction of DNA synthesis was shown to occur no earlier than day 3 (Figure 3.33C). This finding with MCP treatment confirms that the early induction of DNA synthesis in 129S4/SvJae mice occurred after three days of PPs dosing, which is consistent with the work of Ledda-Columbano who showed induction began at 72 hours after feeding CD-1 mice with 0.025% ciprofibrate-supplemented diet [207].

In conclusion, the conflict between this work and Styles's work [164] might be attributed to: a) methodology; b) dose effect or c) strain differences. A possible explanation for the different results that we have obtained is that they were due to a dose effect and/or a strain difference in responsiveness to induction of hepatic replicative DNA synthesis induced by peroxisome proliferators rather than methodology. However, the method that is being used in this study was validated to measure DNA synthesis in 129S4/SvJae mouse liver within 0-48 hours after admin-

istration of compound (Section 3.3).

Section 4.3.3 Dose response of induction of DNA synthesis by ciprofibrate

Dose is another important factor in determining whether a particular peroxisome proliferator can affect the stimulation of cell replication in rodent hepatocytes. A dose-response study was undertaken in order to establish whether there was a dose dependency in the response of mouse liver to induction of DNA synthesis by the peroxisome proliferator, ciprofibrate. In this study, we established a lowest observable effect level (LOEL) for the induction of DNA synthesis by ciprofibrate. The same mouse strain (129S4/SvJae) used in the time course study was gavaged with 0,1,3,10,30,100 or 200 mg kg^{-1} day^{-1} ciprofibrate in corn oil for 3 days, or was gavaged with 0,10,30,100 mg kg^{-1} day^{-1} ciprofibrate for 4 days. We chose the 3 and 4 day time- points to test dose dependency on the basis these two days showed the earliest time point for induction of hepatic DNA synthesis. The choice of dose was based upon the dose which we have characterised the DNA synthesis response, which is 100 mg kg^{-1} day^{-1} of ciprofibrate, a relatively high dose. However, it is also known that 10 mg kg^{-1} day^{-1} of ciprofibrate was used in a mouse bioassay and which causes liver growth (Tim Gray, Sanofi-Aventis, personal communication). It is not possible to increase the dose of ciprofibrate greatly, as the LD50 is ~700 mg kg^{-1} (Tim Gray, Sanofi-Aventis, personal communication), and higher doses would risk non-specific toxicity. However, doses as low as 10 mg kg^{-1} day^{-1} of ciprofibrate also give rise to liver growth.

Within the two time frames, ciprofibrate induced hepatomegaly at 10 mg $kg^{-1}day^{-1}$ and greater (Figure 3.25A). However, there is a discrimination in induction of DNA synthesis between day 3 and 4. In the four day study, an 8-fold induction with 30 mg kg^{-1} day^{-1} was observed in treated animals indicating the start of a proliferative response to ciprofibrate, whereas 100 mg kg^{-1} day^{-1} ciprofibrate (the lowest observable effect level (LOEL)) showed a proliferative response after three days dosing (Figure 3.25B). Ciprofibrate was highly carcinogenic in liver at 10 mg kg^{-}

$^{1}day^{-1}$ in Swiss mice (Sanofi, personal communication), and so it is of interest to compare this dose level with our findings. There is the possibility of strain differences in metabolism, but also the long half-life of ciprofibrate means that the serum level of ciprofibrate would continue to increase after the four days of dosing in our experiments. Therefore, it is possible that the 10 mg $kg^{-1}day^{-1}$ dose level may lead to a mitogenic response in the liver if tested over a longer time period, but the data confirmed that this dose level definitely leads to a hepatic growth response. Ciprofibrate was shown to be mitogenic in a dose-related manner in mice treated for four days. Also, it was shown that 100 mg $kg^{-1}day^{-1}$ is the optimal dose (of those tested) for inducing DNA synthesis. Therefore, the failure to detect ciprofibrate-induced hepatic DNA synthesis within two days (Figure 3.14, 3.21) of dosing is not due to the use of a sub-optimal dose of ciprofibrate.

