Regulation of Antioxidant Enzymes and Bile Acid Transporters in Liver by Rapamycin and Oltipraz

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Doctor of Philosophy

by

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SUMMARY

Ischemia reperfusion (IR) injury is a common phenomenon associated with the surgical treatment of hepatocellular carcinoma (HCC) and metastatic cancer of the liver. Rapamycin, an effective immunosuppressant and inhibitor of growth of cancer cells, is often employed in the management of HCC patients who undergo liver resection or liver transplantation. Rapamycin has also been shown to induce the synthesis of the antioxidant enzymes heme oxygenase 1 (HO-1) and peroxiredoxin 1 (Prx-1). However, rapamycin also inhibits bile flow. Oltipraz, is a very effective inducer of antioxidant enzymes and inhibits cancer cell growth, is currently undergoing clinical trials as a chemopreventive agent for the treatment of HCC. Strategies employing pre-treatment with rapamycin and oltipraz have the potential to reduce IR injury. However, their abilities to induce antioxidant enzymes and potential detrimental effects on both normal and transformed liver cells are not well understood. Therefore, the aim of this study was to characterize the abilities of rapamycin and oltipraz to induce the expression of HO-1 and Prx-1 in normal and transformed liver cells. Another aim of this study was to determine the effects of rapamycin and oltipraz on the expression of bile acid transporters in normal and transformed liver.

To achieve these goals, a rat model of segmental hepatic ischemia and reperfusion, isolated cultured rat hepatocytes and transformed rat liver (H4IIE) cells were employed. Quantitative PCR (qPCR) was conducted for measurement of the expression of mRNA encoding antioxidant enzymes and bile acid transporters. Probe-based strategies, β-actin as reference RNA, and the ΔΔCT method were employed.

Rapamycin increased HO-1 and Prx-1 mRNA expression in rat liver *in vivo* and in cultured rat hepatocytes. On the other hand, rapamycin inhibited expression of mRNA encoding the sinusoidal bile acid transporters Ntcp, Oatp1 and Oatp2 mRNA. But rapamycin induced the canalicular transporter Mrp2 and Bsep mRNA expression in cultured rat hepatocytes. However, in transformed rat liver (H4IIE) cells rapamycin

inhibited HO-1 and Prx-1 expression whereas oltipraz induced HO-1 and Prx-1 mRNA expression. Expression of three bile acid transporters, Bsep, Bcrp and Oatp9, were not detected in H4IIE cells. Moreover, the relative expression of mRNA encoding some bile acid transporters in H4IIE cells were significantly different compared to that in normal rat hepatocytes. Rapamycin induced the sinusoidal transporters Ntcp and Oatp1 mRNA expression but inhibited Oatp2 mRNA expression in H4IIE cells. In contrast, oltipraz inhibited sinusoidal transporters Ntcp, Oatp1 and Oatp2 mRNA in H4IIE cells. Rapamycin and oltipraz both induced Mrp2 mRNA expression in H4IIE cells.

It is concluded that pharmacological pre-treatment with rapamycin may not be so effective in reducing IR injury since the induction by rapamycin of antioxidant enzyme expression in normal liver cells is modest, while in transformed cells expression of antioxidant enzymes is inhibited. The inhibition of bile flow associated with pre-treatment with rapamycin is likely due to inhibition of the expression of sinusoidal BA transporters in normal liver cells. In the clinical treatment of HCC patients with rapamycin, attention needs to be paid to the blood concentration of the drug as different concentrations can have markedly different effects on expression of antioxidant enzymes and BA transporters. The ability of transformed liver cells to transport bile acids is likely impaired compared to that of normal liver cells, and the actions of both rapamycin and oltipraz on the expression of bile acid transporters are mixed. Further studies are warranted to determine conditions under which pre-treatment with oltipraz can be effective to reduce IR injury with minimal effects on bile flow.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Farhana Afroz

Signed:

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Finally, my PhD journey has come to an end after long emotional ups and downs along with some strenuous events. I would like to express my appreciation to several people who have accompanied me to the success of this study. I would like to give my sincere and continuing indebtedness to my principle supervisor Professor Dr. Greg J. Barritt for the opportunity of undertaking my PhD in his laboratory and for his patience guidance, valuable advice and encouragement without it would have been impossible to complete this study. It is my pleasure to express my gratefulness to my co-supervisor Dr. Elke Sokoya for her helpful comments and guidance during my PhD. I would also like to express gratefulness Dr Vincent B. Nieuwenhuijs and Robert T. A. Padbury for their great contribution in my research projects.

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ABBREVIATION

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ABC	ATP-binding cassette
AMPK	5′ Adenosine monophosphate-activated protein kinase
AKT/PKB	Protein kinase B
AhR	Aryl hydrocarbon receptor
BA	Bile acid
BCRP	Breast cancer related protein
Bcl-2	B-cell lymphoma 2
BS	Bile salt
BSEP/Bsep	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
DEPTOR	DEP domain-containing mTOR-interacting protein
eEF2K	Eukaryotic elongation factor-2 kinase
EGFR	Epidermal growth factor receptor
eIF4E	Eucaryotic translation initiation factor 4E
ERK	Extracellular regulated MAP kinase
FDA	Food and drug administration
FXR	Farnesoid X receptor
GSH	Glutathione
HCC	Hepatocellular carcinoma
HIF-1	Hypoxia-inducible factor 1
HNF	Hepatocyte nuclear factors
Hsp70	Heat shock protein 70-alpha
IL-1β	Interleukin o one beta

IRS-1	Insulin receptor substrate 1
LCA	Lithocholic acid
MAPK	Mitogen-activated protein kinase
MDR1/3	Multidrug-resistance 1/3
MEK	Mitogen activated protein kinase
MRP2/Mrp2	Multidrug-resistance-associated protein 2
MRP/Mrp 1-6	Multidrug-resistance-associated protein 1-6
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
NTCP/Ntcp	Na^+ taurocholate co-transporter polypeptide
Nrf2	Nuclear factor-erythroid 2 p45-related factor 2
OATPs/Oatps	Organic anion transporting polypeptides
Oatp1	Organic anion transporting polypeptide 1
Oatp2	Organic anion transporting polypeptide 2
OC	Organic cations
PI3K	Phosphatidylinositol 3-kinase
PIP2/ PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PIP3/PtdIns(3,4,5)P3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PL	Phospholipids
PM	Plasma membrane
PPAR	Peroxisome proliferator activated receptor
PXR	pregnane X receptor
Raptor	Regulatory associated protein of mTOR
RAS	Rat sarcoma virus oncogene
RXR	Retinoid X receptor
Rictor	Rapamycin insensitive companion of mTOR
RSK	p90 ribosomal S6 kinase
S6K	S6 kinase

SLC	Solute carrier
STAT	Signal transducer and activator of transcription
TCA	Taurocholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid
TNF-α	Tumour necrosis factor-alpha
TSC 1/2	Tuberous sclerosis complex 1/2
TUDCA	Tauroursodeoxycholic aid
UDCA	Ursodeoxycholic acid
UICC	International union against cancer
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World health organization

CHAPTER I: GENERAL INTRODUCTION

1.1 Overview

The main aim of this study was to investigate whether pharmacological pre-treatment with rapamycin or oltipraz could be used to reduce ischemia reperfusion injury. The clinical significance of these findings could potentially be used in hepatocellular carcinoma (HCC) patients who will undergo liver surgery. This chapter provides an overview of the liver, hepatocellular carcinoma, currently available treatments and associated problems, the pharmacological agents rapamycin and oltipraz and their associated effects and research aims of this thesis.

1.2 Structure and function of the liver

1.2.1 Anatomy and physiology of the liver

The liver is the largest glandular organ of the human body, weighing nearly 2-5% of the total body weight (Abdel-Mishi and Bloomston, 2010). It is a soft, reddish-brown organ lying mainly in the right hypochondrium, but extending across the epigastrium to the left hypochondrium (Rogers, 1992). According to "classical anatomy", the liver can be divided into a median lobe, two lateral lobes (one right and one left), and one caudal lobe, subdivided into the dorsal and ventral half (Fig. 1.1 a) (Desmet, 2001). According to the functional vascular anatomy, the liver is divided into segment I-VIII (Millward-Sadler *et al.*, 1992; Wright, 1979; Choi & Nguyen, 2005). The liver parenchyma has hexagonal-shaped lobules within each liver lobe, each containing a central vein bounded by six portal triads (Fig 1.1 A. b) (Heath and Young, 2001; Mohammed and Khokha, 2005). The hepatic artery, hepatic vein and bile duct compose each portal triad (Fig 1.1 A. c). A thin transparent capsule, called the Glisson capsule, covers the organ.