Section 4.3.4 Strain differences in the induction of DNA synthesis

Mouse strain differences are known to cause significant phenotypic effects (*e.g*. variation in susceptibility to liver-tumor induction by genotoxins [114] [115]), but there is little direct evidence of strain differences significantly affecting peroxisome proliferation [11] [3]. Styles found rapid induction of hepatocyte DNA synthesis by PPARα ligands in Alderley Park (AP) [113] and heterozygous Snell dwarf mice, which originated on the C57Bl/6J mouse background [164]. In this study, the induction of liver growth was demonstrated in 129S4/SvJae mice, suggesting that a strain difference might be responsible for the discrepancy from Styles's work. AP mice are an outbred stock of Swiss origin [187] [188], and so we directly tested in AP mice using the same dose of the same PPARα ligand described by Styles *et al*. [113]. Figure 3.36B shows that the induction of hepatocyte DNA synthesis response is delayed until three/ four days after administration of MCP in 129S4/SvJae and in AP mice, thus showing no effect of strain in the response of the mouse to PPARα ligands. Control and treated AP mice demonstrated a higher liver: body weight ratio and a greater maximal response than 129S4/SvJae mice (Figure

3.36A). AP mice had a hepatocyte labelling index higher than that of 129S4/ SvJae mice at all time points (Figure 3.36B). However, because the basal levels of LIs in control mice were higher than in 129S4/SvJae mice, AP mice demonstrated a significantly increased LI at day four only, whereas 129S4/SvJae mice showed a significant increased in LIs at day three and four. The LI for AP mice was 4-fold higher than the value for the 129S4/SvJae mice at day 4. In addition, no toxic effect was observed during the dosing of AP mice as demonstrated by the liver marker enzyme ALT (Figure 3.34B) and body weight (Figure 3.34A). Moreover, unpublished results from our laboratory showed the kinetics of induction of liver growth and DNA synthesis in C57BL/6J mice (which the same strain used by Styles [164]) after treatment with ciprofibrate (Amer, personal communication) were similar to that seen in 129S4/SvJae mice. The findings in 129S4/SvJae, AP and C57BL/6J mice confirm that strain differences do not affect the kinetics of induction of liver growth or hepatic DNA synthesis, and are consistent with previous examinations of strain differences in peroxisome proliferation in mice [11] [3], which show minimal effect due to the strain. Thus, we have been unable to replicate the results of Styles in the AP mouse strain; while it is possible that the AP mice, being outbred, may have undergone strain drift, the C57BL/6J mice are inbred, and were unlikely to show significant strain drift. Moreover, the concordance between the results obtained in 129S4/SvJae and AP and mice in our hands excludes the possibility that the discordance between our data, and those of Styles [113] [164], arises from mouse strain differences.

Section 4.3.5 PPARα receptor is required for the action of peroxisome proliferators

129S4/SvJae mice were chosen as a model in this study due to the availability of a congenic strain of 129S4/SvJae-PPAR $^{tm1Gonz/tm1Gonz}$ mice, a null allele of the PPARα gene[81], which is required to test whether the action of peroxisome proliferators is mediated by the PPARα. The hypothesis that the effects of a specific peroxisome proliferator were mediated by the PPARα

was tested with 100 mg kg^{-1} day^{-1} ciprofibrate for 2 and 6 days. This experiment showed no induction in liver growth or hepatic DNA synthesis occurred in ciprofibrate-treated PPARα null mice, which proves that PPARα is required for mediating the pleiotropic response resulting from the action of the peroxisome proliferator ciprofibrate (Figure 3.23). This finding extends published reports [81] [82] by showing that PPARα is required for the early induction of DNA synthesis in liver and also excludes off-target effects of ciprofibrate as a cause of the DNA synthesis in liver.

Section 4.3.6 Sex differences in the induction of liver growth by ciprofibrate

To complete the characterisation of hepatic DNA synthesis in both sexes of 129S4/SvJae mice, the study was extended to female mice to find out if there were sex differences in the augmentative liver growth induced by peroxisome proliferators. Several studies have reported that male rats were more responsive to various effects of fibrates than female rats, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities [189] [190] [191] [89] [192] [193]. Furthermore, studies in Sv/129 mice given the peroxisome proliferator trichloroethylene show that liver growth and peroxisome proliferation in males is more responsive than in females [88]. However, no information was available about sex-related differences in the induction of hepatic DNA synthesis by peroxisome proliferators. For this reason, female 129S4 mice were tested with ciprofibrate (100 mg kg^{-1} day^{-1}) at the same dose as given to male mice for three and four days. Figure 3.30 showed that liver/body weight ratio was significantly higher in treated male over treated female mice, which confirm the published report on Sv/129 mice [88]. In contrast, induction of DNA synthesis by ciprofibrate does not show any sex-related differences after three or four days dosing (Figure 3.31).

Section 4.3.7 Differences between the current protocol and Styles' protocol

Styles [113] [164] reported substantively different kinetics of induction of hepatic DNA synthe-

sis by PPARα ligand, compared to this study. There were numerous possible variables that could explain the differences between the experiments of Styles, and this study. However, systematic investigation of these variables has excluded:

(1) The validity of the DNA labelling methodology (Figure 3.16B).

(2) Dose of PPARα ligand (ciprofibrate) being either too high, or too low (Figures 3.14A and 3.25B).

(3) Off-target effects of ciprofibrate, as judged by the similar effect of methylclofenapate to ciprofibrate, and the absence of effect of ciprofibrate in PPARα null mice.

(4) Strain differences in response, as the same mouse strains used by Styles fails to show an early induction of DNA synthesis.

Thus, it is difficult to explain the difference in kinetics of induction of DNA synthesis reported by Styles [113] [164], and in this study. The principal remaining variable is that this study uses immunohistochemical detection of incorporated BrdU, whereas Styles detected incorporated BrdU by isolation of hepatocytes, and flow cytometry of the isolated hepatocytes [194]. The experimental methodology in this thesis has an internal control (labelling of gut nuclei) to control for correct dosing of BrdU, and the methodology has been shown to detect high level induction of hepatic DNA synthesis as reported by other authors (Section 3.3). Immunohistochemical detection of BrdU to detect hepatic DNA synthesis has been independently validated [166], and the method is specific for hepatocytes as a result of morphologically identifying the cell. Moreover, the method involves few steps that could affect the representation of liver cells (*i.e.* the liver is directly fixed, and then embedded), and the method is.in widespread use [195] [196] [46] [197].

By contrast, flow cytometry requires significant sample processing (Figure 4.1 steps 1-8) prior to analysis of the BrdU labelling index. Figure 4.1 outlines the flow cytometry protocol used by Styles for determination of DNA content and S-phase in hepatocytes [194] [113] [164] There were numerous stages during this protocol which show limited or inadequate controls. For example, during liver isolation (step 2), collagenase (747μg ml^{-1}) is used to isolate liver cells. Collagenase is known to destroy membrane structures [198], and there is no control to determine whether collagenase treatment differentially recovered the liver cells during the hepatocyte isolation procedure, either between different hepatocyte preparations, or systematically between

control and treated animals.