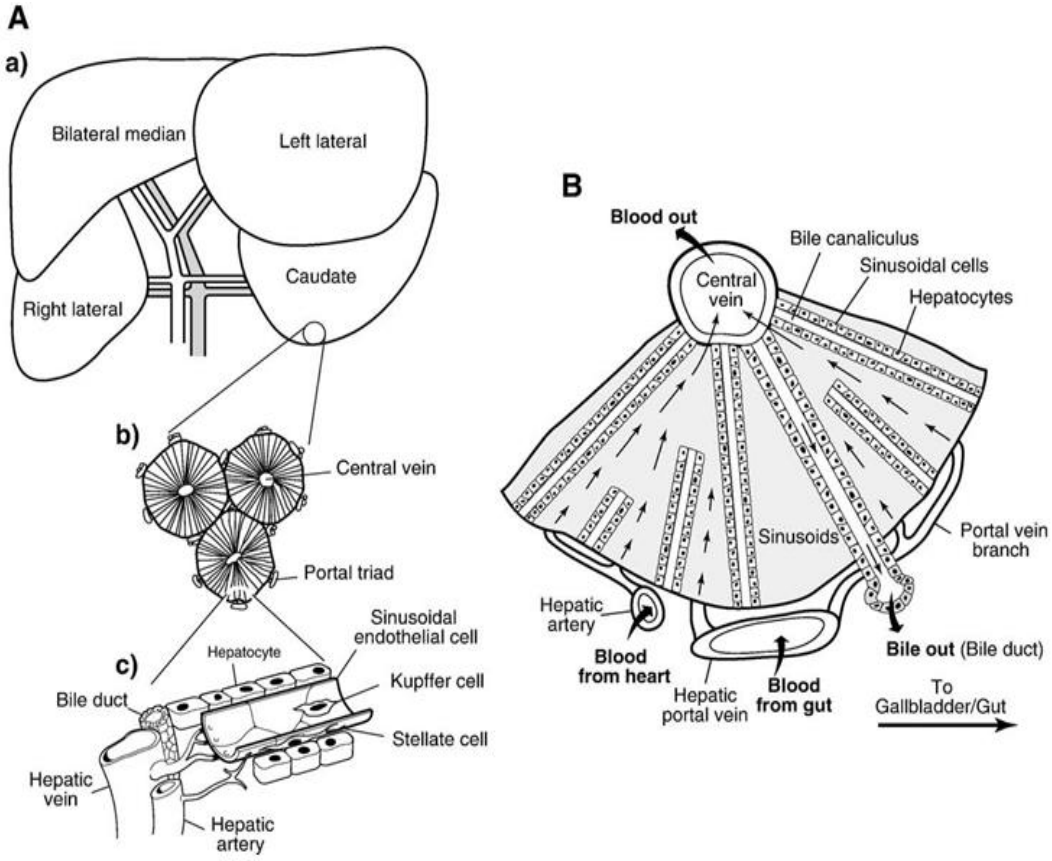


Figure 1.1. Liver anatomy and hepatocyte organisation. (A) Drawing of the major lobes of the rat liver (a), the relationship between the central vein and portal triads (b), and the arrangement of the hepatic vein, hepatic artery, bile duct, hepatocyte plate and sinusoidal space (c) is presented. (B) A schematic drawing of the hepatocyte plates showing the direction of blood flow from the hepatic portal vein and hepatic artery to the central vein, and the direction of bile flow through the bile canaliculus. This figure has been taken from Barritt *et al.*, 2008.

The hepatocyte (parenchymal cell) is the predominant cell type in the liver, representing approximately 70% of all cells in the liver (Millward-Sadler *et al.*, 1992; Wright, 1979; Schiff *et al.*, 2007; Mohammed and Khokha, 2005; Boyer, 2003). Besides hepatocytes, the liver comprises endothelial cells, biliary epithelial cells (cholangiocytes), hepatic stellate cells, Kupffer cells (macrophages) and oval cells, which also play important roles in the liver. Hepatocytes are specialised epithelial cells arranged in single cell plates (Fig. 1.1 B). Blood perfuses into the hepatocyte plates through the basal part or sinusoidal part from the gut (hepatic portal vein) and the hepatic artery drains into the central vein (Fig. 1.1 B). The bile canaliculi (enclosed by the adjacent membranes of two hepatocyte lines) accumulate bile fluid from each hepatocyte and excrete this fluid to the bile ducts (Figs. 1.1 A and 1.2). The (i) vascular system, (ii) hepatocytes and hepatic lobule, (iii) hepatic sinusoidal cells, (iv) biliary system and (v) stroma (Ishibashi

et al., 2009) are assembled by these types of cells. Substances released from neighbouring nonparenchymal cells regulate hepatocyte function. (Hoehme *et al.*, 2010; Matsumoto *et al.*, 2001).

Hepatocytes are different from the other liver cells because they express special characteristics on polarisation and intracellular organization (Barritt *et al.*, 2008; Berry *et al.*, 1991; Hubbard *et al.*, 1994; Zegers and Hoekstra, 1998; Boyer, 2003; Wakabayashi *et al.*, 2006). Each hepatocyte plasma membrane is divided into the sinusoidal (basal), contiguous (lateral), and canalicular (apical) domains (Fig. 1.2). The sinusoidal domain occupies more than 70% of the surface area, facing the blood circulation, comprising many receptors, ion channels and transport systems, which mediate exchange of nutrients and other solutes between the hepatocytes and the systemic circulation. The contiguous domain is mainly occupied by tight junctions, desmosomes, and gap junctions (Fig. 1.2). Gap junctions facilitate the movement of molecules between adjacent hepatocytes (Barritt *et al.*, 2008; Berry *et al.*, 1991; Hubbard *et al.*, 1994; Zegers and Hoekstra, 1998; Boyer, 2002; Wakabayashi *et al.*, 2006). Tight junctions divide the canalicular domain from the sinusoidal and lateral domains. The canalicular domain is responsible for the secretion of bile acids and polymeric immunoglobulin A into the bile (Nathanson and Boyer, 1991).

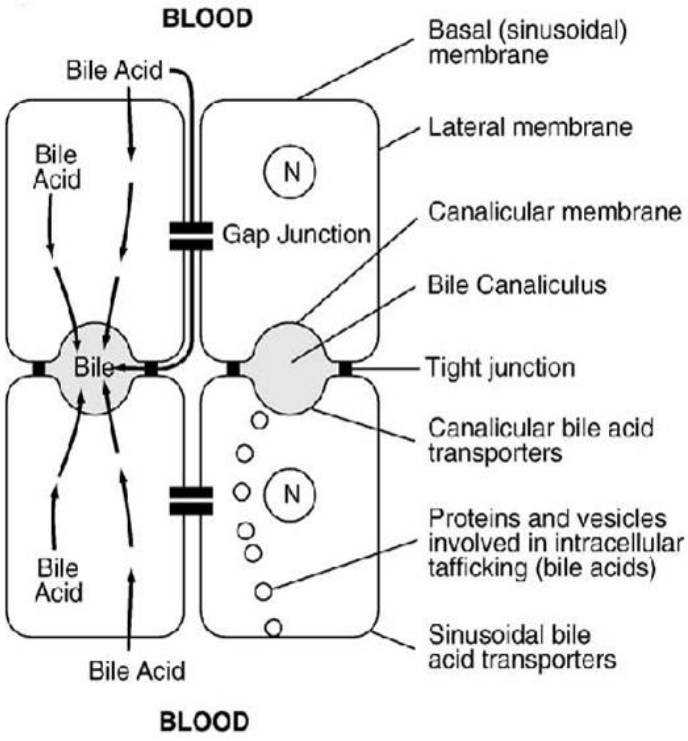


Figure 1.2 Schematic representation of the hepatocyte spatial polarity. The scheme shows the different domains of the hepatocyte and the pathways of bile acid movement and vesicle trafficking in hepatocytes within the hepatocyte plate. The figure has been taken from Barritt *et al.*, 2008.

1.2.2 Functions of the liver

The liver acts as a primary organ of metabolic homeostasis of the body. It plays a central role in the uptake, storage, metabolism and release of carbohydrates, amino acids, lipids, vitamins into the blood and bile. It also plays an important role in the detoxification of drugs and other xenobiotics (Berk *et al.*, 1987; Barritt *et al.*, 2008; Ramadori *et al.*, 2008; Bailey *et al.*, 2007; Aomataris *et al.*, 2008; Nelson *et al.*, 2008; Tsuchiya *et al.*, 2010). Moreover, this organ is responsible for the production of bile acid, bile fluid and protein synthesis and their trans-cellular movement (Barritt *et al.*, 2008; Leite and Nathanson, 2001; Boyer, 2003). Bile flow is an indicator of healthy liver function (Leite and Nathanson, 2001).

During liver injury and inflammation, a large amount of reactive oxygen species (ROS), eicosanoids, nitric oxide, carbon monoxide, TNF-α, and other cytokines are generated thereby contributing to the early phase of liver inflammation. Moreover, during liver injury and inflammation, Kupffer cells secrete enzymes and cytokines that may damage hepatocytes, and are active in the remodelling of the extracellular matrix (Kageyama *et*

al., 2015; Quesnelle *et al.*, 2015; Tanaka *et al.*, 2006; Kmiec, 2001; Ramadori *et al.*, 2008). In humans, the liver is capable of regenerating lost tissue. Approximately 25% of intact liver can regenerate into a whole liver again (*Häussinger, 2011).*

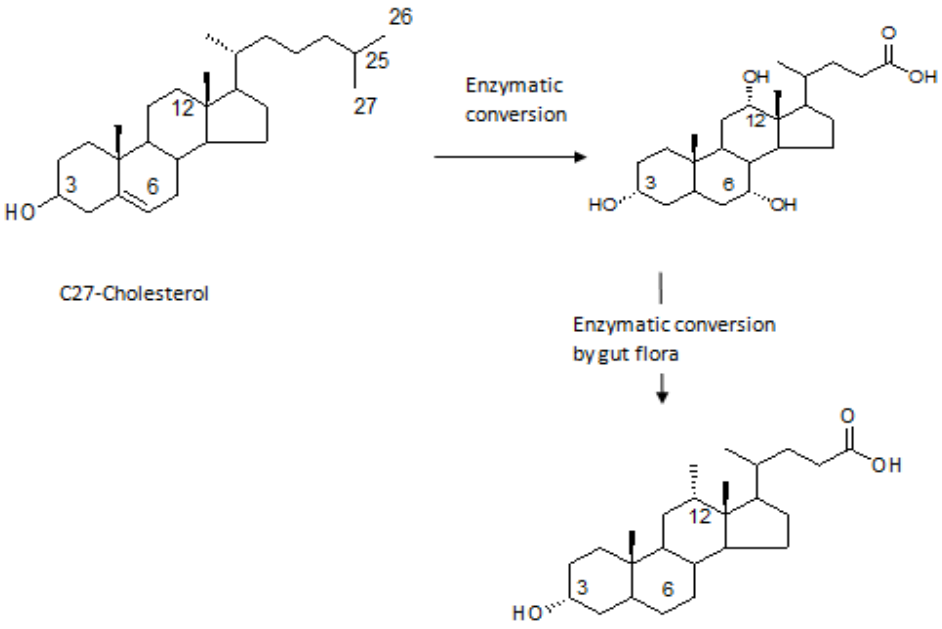
1.3 Role of liver in the synthesis and re-circulation of bile acids

1.3.1 Composition of bile fluids

Hepatocytes play a central role in the synthesis and movement of bile acids from the portal blood to the gallbladder and intestine. Bile acids are steroid acids that are made from the catabolism of cholesterol. Bile is comprised 85% water. The residual solute is a complex combination of bile salts (67%), phospholipids (22%), cholesterol (4%), minerals, electrolytes, bilirubin and biliverdin pigments. Bilirubin and biliverdin give it a yellow-green or orange color (Ganong & Barrett, 2005; Hall & Guyton, 2011). Secretory immunoglobulin A and small amounts of mucus give the bile bacteriostatic functions (Sung *et al.*, 1992; Wang *et al.*, 2009).