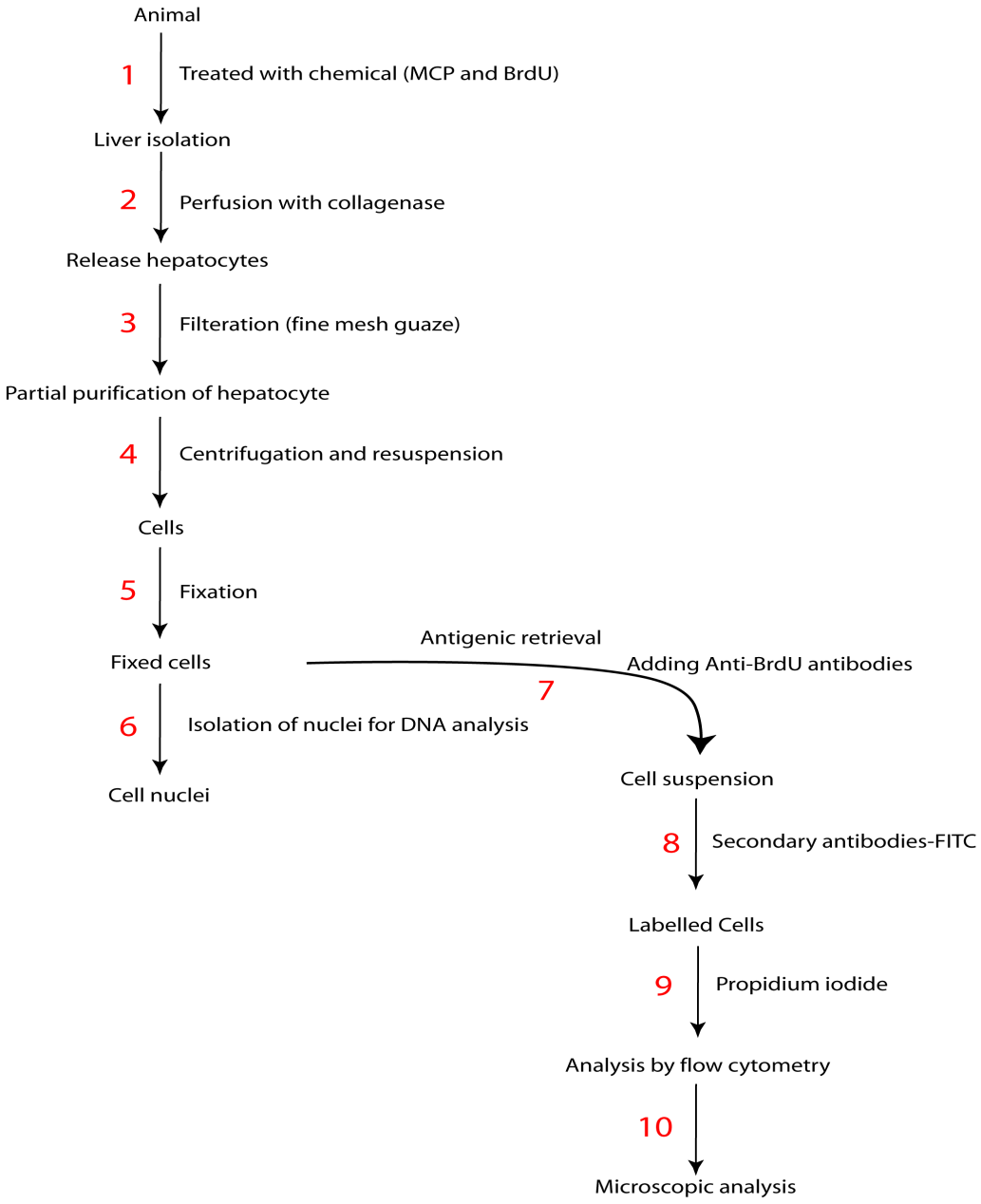

Figure 4.1 The flow cytometry protocol. This protocol outlined the method that has been used by Styles et al to determine DNA content and S-phase in hepatocyte and analysis by two-parameter flow cytometry using an Ortho 2150 Cytofluorograf

For measuring DNA content by flow cytometry, propidium iodide (PI) was used for this purpose (step 9). Propidium iodide is known to bind to RNA as well as DNA [199]. This fact can overestimate the actual level of DNA on the cell. Styles used RNase treatment to get rid of RNA.

However, it is unclear whether the altered hepatocyte size after treatment with PPARα ligands would affect the propidium iodide staining used in this methodology, and also there were no controls to ensure that RNase has degraded the intact RNA, or to determine if there is any DNAase contamination of the RNAase, which would degrade nuclear DNA. Both such artefacts could significantly affect the determination of propidium iodide fluorescence, and hence the derived DNA content in the cells.

Signal-to-noise (S/N) ratio also is another factor that needs to be considered in term of sensitivity of the method. In Styles's procedure, partial hepatectomy was used as a positive control to validate the method [194]. However, the data was gated, and the authors did not show the non-gated signal. Thus it was not possible to determine the signal/noise ratio of Styles'method for detecting the content of DNA in cells, the proportion of the 2n and 4n cells in all cells, or whether these change between preparations or treatment groups. This is a serious limitation in interpreting the results of their positive control experiment that validates the method's ability to measure DNA synthesis. Moreover, the S/N ratio for detection of BrdU was poor, with a maximal 5-fold difference in BrdU signal between labeled and unlabelled cells, and overlapping BrdU signal between "labeled" and "unlabelled" cells. Moreover, the data presentation allows no analysis of the density of data within different fluorescence, calling into question the reliability of the determination of immunofluorescent detection of BrdU in this methodology. This contrasts markedly with the high signal-to-noise ratio obtained using immunohistochemistry.

In modern flow cytometry, forward scatter and side scatter profiles are used to differentiate the cells within heterogeneous population based on size and complexity of the cells. This would help to eliminate any background noise resulting from non hepatocytes. In Styles' method, fluorescence technique was used only to determine the DNA content and S-phase without differentiating the cells based on size. Therefore, non-parenchymal cells were another source of

interference in hepatocyte estimation, particularly if these show differential recovery between control and treated animals.

All of these issues could introduce artefactual error into the determination of the proportion of labelled hepatocytes by flow cytometry. There are therefore severe difficulties in interpreting the flow cytometric data, in the absence of adequate control experiments and data to validate the methodology. Notably, other investigators using the same methodology have reported different effects on hepatocyte ploidy in rat to those reported by Styles [200] [194], which shows difficulty in reproducing results obtained by this methodology. The concordance between inadequate control experimentation and lack of reproducibility provide compelling reasons to believe that these experiments cannot be relied upon.

The reason for the difference in results remains obscure, but we have excluded the choice of PPARα ligand, dose, sex and strain differences as possible variables. Further, our results are consistent with other reports [207], and so we conclude that the induction of hepatocyte DNA synthesis is delayed until three days after administration of PPARα ligand, and we propose that the results obtained by Styles are an artefact arising from the cell isolation and flow cytometric analysis of liver cells.

Polyploidy was investigated to determine whether induction of liver growth by peroxisome proliferators might affect the ploidy status in the liver, and cause divergent results between Styles's work and this work. Styles *et al*. [113] hypothesised that induction of liver growth by MCP caused conversion of some 2x2N cell to 4N in rat and mice by amitotic cytokinesis, and the DNA synthesis occurred mainly in the 2x2N cell [113]. In contrast, Miller *et al* (1996) reported that there was no significant alteration in these cell populations, and DNA synthesis occurred mainly in 4N cells not in 2x2N [200]. In principle, increasing nuclear ploidy during augmenta-

tive liver growth either in 4N (amitotic cytokinesis) or in 2x2N (no cytokinesis) would be associated with increasing of BrdU-containing hepatocytes. Both techniques, flow cytometry and immunohistochemistry, are still able to detect the incorporated BrdU during DNA synthesis in the same sensitivity. Therefore, polyploidy is unlikely to be a reason for the divergence of the results between Styles' work and this work.

Section 4.3.8 Species differences in the induction of liver growth by ciprofibrate

It would be impossible in the mouse model to define a narrow time period when the "immediate early" genes are switched on to signal DNA synthesis, because the DNA synthesis response was delayed by 72 hours. To identify the switch "on/off" gene(s), the DNA synthesis response must be a strong response, and arise rapidly (by ~24 hours) after treatment with peroxisome proliferator; given that it takes mammalian cells ~20 hours to prepare for DNA synthesis, it follows that the signalling which gives rise to this process must occur in a synchronous manner in the first four hours after treatment with compound. Under those circumstances, it would be possible to identify "immediate early" genes in the first four hours after treatment which are linked to the induction of hepatic DNA synthesis. This approach was used to investigate the signalling mechanisms during liver regeneration after partial hepatectomy [6] [19].

A species differences was also considered as a factor that might control early induction in liver growth. It is not known whether rats have a different time-course of induction of hepatic DNA synthesis to mice, but it had been assumed that induction of hepatocyte DNA synthesis by PPARα ligands was similar between mouse and rat [113]. The only paper to undertake a detailed and contemporaneous comparison of mouse and rat responses is by Styles [113], and we have been unable to repeat their mouse data. There are some reports that show that induction of hepatic DNA synthesis by peroxisome proliferators is rapid (~24 hours) in rat, *e.g.* [201] [200] [202] [113]. In the current study, F-344/ NHsd rats were used to characterise the time-course of