1.3.2 Classification and properties of bile acids

According to their hydrophobicity, bile acids can be categorised into two groups. The more hydrophobic bile acids (such as taurolithocholic (TLCA), lithocholic (LCA), cholic (CA) and taurocholic (TCA) acids), are called cholestatic bile acids; whereas the less hydrophobic bile acids (such as taurodeoxycholic (TDCA), tauroursodeoxycholic (TUDCA) and ursodeoxycholic (UDCA) acids), are called choleretic bile acids. Hydrophobicity and solubility of bile acids depend on the position of hydroxyl groups in the α or β orientation of the steroid at positions 3, 6, 7, and 12 (Fig. 1.3). The structural differences of bile acids exhibit important properties with regards to their specificity of receptor activation (Russell 2003; de Aguiar Vallim *et al.*, 2013; Chiang, 2009; Hofmann *et al.*, 2010; Lieberman *et al.*, 2006).



Primary Bile Acids	Hydroxyls	Secondary Bile Acids	Hydroxyls
Cholic Acid	**3α, 7 α, 12 α**	**Deoxycholic Acid**	**3α, 12α**
Chenodeoxycholic Acid	**3 α, 7 α**	**Lithocholic Acid**	**3α**
α-Muricholic Acid (rodents)	**3 α, 6β, 7 α**	**Ursodeoxycholic Acid**	**3α, 7β**
β-Muricholic Acid (rodents)	**3 α, 6β, 7 α**	**ω-Muricholic Acid (rodents)**	**3α, 6α, 7β**

Figure 1.3 Synthesis of Bile Acids. Cholesterol is catalysed and produced primary and secondary bile acids by liver enzymes and gut flora (Hofmann, 2010).

1.3.3 Synthesis of bile acids

Bile acids are divided into primary and secondary bile acids. Primary bile acids are produced from the alteration of the cholesterol steroid ring. Cholesterol is oxidized and the side chain is cleaved by cytochrome P450 and followed conjugation with chenodeoxycholic acid and cholic acid in human (Fig. 1.3). Bacterial activation forms the secondary bile acids by dehydroxylation of primary bile acid into deoxycholic acid and lithocholic acid (Russell, 2003; 2009; de Aguiar Vallim *et al.*, 2013; Hofmann, 1999; Chiang, 2009).

1.3.4 Function of bile acids

Bile acids regulate a number of functions in the body. Firstly, bile acids play a vital role in forming the structure of micelles in the small intestine that mediate solubilisation, digestion, absorption of fat-soluble vitamins and dietary lipids (Hofmann, 1963; *Hofmann and Borgström, 1964*). Secondly, the hepatic conversion of cholesterol to bile acids and the following excretion of bile acids in the feces comprise the key pathway for cholesterol secretion (de Aguiar Vallim *et al.*, 2013). Thirdly, bile acids solubilise cholesterol in the bile, thereby, preventing the precipitation of cholesterol in the gall bladder (Hofmann, 1999). Finally, bile acids assist in the digestion of dietary triacylglycerols as emulsifying agents that make fats accessible to pancreatic lipases (Lieberman *et al.*, 2006; Fiorucci *et al.*, 2009). Recent findings have confirmed that bile acids are involved in the regulation of their own metabolism and transportation via the enterohepatic circulation, lipid metabolism, glucose metabolism, signalling events in liver regeneration, and the regulation of overall energy expenditure (Nagahashi *et al.*, 2015; Wiemuth *et al.*, 2012; Magotti *et al.*, 2015).

1.3.5 The enterohepatic circulation of bile acids

The enterohepatic circulation is defined as the circulation of bilirubin, biliary acids, drugs and metabolites from the bile into the liver, their extraction by hepatocytes, followed by excretion into the small intestine by the active transporters and re-absorption by the enterocyte and movement back to the liver. In adults, 12-18 g of bile acid is produced every day but pool size ranges from 4–6 g, suggesting that bile acids are recycled. In the enterohepatic circulation, new bile acid is synthesised at a low rate (Hofmann, 1999).

To maintain the enterohepatic circulation, liver parenchymal cells transport 95% of the bile acids from the portal blood into bile. Bile acids are transported from the blood across the basal (sinusoidal) and basolateral membranes of hepatocytes, through the cytoplasmic space, and then across the canalicular membrane into the bile canaliculus (Fig. 1.4) (Boyer, 2003). Before bile acid secretion, the bile acids are conjugated with glycine or taurine that creates a lower pKa and increases solubility. Thus, micelle formation happens in the acidic milieu of the duodenum. Bile salts need transmembrane transporters to move them across membranes. Nonconjugated bile acids can diffuse across membranes but hepatocellular uptake of bile acids occurs against an

electrochemical gradient (Weinman & Maglova, 1994; Lidofsky *et al.*, 1993) via sodium-dependent and independent mechanisms by Na^+ taurocholate co-transporter (NTCP) and organic anion transporting polypeptides (OATPs) transport system (Fig. 1.4) (Dawson *et al.*, 2009) and transport across the hepatocytes to the canalicular membrane for secretion into bile (Hofmann & Hagey, 2008). Active transport systems including canalicular transporter e.g. bile salt export pump (BSEP); multidrug-resistance-associated protein 2 (MRP2) are required for the excretion of bile salts from the hepatocytes into the canaliculi to cross the higher concentration gradient (Stieger *et al.*, 1992; Stieger *et al.*, 2007; Akita *et al.*, 2001; Nies & Keppler, 2007). Bile is moved along the bile canaliculus and bile ducts until it is secreted into the common bile duct. F-actin, which regulates contraction of the bile canaliculus, and cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_{cyt}$) play an important role in this circulation (Nieuwenhuijs *et al.*, 2006).

After secretion of bile acid into the intestine, bacterial activation generates the secondary bile acids by dehydroxylation of primary bile acid into deoxycholic acid and lithocholic acid. These bile acids are transported back into the blood, returned to the liver, and re-secreted by enterohepatic circulation (Russell, 2003; Chiang, 2009).

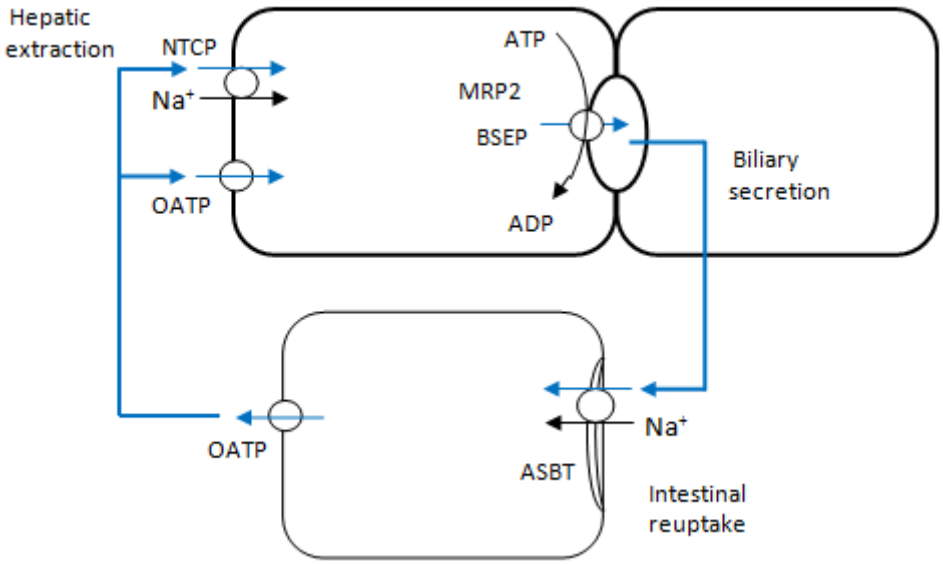


Figure 1.4: Schematic diagram of enterohepatic circulation of bile acid. Bile acids are transported from the blood across the basal (sinusoidal) and basolateral membranes of hepatocytes, through the cytoplasmic space, and then across the canalicular membrane into the bile canaliculus. After secretion of bile acid into the intestine, bacterial activation forms the

secondary bile acids and these bile acids are returned back in the liver, and re-secreted. This figure has been compiled from Trauner and Boyer, 2003.

1.3.6 Significance of enterohepatic circulation

Bile acid homeostasis plays an important role in maintaining normal physiology in mammals. Interruption of bile flow is associated with cholestasis, gallstones, inflammation, malabsorption of lipids and fat-soluble vitamins, bacterial overgrowth in the small intestine, atherosclerosis, neurological diseases, and various inborn errors such as progressive familial intrahepatic cholestasis types I-III (PFIC I-III) (Li & Chiang, 2014; de Aguiar Vallim *et al.*, 2013; Hofmann, 1999; Hofmann & Borgström, 1964; Fiorucci *et al.*, 2009; Carey *et al.*, 1972; Hofmann, 1963). This reduced bile flow increases the toxic bile acid in the liver (Weerachayaphorn *et al.*, 2014). ROS can be generated as a result of increased toxic bile acid in the liver leading to oxidative stress and progressive liver damage (Copple *et al.*, 2010; Xu *et al.*, 2010). Bile acid transporters play a key role in regulating enterohepatic circulation.

1.4 Hepatic bile acid transporters, synthesis and regulation

1.4.1 Overview of the hepatic bile acid transporters

Bile acid transporters are particular proteins that mediate the movement of bile acids into and out of hepatocytes by active and passive mechanisms (Fig. 1.5) (Agellon & Torchia, 2000; Kullak-ublick *et al.*, 2004; Meier & Stieger, 2002; Trauner and Boyer, 2003; Alrefai and Gill, 2007). Transporters on the surface of hepatocytes are classified into the following groups on the basis of functional characteristics: (i) sinusoidal /influx transporters, and (ii) canalicular/efflux transporters (Trauner and Boyer, 2003; Alrefai and Gill, 2007).

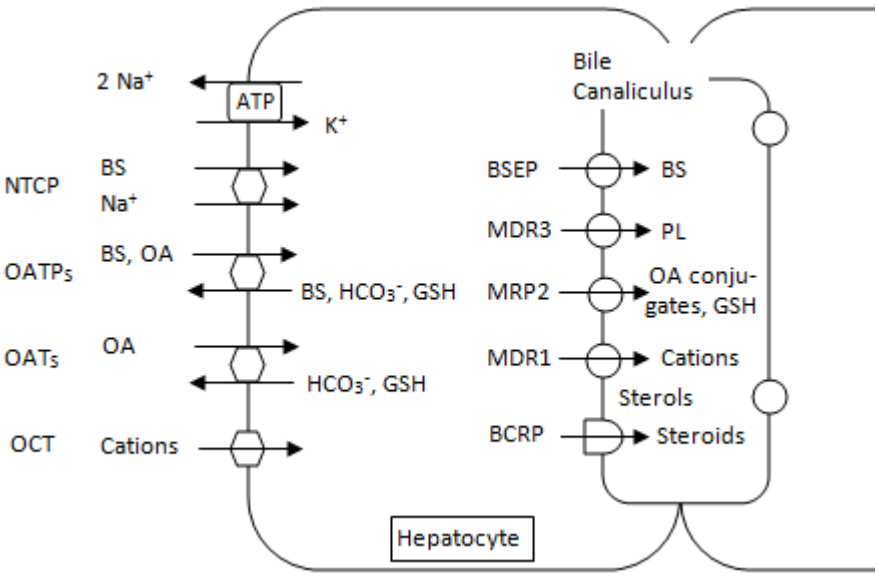


Figure 1.5: Schematic representation of bile acid transportation across the hepatocytes. Transport proteins are illustrated as circles with arrows presenting the direction of transport. The 'half ABC transporters' are shown as semi-circles. The symbols are Na^+ taurocholate co-transporter (NTCP); bile salt (BS); Na^+-independent organic anion transporting polypeptides (OATPs); OC transporter (OCT); Organic cations (OC); OA transporters family (OATs); Bile salt export pump (BSEP); Glutathione (GSH); multidrug-resistance-associated protein 2 (MRP2); phospholipids (PL); multidrug-resistance 1 (MDR1); multidrug-resistance 3 (MDR3); breast cancer related protein (BCRP). This figure has been compiled from Trauner and Boyer, 2003; Alrefai and Gill, 2007.

1.4.2 Sinusoidal/basolateral/influx transporter

The sinusoidal bile acid transporter plays the first step in the delivery of bile acids into the hepatocytes from blood stream (Meier, 1995; Klaassen and Aleksunes, 2010). These bile acid transporters extract conjugated bile acids from the portal blood through hepatic lobules and transport across the sinusoidal or basolateral membrane (Trauner and Boyer, 2003; Meier, 1995) (Fig. 1.5). Bile acid collection from the blood stream generally involves two major ways: (a) Na^+-dependent and (b) Na^+-independent influx of bile acids into the hepatocytes (Kullak-ublick *et al.*, 2004; Trauner and Boyer, 2003).

Na^+-dependent bile acid transporter: Na^+ taurocholate co-transporter (NTCP) is an Na^+-dependent bile acid transporter in hepatocytes. NTCP transports both conjugated and unconjugated bile acids but it mainly transports conjugated bile acid taurocholic acid (TC) with a Km ranging between 15-56 µM (Meier, 1995). This influx process is

activated by Na^+/K^+ ATPase activity and Na^+ inward gradient (Meier & Stieger, 2002; Trauner and Boyer, 2003).

Na^+-independent bile acid transporter: This transport process accounts for most of the intake of unconjugated bile acids from blood stream (Meier, 1995). A wide range of diverse amphipathic organic anions utilise this Na^+-independent transport process (Trauner and Boyer, 2003; Meier, 1995). Several members of the organic anion transporting polypeptides super family of transporters (OATPs) are responsible for the uptake of Na^+-independent bile acids and organic anions into hepatocytes (Kullak-ublick *et al.*, 2004; Trauner and Boyer, 2003; Meier, 1995). Intracellular $HCO3^-$ and glutathione (GSH) are also exchanged along with organic anion by OATPs transporters (Trauner and Boyer, 2003).

1.4.3 Canalicular/efflux transporter

The liver canalicular bile acid transporter is a significant part of the enterohepatic circulation and acts as the rate limiting step in bile formation and hepatic excretion (Trauner and Boyer, 2003). A large bile acid concentration gradient of 100-1000 fold is needed to excrete bile acids into canaculi (Suchy & Ananthanarayanan, 2006). To overcome this large concentration gradient, the canalicular transporters require ATP hydrolysis (Trauner and Boyer, 2003; Suchy & Ananthanarayanan, 2006). The key transporters that account for hepatic bile acid excretion involve Bile Salt Export Pump (BSEP) and Multidrug Resistance Protein 2 (MRP2) (Fig. 1.5) (Trauner and Boyer, 2003; Suchy & Ananthanarayanan, 2006; Arrese & Ananthanarayanan, 2004; St-Pierre *et al.*, 2001). BSEP transports conjugated monovalent bile acids (Byrne *et al.*, 2002; Noé *et al.*, 2002), while MRP2 transports divalent bile acids (Trauner and Boyer, 2003).

1.4.4 Regulation of hepatocyte bile acid transport

Since bile acid transporters play a significant role in physiological and pathophysiological processes, it is important to understand the regulation of these bile acid transporters at the molecular and cellular level (Alrefai *et al.*, 2005). Regulation of hepatic bile acid transport depends on the regulation of transporter activity, post translational modification and regulation of synthesis. Regulation of synthesis is very important to maintain bile flow in the hepatocytes (Boyer, 2003).

1.4.5 Extracellular signals that regulate bile acid transporter synthesis

Bile acids are toxic to cells. Therefore, their blood concentrations are tightly regulated (Weerachayaphorn *et al.*, 2014; Alrefai and Gill, 2007). When bile acid levels are too high, feedback inhibition in the liver reduces synthesis of bile acids (Kim *et al.*, 2007; Zollner *et al.*, 2010). Bile acid synthesis is regulated through the activation of extracellular signals (de Aguiar Vallim *et al.*, 2013). Transcription factors such as FXR (Alrefai *et al.*, 2005; Boyer, 2003), sterols such as 25-hydroxycholesterol (Alrefai *et al.*, 2005), bile acid responsiveness of bile acid transporter genes (Neimark *et al.*, 2004), proinflammatory cytokines such as IL-1β and TNF (Chen *et al.*, 2002), glucocorticoids (GC) (Nowicki *et al.*, 1997; Jung *et al.*, 2004), 1α, 25 dihydroxyvitamin D (Chen *et al.*, 2006) regulate the synthesis of bile acid transporters.

1.4.6 Roles of transcription factors

A number of hepatic transcription factors are responsible for upregulating bile acid transporter expression (Handschin and Meyer, 2003; Xu *et al.*, 2005; Klaassen and Slitt, 2005). These transcription factors are NFE2-related factor 2 (Nrf2), hepatocyte nuclear factors (HNF), farnesoid X receptor (FXR), aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator activated receptor (PPAR). These transcription factors (except AhR) function by forming heterodimers with retinoid X receptor α (RXRα) (Tab. 1.1) (Alrefai and Gill, 2007).

Table 1.1: List of transcription factors that regulate mRNA and protein expression in rodents and humans (Klaassen and Aleksunes, 2010).

Transcription Factors	Sinusoidal transporters		Canalicular transporters	
	Rodent (*In vivo*)	Human (*In vitro*)	Rodent (*In vivo*)	Human (*In vitro*)
Nrf2			↑ Mrp1-4	↑ MRP2-3
			↑ Mdr1a/1b	↑ MDR1
				↑ BSEP
				↑ BCRP

HNF1α	↑ Ntcp	↑OATP1B1		
		↑OATP1B3		
		↑OAT1		
		↑OAT3		
HNF4α	↑ Ntcp	↑OAT1		
		↑OAT2		
		↑OCT1		
FXR	↑Ntcp	↓OATP1B3	↑ Bsep	↑ BSEP
			↑ Mrp2	
			↑ Ostα/β	
AhR	↓ Oatp1a1	↓NTCP		↓ BSEP
	↓ Oatp1a4	↓OATP1B1		↑ MDR1
	↑ Oatp2b1	↓OATP1B3		
	↑ Oatp3a1	↓OAT2		
		↓OCT1		
CAR	↓ Oatp1a1	↓NTCP	↑ Mrp2-6	↑MRP2-3
	↑ Oatp1a4	↓OATP1B3		↑BSEP
		↓OAT2		↑MDR1
		↓OCT1		↑BCRP
PXR	↑ Oatp1a4	↓NTCP	↑ Mrp3	↑MRP2-3
		↑OATP1A2	↑ Mdr1a/1b	↓BSEP
		↓OAT2	↑ Abca1	↑MDR1
		↓OCT1	↑ Abcg5/8	↑BCRP
PPARα	↓ Oatp1a1		↑ Mrp3-4	
	↓ Oatp1a6		↑ Mdr1a/1b	
	↓ Oatp2a1		↑ Mdr2	
	↓ Oatp4a1		↑ Bcrp	

Some studies have reported that the expression of bile acid transporters is altered in hepatocellular carcinoma cells. Sinousoidal bile acid transporter expression (e.g. OATP1B1) is decreased while canalicular bile acid transporter expression (e.g. MRP2)

is increased in human hepatoceluar carcinoma (HCC) cells (Zollner *et al*., 2005; Vavricka *et al*., 2004; Nies *et al*., 2001; Cui *et al*., 2003; Tsuboyama *et al*., 2010). Thereby, alteration of bile acid transporter expression is an important issue for the treatment of HCC patients.