induction of hepatocyte DNA synthesis by ciprofibrate. The Fischer F-344 strain was selected since this strain is an inbred rat characterised by homozygosity in most genetic loci [203]. F-344 rats were dosed with 50mg $kg^{-1}day^{-1}$ ciprofibrate for 1, 2 and 4 days. No liver injury was observed in the treated rats, as assessed by serum ALT, after dosing with ciprofibrate, demonstrating that the DNA synthesis is not regenerative (Figure 3.37B). Liver-to-body weight showed an early induction at day one (Figure 3.38A), and induction of DNA synthesis significantly increased by day 2, demonstrating that induction of DNA synthesis in rat was earlier than mouse (Figure 3.38B). However, there was a statistically significant body weight loss in the rats (~ 2-4%). Therefore, it is difficult to draw an unambiguous conclusion from this experiment, since the early induction of DNA synthesis may have been affected by the decrease in body weight. Nonetheless, there was a large and significant induction of hepatocyte DNA synthesis at 48 hours after dosing, and it is unlikely that this induction of hepatic DNA synthesis is caused by the loss in body weight. This result has since been confirmed by further work in our lab (A. Amer, personal communication), who showed that the induction of DNA synthesis is significantly increased at 24 hours after dosing. Moreover, the results in the rat study are consistent with published work that PPARα ligands cause rapid (*ca.* 24 hours) induction of DNA synthesis in rat hepatocytes *in vitro* [204], and the finding that the PPARα ligands, nafenopin and Wyeth-14,643, causes induction of hepatic DNA synthesis at 24 hours after dosing in Wistar and Fisher 344 rats, respectively [201] [200].

These data therefore show that the Fischer 344 rat shows a much more rapid induction of hepatic DNA synthesis in response to PPARα ligands in comparison to the mouse. Although there are reports in the literature of different time courses of induction of hepatic DNA synthesis by PPARα ligands in different species, it is essential to use the same methodology in the same laboratory to obtain a reliable comparison of mouse and rat. Our results therefore show a species

difference between mouse and rat in the kinetics of hepatic response to PPARα ligands.

The delayed response of the mouse to induction of hepatocyte DNA synthesis is reminiscent of the response to partial hepatectomy, where mouse hepatocyte DNA synthesis commences 12-16 hours later than in the rat; after partial hepatectomy, it has been shown that the faster response of the rat hepatocyte is maintained by an intrinsic program inside the hepatocytes (cell autonomy) [205]. Weglarz *et al*. showed that timing and magnitude of DNA synthesis is instructed by intrinsic factors within hepatocytes. These factors maintain the species-specific response of hepatocytes after partial hepatectomy. Other growth factors such as HGF and TGFα drive the cell cycle progression and increase hepatocyte turnover, but do not show any significant alteration in the timing of cell cycle progression after partial hepatectomy ([15] [205]).

The species difference in response is unlikely to be due to a species difference in the amount of the PPARα, since the receptor is present at high levels in mouse, compared to other rodents [101]. Although PPARα is present in both responsive and non-responsive species, the response of species to Peroxisome proliferators may vary due to the relative level of PPARα present in the liver [94]. Choudhury *et al*. examined the level of PPARα in both mouse and guinea pig liver, showing that PPARα in mouse was 10 times higher in mouse, compared to guinea pig. A study by Palmer *et al*. (1998) showed that PPARα mRNA was found to be 1 order of magnitude higher in mouse than that observed in humans [76]. Thus, these studies confirm that the species differences in the induction DNA synthesis between mouse and rat is not a consequence of low level of PPARα in mouse liver.

Section 4.4 Future work in the induction of liver growth by peroxisome proliferator

Data presented here characterises the kinetics of induction of DNA synthesis by peroxisome proliferators, which is important to investigate the mechanism of augmentative liver growth.

Future work requires the identification of liver cell populations undergoing DNA synthesis (polyploidisation) by using flow cytometry analysis, or lobular localisation of cells that have undergone DNA synthesis. In addition, the genes and proteins induced by specific peroxisome proliferators have not been identified. It is essential to set up an experiment to identify the genes that are induced by peroxisome proliferators that cause hepatic replicative DNA synthesis (*i.e.* the regulatory mechanisms involved in liver growth). The DNA synthesis response must be a strong response, and arise rapidly (by ~24 hours) after treatment with peroxisome proliferator, then it would be possible to identify "immediate early" genes in the first four hours after treatment which are linked to the DNA synthesis response by using microarray analysis. I have now established a model system which has great promise for meeting these criteria.

Chapter 5 References

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