1.5 Hepatocellular Carcinoma (HCC)

1.5.1 Incidence of hepatocellular carcinoma (HCC) in the population

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is a diverse, histologically distinct primary hepatic neoplasm (Kumar *et al.*, 2014). HCC occupies the fifth position in most common cancers diagnosed worldwide in terms of incidence (626000 new cases per year, considering for 5.7% of all new cases) and the third leading cause of death from cancer (El–Serag & Rudolph, 2007; Ashworth & Wu, 2014; Germano and Daniele, 2014; Parkin *et al.*, 2005).

1.5.2 The anatomical, histological and clinical features of HCC and stages of progression

HCC usually forms soft masses with a heterogeneous macroscopic shape, polychrome with foci of hemorrhage or necrosis (Wanless, 1995). HCC (nodule) can be single or multiple in size ranging from 1.0 to 30 cm. According to the size and number of the tumor, three main patterns are described: (i) the nodular and expanding pattern, (ii) the infiltrative or massive pattern, and (iii) the less frequent diffusion pattern (Okuda *et al.*, 1984).

It has been suggested by the World Health Organization (WHO) that HCC might be categorised into histological types based on the tumor cell structural organisation: (i) trabecular or sinusoidal type; (ii) pseudoglandular or acinar type; and (iii) compact or scirrhous sclerosing agent type (Paradis, 2013). According to the degree of tumor cell differentiation, it could also be graded by well, moderately, and poorly differentiated HCC (Fig. 1.6) (Farazi and DePinho, 2006). The UICC (International Union against Cancer) classification is another clinically used staging method. This method is based on tumor number, size, vascular invasion and metastasis. Before a treatment strategy is planned, it is important to account the severity of liver disease. Child Pugh scoring is used to determine the severity of liver disease on the basis of serum albumin, bilirubin,

prothrombin time, ascites, and encephalopathy (Farazi and DePinho, 2006; Paradis, 2013).

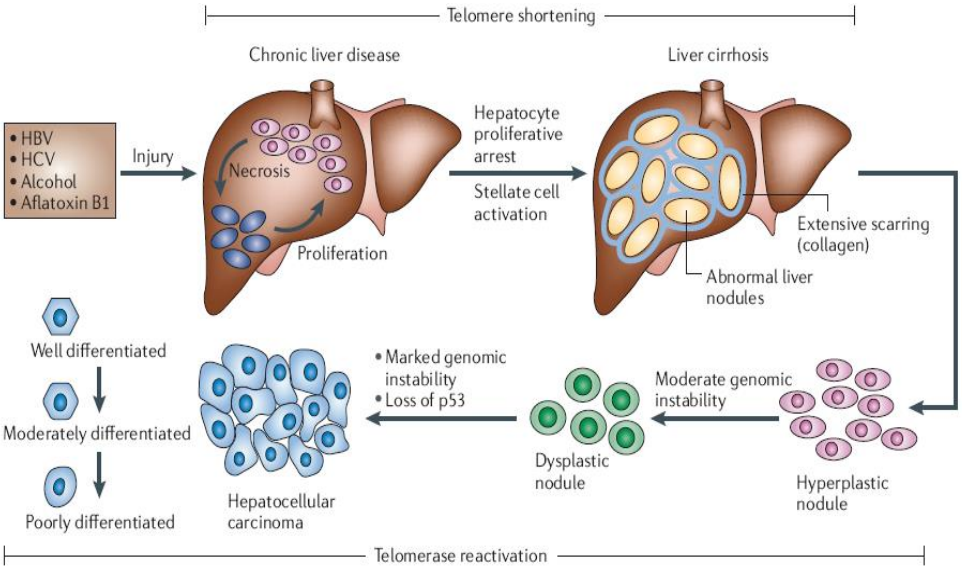


Figure 1.6: Histopathological progression and molecular features of HCC. There is necrosis followed by hepatocyte proliferation after hepatic injury mediated by any one of several factors (hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol and aflatoxin B1). Cirrhosis is distinguished by abnormal liver nodule formation enclosed by collagen deposition and scarring of the liver. Subsequently, hyperplastic nodules are observed, followed by dysplastic nodules and ultimately HCC, which can be further classified into well, moderately and poorly differentiated tumours. This picture has been taken from Farazi and DePinho, 2006.

1.5.3 Hepatocarcinogenesis

Initiation

Hepatocarcinogenesis is a complex multistep process involving a number of genetic and epigenetic alterations (Chuma *et al.*, 2015(Pitot, PERAiNO, Morse Jr, & Potter, 1964; Tillitt *et al.*, 1991), Farazi and Depinho, 2006). The most prominent aetiological factors associated with HCC development involve hepatitis B and C viral infection, chronic alcohol consumption, aflatoxin-B1 contaminated food and all cirrhosis inducing conditions (Farazi and Depinho, 2006; Farazi *et al.*, 2003; Thorgeirsson and Grishma, 2002) (Fig. 1.7).

Progression

There are various genetic events involved in the progression of HCC, which are the alteration of the tumor suppressor p53, overexpression of various ErbB receptor family

members, mutations in β-catenin and overexpression of the MET receptor (Fig. 1.7) (Minouchi *et al.*, 2002; Nose *et al.*, 1993; Nishida *et al.*, 1993; Hosono *et al.*, 1993; Ishizaki *et al.*, 2004; Edamoto *et al.*, 2003; Ito *et al.*, 2001; Höpfner *et al.*, 2004; Daveau *et al.*, 2003; Sakata *et al.*, 1996). Moreover, different cancer relevant genes may be targeted for the aberrant DNA methylation on the epigenetic level in human HCC (Thorgeirsson & Grisham, 2002; Kanai *et al.*, 2000; 1999; 1996; Jian *et al.*, 2003). ROS may induce the epigenetic changes in hepatocellular carcinoma (HCC) (Lim *et al*., 2008).

Under normal physiological conditions, ROS are produced as a byproduct of normal respiration (Cedar, 1988). At low levels, ROS have many physiological functions including modulation of activities of redox-sensitive transcription factors, activation and modulation of signal transduction pathways and regulation of mitochondrial enzyme activities (Jones and Baylin, 2007; Esteller, 2002). ROS are toxic when their levels are high. Excessive ROS are produced when cells are exposed to different insults e.g. inflammation, injury, host-viral interactions, viral integration (McClain *et al.*, 2002; Campbell *et al.*, 2005; Osna *et al.*, 2005; Marrogi *et al.*, 2001; Lim *et al*., 2008; Oberly, 2002). Overproduction of ROS promotes tumor progression by DNA damage and altering cellular signaling pathways (Fig. 1.7) (Lim *et al*., 2008; Liu *et al*., 2005). It was recently reported that ROS are involved in tumor metastasis by epithelial-to-mesenchymal transitions, migrations, invasion, and angiogenesis (Batlle *et al*., 2000). ROS also regulate mitogen activated protein kinase (MAPK), expression of matrix metalloproteinases (MMPs), and Ras pathway activation and downregulates E-cadherin expression by hypermethylation of promoter region in HCC cells (Szpaderska *et al*., 2001; Giannelli *et al*., 2005; Mori *et al*., 2004). This decreased E-cadherin expression is directly correlated with metastasis, epithelial-to-mesenchymal transitions and poor prognosis in HCC (Endo *et al*., 2000; Anthony, 2001). Thus, ROS play an important role in progression of hepatocarcinogenesis.

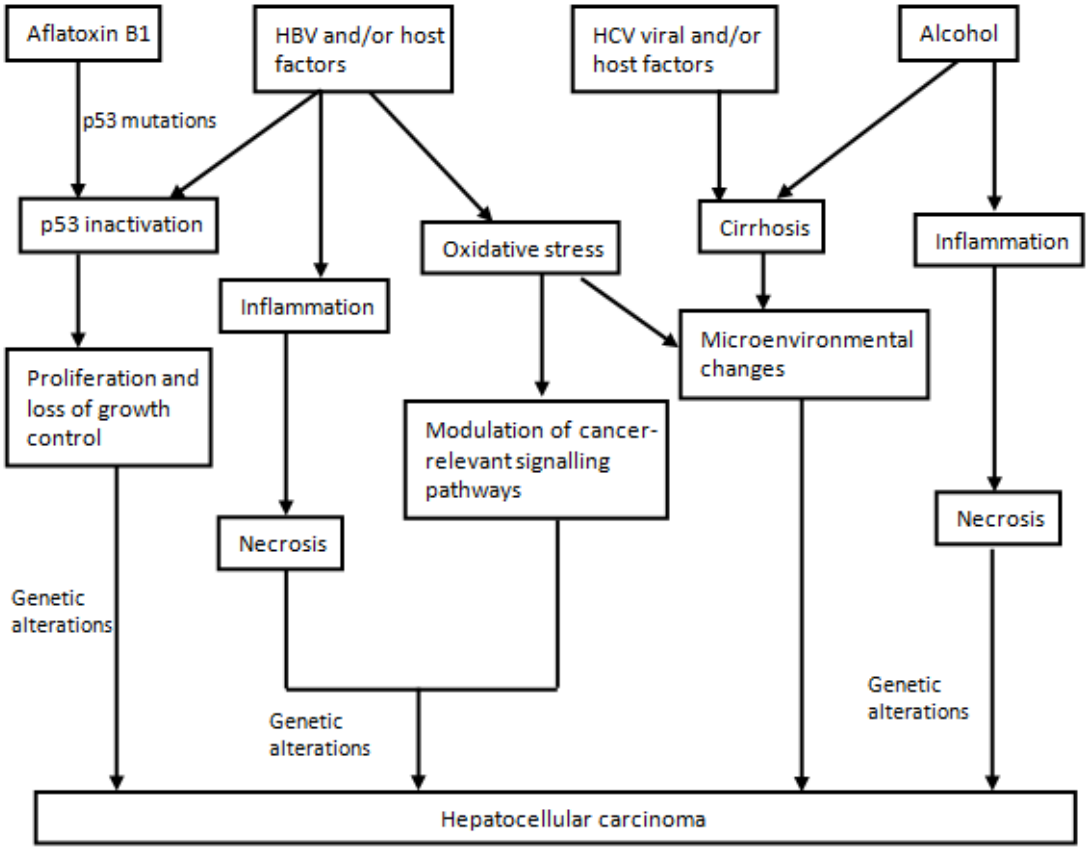


Figure 1.7: Proposed mechanisms of hepatocarcinogenesis. The proposed mechanisms of hepatocarcinogenesis for the various aetiological factors are shown. This figure has been compiled from Farazi and Depinho, 2006; Thorgeirsson & Grisham, 2002.

1.5.4 Treatments for hepatocellular carcinoma (HCC)

Treatment options for HCC and prognosis are dependent on many factors including the size, number, and distribution of tumors, the status of distant metastases, the relationship of the tumor to hepatic vasculature, the severity of liver disease (Child-Pugh score), the functional status of the patient, the suitability of the patient for liver transplantation and local expertise (Bruix & Sherman, 2011; Paradis, 2013). The following options are available for the treatment of HCC patients: (i) surgical resection, (ii) liver transplantation, (iii) cryosurgery, (iv) hepatic artery chemoembolization, (v) percutaneous ethanol, (vi) radiofrequency ablation (surgical and percutaneous), and (vii) cisplatin gel injection, (viii) chemotherapy and (ix) radiotherapy (Bruix & Sherman, 2011; Germano and Daniele, 2014; Ueda *et al.*, 1994).

Surgery is the best practiced option for noncirrhotic patients with hepatocellular carcinoma and for cirrhotic patients with fresh synthetic functions (Teoh, 2011; Bahde

& Spiegel, 2010; Toso *et al.*, 2010; Kist *et al.*, 2012). The liver has the ability to regenerate if a fraction of it is discarded. Up to 75% of the liver can be removed from a healthy liver, and the rest part can redevelop its normal size within six months (*Häussinger, 2011*). However, the capacity to regenerate cirrhotic liver is limited (Sherman *et al.*, 2012). Unfortunately, all patients are not eligible for liver resection; only 20% of patients are potentially resectable (Ang *et al.*, 2015).

1.5.5 Problems associated with treatment options

As mentioned previously in terms of overall survival and recurrence rate, liver resection or transplantation is the most efficient therapeutic practice for patients with liver cirrhosis and early stage hepatocellular carcinoma (HCC) (Sherman *et al.*, 2012). Moreover, tumor recurrence or HCC is a major concern for long-term survival after curative transplantation or resection of HCC (Castroagudin *et al.*, 2011; Toso *et al.*, 2010; Ferreiro *et al.*, 2014; Ashworth and Wu, 2014). Usually, tumor recurrence happens in 4-21% of recipients and HCC recurrence rate is 9.6% (Castroagudin *et al.*, 2011). For improving prognosis, it is important to prevent the recurrence of tumor and HCC after transplantation or resection, but at this moment there is no standard therapy for preventing tumor regrowth or HCC recurrence (Kaibori *et al.*, 2014; Ferreiro *et al.*, 2014).

Although recurrence of HCC is unavoidable, some strategies may be applied to reduce its rate and impact (Castroagudin *et al.*, 2011). Modulation or minimisation of immunosuppressive therapy may influence tumor progression, reducing the negative impact of recurrence on post-transplant survival. Rapamycin is an alternative immunosuppressive agent as rapamycin can inhibit angiogenesis and proliferation of neoplastic cells, resulting inhibition of tumor regrowth (Cholongitas *et al.*, 2014; Neuhaus *et al.*, 2001). The impact of rapamycin on liver and HCC will be discussed later in details.

Ischemia reperfusion injury is the common side effect associated with liver resection or transplantation for the treatment of HCC, metastatic cancer and advanced liver diseases during liver surgery. The effect of ischemia reperfusion injury is described in detail below.

1.6 The problems of liver surgery ischemia reperfusion injury

1.6.1 Overview of ischemia reperfusion injury

Ischemia and reperfusion injury exhibits a complex series of events that result in cellular and tissue injury during surgery and transplantation of organ (Quesnelle *et al.*, 2015; Kageyama *et al.*, 2015; Teoh, 2011; Jin *et al.* 2010). Liver surgery usually requires clamping of blood vessels and cessation of blood flow. After blood flow is resumed, damage to the liver (called ischemia reperfusion injury) ensues. Release of ROS, cytokines and up regulation of adhesion molecules with consequent cellular and organ dysfunction (Vardanian *et al.* 2008; Hoekstra *et al.*, 2008) occurs during ischemia reperfusion injury. Toledo-Pereyra and his associates first recognised this form of injury in 1975 (Toledo-Pereyra *et al.*, 1975).

1.6.2 Classification and characteristics of ischemia reperfusion injury

There are three forms of ischemia identified in hepatic ischemia reperfusion injury during liver resection and transplantation-cold, warm, and rewarming ischemia reperfusion injury (Teoh *et al.* 2011, Selzner *et al.* 2003). Nonparenchymal cells, particularly the sinusoidal endothelial cells (SEC), are damaged in the cold ischemic injury while hepatocytes are mainly damaged in the warm ischemic injury, continued by massive death of liver cells (Selzner *et al.*, 2003; Glantzounis *et al.*, 2005; Massip-Salcedo *et al.*, 2007). Thus, generation of different types of ROS following cellular damage is the long implication in reperfusion injury in liver surgery (Fig 1.9) (Glantzounis *et al.*, 2005; de & Rauen, 2007; Czaja, 2002; Kohli *et al.*, 1999). It is noteworthy that both necrosis (Bradford *et al.*, 1986; Currin *et al.*, 1991; Gujral *et al.*, 2001; Jaeschke & Lemasters, 2003) and apoptosis (Kohli *et al.*, 1999; Sasaki *et al.*, 1996; Gao *et al.*, 1998) play major roles mediating the cell damage in ischemia reperfusion injury in liver surgery.

1.6.3 Mechanism of ischemia reperfusion injury

The pathophysiology of ischemia reperfusion injury is complex involving various biochemical pathways, some of which have not yet been fully explored (Glantzounis *et al.*, 2005; Husted *et al*, 2006; Selzner *et al.*, 2003; Massip-Salcedo *et al.*, 2007). There are two distinct phases identified that are responsible for this special damage: the early (initial) phase (0-2 hours after reperfusion) and the late phase (6-48 hours after

reperfusion) (Fig. 1.8) (Teoh, 2011; Diesen & Kuo, 2011). The early phase is associated with activation of Kupffer cells by complement enhancement (Teoh, 2011), CD^{4+}, T-lymphocytes, and slow recommencement of normal blood flow species (Fig. 1.8) (Jaeschke & Farhood, 1991). Activation of Kupffer cells subsequently generate a high level of ROS; reactive nitrogen species (RNS) and tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and other pro-inflammatory cytokines (Jaeschke & Farhood, 1991; Tsukamoto, 2002, Glantzounis *et al.* 2005) (Fig. 1.8). ROS and cytokines exhibit a direct deleterious effect on the hepatocytes and the endothelial cells, including abnormal Ca^{2+} entry and Ca^{2+} uptake by mitochondria, suppression of the transcellular movement of bile acids and inhibition of the discharge of bile fluid to the bile canaliculus. It is presumed that these early occurrences most likely exhibit a critical function in determining the following short and long term patterns of hepatocyte damage (Selzner *et al.*, 2003; Glantzounis *et al.*, 2005; Husted *et al*, 2006; Nieuwenhuijs *et al.*, 2006). Cytokines mediate neutrophil activation and growth in the late phase, adding to the release of more ROS and proteases, causing damage to hepatocytes (Teoh, 2011; Massip-Salcedo *et al.*, 2007; Jaeschke & Farhood, 1991). Neutrophils are the key cells in the late phase of ischemia reperfusion injury (Tapuria *et al.* 2008). Damage to hepatocytes in the late phase of ischemia reperfusion injury is more severe compared with that in the early phase of ischemia reperfusion injury (De Groot & Rauen, 2007).

Liver injury due to ischemia reperfusion is induced when the organ is rapidly depleted of oxygen and then reoxygenated, resulting in insufficient oxygen supply to the cell. As a result, there is a reduction of adenosine triphosphate (ATP) synthesis (Gonzalez-Flecha *et al.* 1993). The membrane potential is disrupted by reduction of ATP-dependent sodium (Na^+) /potassium (K^+) ATPase activity, ensuing in total sodium influx, creating cell swelling and cell death (Blum *et al.* 1991). Anaerobic glycolysis and lysosomal leakage of H^+ generate low intracellular pH in this milieu (Xia *et al.*, 1996; Teoh & Farrell, 2003; Woolf, 1998) which subsequently develops metabolic acidosis functions as a protective measure against necrotic cell death of hepatocytes. Revival of normal pH on reperfusion stimulates cell injury and enhances cell death. In addition to these described steps, reperfusion of hypoxic liver tissue can stimulate an inflammatory response wherein various intracellular signal pathways are involved (Teoh & Farrell, 2003).

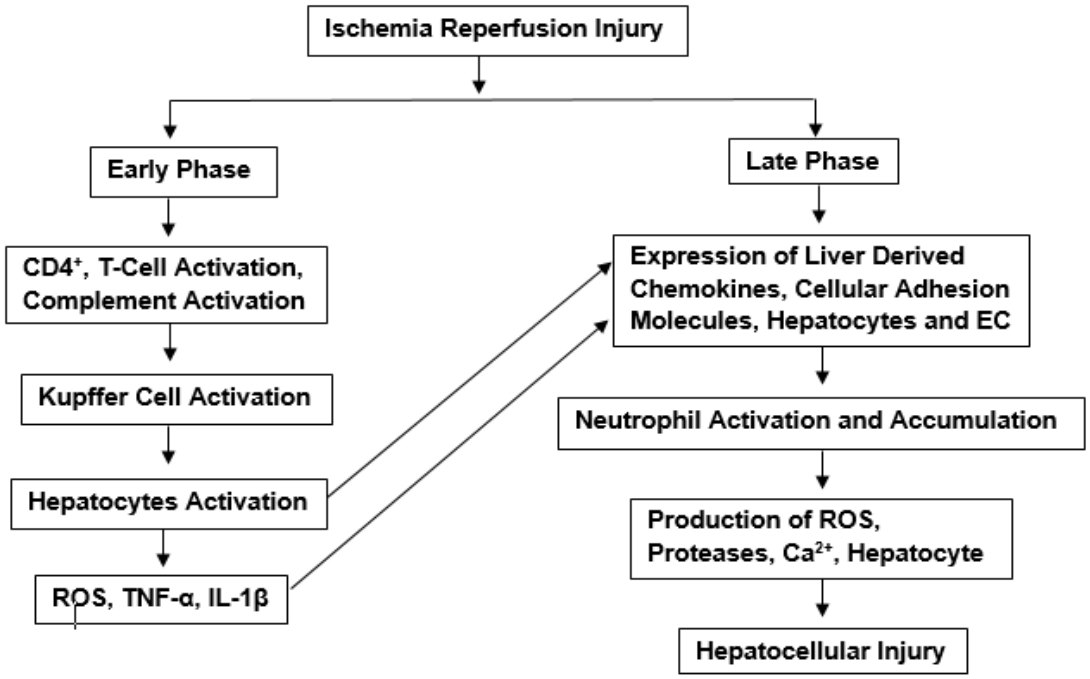


Figure 1.8: Mechanisms involved in the pathophysiology of ischemia reperfusion injury. In the early phase of ischemic injury, hepatocytes produce small amounts of ROS that activate neutrophils. Hepatocellular ROS also mediate to release HMGB1, a damage-associated molecular pathogen that stimulates Kupffer cells associated with $CD4^{+}$T cells. Stimulated Kupffer cells and neutrophils generate toxic levels of ROS, such as hydrogen peroxide and hyperchlorous acid to induce necrotic pathways. EC, endothelial cell; ROS, reactive oxygen species; HMGB1, high mobility group box 1. This figure has been compiled from Selzner *et al.*, 2003, Kaibori *et al.*, 2015.

1.6.4 Bile flow as an important marker of ischemia reperfusion injury in liver

Impaired bile flow indicates the functional capacity of the liver and is considered to be a reliable indicator of hepatic ischemia reperfusion injury (Sumimoto *et al.*, 1988; Accatino *et al.*, 2003; Bowerd *et al.*, 1987). Bile flow enables a useful monitoring of early stages of ischemia reperfusion damage (Selzner *et al.*, 2003) while apoptosis and liver enzymes cause huge cellular damage. Ischemia reperfusion injury is associated with an unexpected and usually temporary decrease in bile secretion. Accatino and co-workers have demonstrated in a partial (70%) liver ischemic model that bile flow is provisionally decreased for 24 hr after 30 min of warm ischemia (Accatino *et al.*, 2003). These variations in bile flow are linked to increased serum alanine and aspartate aminotransferase activities, induced liver myeloperoxidase activity, thereby, increased serum bile salt concentrations. All changes are reversible within one to three days

(Selzner *et al.*, 2003). The exact mechanisms behind the ischemia reperfusion injury induced inhibition in bile flow are not fully understood.

1.6.5 Strategies to reduce ischemia reperfusion injury

Generation of ROS and oxidative stress play a key role to provoke hepatic ischemia reperfusion injury and associated problems. For successful targeting of ROS, it is essential to account for the sources of ROS formation, in what specific location, at what time in pathogenesis and how much oxidative stress is produced (Jaeschke and Woolbright, 2012). Ischemia reperfusion injury on the basis of ROS generation can be categorised into three distinct ways: (i) surgical interventions or preconditioning, (ii) use of pharmacological agents, and (iii) gene therapy (Jaeschke and Woolbright, 2012; Selzner *et al.*, 2003).

Surgical intervention/preconditioning

The most common clinical approach to reducing ischemia reperfusion injury is ischemic preconditioning. The liver is pre-exposed to a brief period of ischemia followed by reperfusion (Nieuwenhuijs *et al.*, 2007; Kageyama *et al.*, 2015; Selzner *et al.*, 2003). These methods have been shown to reduce inflammatory response and oxidative stress. Induction of antioxidant enzymes (e.g. HO-1) is one of the popular mechanisms involved in preconditioning therapies (Kageyama *et al.*, 2015; Jaeschke and Woolbright, 2012). Although current progresses in surgical practices that permit liver resection to be executed without portal triad clamping and following substantial blood loss, might still provide ischemic preconditioning or intermittent clamping outmoded in the future (Kageyama *et al.*, 2015; Rahbari *et al.*, 2008; Belghiti *et al.*, 1999; Desai *et al.*, 2008).

Use of pharmacological agents

Current pharmacological strategies include antioxidants, adenosine agonists and nitric oxide donors, inhibitors of intracellular proteases, matric metalloprotease inhibitors, prostaglandins, and inhibitors of TNF-α action have been employed with some clinical benefits. Current studies emphasise the induction of antioxidants (e.g. HO-1; Prx-1) to protect or neutralise oxidative stress (Wang *et al.*, 2014; Kageyama *et al.*, 2015; Selzner *et al.*, 2003; Kist *et al.*, 2012). This preconditioning technique impedes different pathways that direct to cell death and thus, prevents the liver from function loss. Many

drugs have been examined to reduce ischemia reperfusion injury in experimental paradigms (Selzner *et al*, 2003; Nieuwenhuijs *et al.*, 2006; Gems & Partridge, 2008) but none have yet demonstrated evidence of clinical benefit (Selzner *et al*, 2003; Theodoraki *et al.*, 2011; Gurusamy *et al.*, 2010). In many cases, the mechanism of action has not been explored and the specificity of the pharmacological agent is too broad, thereby producing deleterious systemic side effects (Clavien et. al. 2004). However, a small number of pharmacological agents are currently nearing clinical application (Jaeschke and Woolbright, 2012; Selzner *et al*, 2003).

Gene therapy

Finally, gene therapy emerges to present a new elegant option to alleviate protective mechanisms (Selzner *et al*, 2003; Jaeschke and Woolbright, 2012). Pretreatment of donor with adenoviral vectors carrying the antioxidant gene (e.g. heme oxygenase 1) is a promising approach in this method which can significantly recover several parameters after warm ischemia and liver transplantation (Selzner *et al*, 2003). Treatment of a healthy donor with adenoviral vectors with its possible negative side effects is presently morally unacceptable. While experimental verification exhibits the effectiveness of gene therapy, there are still several ethical considerations to be conquered (Selzner *et al*, 2003; Glantzounis *et al.*, 2005; Li *et al.*, 2007). However, beneficial effects of gene transfer have been reported, notably improved graft survival in a rat ischemia reperfusion model (Coito *et al.*, 2002).

Induction of antioxidant enzymes (e.g. HO-1; Prx-1) by pharmacological agents to restore redox balance in HCC patient is a potential strategy in clinical practice (Wang *et al.*, 2014; Kageyama *et al.*, 2015; Selzner *et al.*, 2003).

1.6.6 The induction of antioxidant enzymes as a strategy to reduce ischemia reperfusion injury

The major antioxidant enzymes are heme oxygenase 1 (HO-1), glutathione reductase and peroxidise, peroxiredoxins, catalase, and the superoxide dismutases. HO-1 and Prx-1 are induced by various conditions such as hypoxia (Murphy *et al.*, 1991; Bonkovsky *et al.*, 1986; Selzner *et al.*, 2003), hyperthermia (Raju and Maines, 1994) and ischemia reperfusion injury (Selzner *et al.*, 2003; Kist *et al.*, 2012; Wilson *et al.*, 2011; Jaeschke and Woolbright, 2012) in a variety of organs (Kageyama *et al.*, 2015). Several studies

showed that induction of HO-1 is essential to reduce the inflammation and preventing cell death or apoptosis (Wang *et al.*, 2014) in hepatic ischemia reperfusion injury and liver transplantation (Kageyama *et al.*, 2015; Kobayashi *et al.*, 2005; Kato *et al.*, 2001). Many known pharmacological agents and inducers such as rapamycin, simvastatin, everolimus, chromium mesoporphyrin, ZnPP, CoPP, SnPP, hemin can induce HO-1 expression in various organs (kidney, heart, limb and lung) during ischemia reperfusion injury in different experimental models (Feitoza *et al.*, 2007; Lai *et al.*, 2008; Nie *et al.*, 2002; Jaeschke and Woolbright, 2012) but inducers such as cobalt protoporphyrin (CoPP) and hemin are toxic to humans (Wang *et al.*, 2011). Also, studies indicate that the protection by upregulation of HO-1 expression might be limited to a narrow threshold and that excessive overexpression is directly related to increased injury (Yun *et al.*, 2010; Suttner and Dennery, 1999; Suttner *et al.*, 1999; Eipel *et al.*, 2007). Moreover, research has shown that HO-1 is often overexpressed in human HCC and that experimental down regulation reduced orthotopic tumor growth in mouse liver (Saas *et al.*, 2008). This may possibly challenge the significant benefits of patients with HCC could undergo for surgical interventions. However, beneficial effects of HO-1 upregulation on histological injury and inflammatory biochemical markers in ischemia reperfusion and on graft longitivity in human liver transplantation have been observed repetitively (Kato *et al.*, 2001; Wang *et al.*, 2001; Amersi *et al.*, 1999; Geuken *et al.*, 2005; De Rougemont *et al.*, 2009). Therefore, if pharmacological novel compounds can be identified that induce HO-1 and are non-toxic to humans, this might offer a novel strategy to attenuate ischemia reperfusion injury. Rapamycin, also known as sirolimus, is an accepted drug that is capable of inducing HO-1 expression in kidney, lung and liver (Zhou *et al.*, 2006; Visner *et al.*, 2003). The effect and mechanism of rapamycin action on liver will be discussed later.

Gene therapy approach has been used for the induction of HO-1 in Zucker rats (Amersi *et al.*, 1999; Coito *et al.*, 2002). Research has shown that pre-treatment of donor rats with adenoviral vectors carrying the HO-1 gene are able to potentially progress a number of parameter after warm ischemia and orthotopic liver transplantation (Jaeschke and Woolbright, 2012). By this treatment edema, necrosis and macrophage infiltration are decreased significantly whereas survival rate of recipient is increased from 40% to 80%. In addition, proteins with antiapoptotic activities (e.g., Bag-1 and

Bcl-2) are increased whereas inducible nitric oxide synthetase is reduced (Coito *et al.*, 2002) but the mechanism involved is still unclear.

Reaction catalyzed by Heme Oxygenase-1

HO-1, known as heat shock protein 32, is encoded by the *Hmox 1* gene exhibiting endogenous catalyzing enzyme (Goncalves *et al.*, 2006). It is responsible for the rate - limiting step in the oxidative detoxification of excess heme, by cleaving the α-methane bridge into equimolar amounts of free iron, biliverdin and carbon monoxide (CO) (Fig. 1.9) (Maines, 1997) which are being released in large amounts from hemoproteins during liver surgery. The released CO interacts with hemoglobin forming carboxyhemoglobin, inhibiting its oxidation to methemoglobin and, resulting in release of free heme moieties (Richards *et al.*, 2010). Biliverdin catalyses guanylyl cyclase by CO, has vasodilation effects, preventing platelet accumulation and regulates bile flow (Lai *et al.* 2008, Zeng *et al.* 2010). Biliverdin is again catalysed to bilirubin, which also acts as a strong antioxidant. Soares *et al.* reported that discharge of free Fe^{2+} radicals in the cell are directed to increase cellular extrusion of Fe^{2+} by HO-1, thereby, protecting liver by preventing ROS production (Soars *et al.*, 2009, Richards *et al.*, 2010; Yun *et al.*, 2010; Ferris *et al.*, 1999).

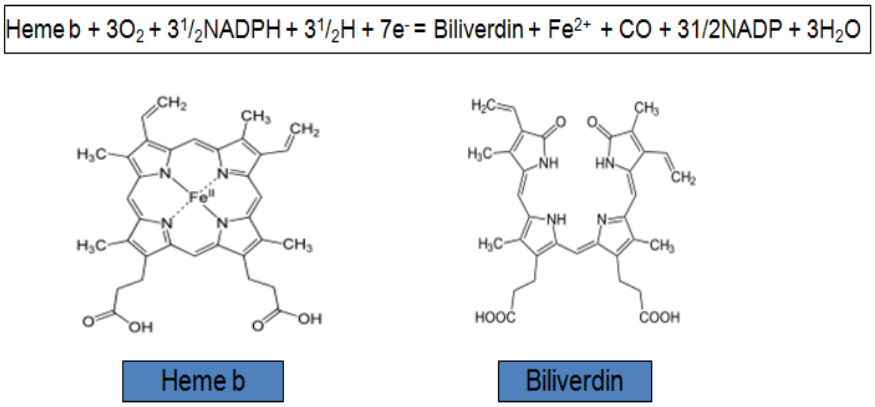


Figure 1.9: Catalytic activity of HO-1. Heme is cleaved by HO-1 and produces equimolar concentrations of biliverdin, Fe^{2+} and CO. This figure has been taken from Yoshida and Migita., 2000.

The promising roles of HO-1 in ischemic tissue comprise protection against oxidative stress, repair of damaged proteins, repression of pro-inflammatory cytokines, and repair of the ion channel (Morimoto & Santoro, 1998; Chi & Karliner, 2004). However, in

spite of these benefits of heat shock preconditioning, its application in the clinical setting is limited.

Reaction catalyzed by Peroxiredoxin-1

Peroxiredoxin 1 (Prx-1) is an intracellular antioxidant protein belonging to the peroxiredoxin family that is abundantly expressed in liver cells (Immenschuh *et al.*, 2003; Bae *et al.*, 2011). It acts along with HO-1 in reducing oxidative stress and is a regulator of signal transduction (Wen and Van Etten 1997; Kang *et al.*, 1998; Rhee *et al.*, 2005; Chang *et al.*, 2002; Immenschuh *et al.*, 2003). Each peroxiredoxin reduces H_2O_2, and other peroxides, by using thioredoxin which is essential for reducing equivalent (Cesaratto *et al.*, 2005; Kang *et al.*, 2005). Cys^{52} and Cys^{173} are the two active sites of cysteine residues. Cys^{52} activates the catalytic mechanism of Prx-1 action as a peroxidatic cysteine and Cys^{173} acts as a resolving cysteine (Aran *et al.*, 2009). Cys^{52} is catalyzed and formed first to sulfinic acid ($-SO_2H$) then converted to sulfonic acid ($-SO_3H$) that is oxidized to form inactive over-(hyper)-oxidized Prx1 in presence of ROS (Wilson *et al.*, 2011; Yang *et al.*, 2002). Reactivation of this hyperoxidized complex is reversible by the activity of enzyme sulfiredoxin (Srx) (Bae *et al.*, 2011), thus preserving the catalytic activity of Prx1 (Soriano *et al.*, 2009). On the other hand, sulfonic oxidation is irreversible (Soriano *et al.*, 2009; Lim *et al.*, 2008). In addition, some reports also demonstrated that oxidized Prx1 and its multimers are chaperone proteins, and defend cells from stress-induced damage (Kang *et al.*, 2005; Lim *et al.*, 2008). Overoxidation of Cys^{175} in rapeseed Prx1 is essential for the integration of a phosphoryl group from ATP to develop sulfinic-phosphoryl [Prx-(Cys^{175})-$SO_2PO_3^{2-}$] and the sulfonic-phosphoryl [Prx-(Cys^{175})-$SO_2PO_3^{2-}$] anhydrides (Aran *et al.*, 2008; Wilson *et al.*, 2011).

Prx-1 has multifunctional antioxidant properties that exhibit peroxidase enzyme activity (Wilson *et al.*, 2011; Bae *et al.*, 2011; Kang *et al.*, 1998). Thus, Prx-1 similar to other antioxidant enzymes may contribute in the cellular defence mechanisms against oxidative stress (Wilson *et al.*, 2011; Oberley 2002; Shen and Nathan 2002). The antioxidant property of Prx-1 is evidenced by its high binding affinity with the pro-oxidant heme (Kang *et al.*, 2005). Since non-protein bound heme generates free radicals (Katori *et al.*, 2002), Prx-1 is required to exert the pro-oxidant function of heme by non-covalent binding (Vincent 1989; Immenschuh *et al.*, 2003). Thus, the structure of the

Prx1-heme complex is thought to be an element of the defensive mechanism involving Prx-1. However, Prx-1 serves as a transporter of heme for intracellular transportation (Muller-Eberhard and Nikkila 1989; Boyer and Olsen 1991). This hypothesis is also nourished by the localisation of Prx-1 in mitochondria and peroxisomes which restrain considerable amounts of cytochromes and catalase, two hemoproteins (Immenschuh *et al.*, 2003). Furthermore, Prx1 can control signal transduction pathways and alter cell proliferation and differentiation (Jin *et al.*, 1997; Wen and Van Etten, 1997; Kang *et al.*, 1998; Seo *et al.*, 2000; Chang *et al.*, 2002). Thus, Prx-1 provides an important role for preventing cellular damage during ischemia reperfusion injury. Pharmacologically-induced increases in Prx-1 expression have been shown to protect liver from ischemia reperfusion injury (Wilson *et al.*, 2011; Kist *et al.*, 2012), indicating that prê-treatment with lead compounds which induce Prx-1 is a viable strategy for reduction of liver ischemia reperfusion injury.

Regulation HO-1 and Prx-1 expression

Antioxidant genes (HO-1 and Prx-1) and gene products are highly protective against ischemia reperfusion injury. These genes involve scavenging ROS either indirectly or directly. (Jaeschke and Woolbright, 2012). These genes are regulated by the transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-Kelch-like-ECH-associated protein 1 (Keap1) pathway (Klaassen and Reisman, 2010; Kensler *et al.*, 2007). Oxidative stress or hypoxia can activate Nrf2 by dissociating Keap1 and Nrf2 translocation to the nucleus resulting gene expression (Jaeschke and Woolbright, 2012; Baird and Dinkova-Kostova, 2011).

1.7 Rapamycin in the treatment of liver surgical and HCC patients

Rapamycin, also known as sirolimus, is a macrolide isolated from a fungal strain *Streptomyces hygroscopicus*, found in the soil of Rapa Nuli in 1969 (Vezina *et al.*, 1975). Rapamycin was intially developed for its antifungal properties but later it has been used as anticancer drug for its immunosuppressive and antiproliferative properties (Martel *et al.*, 1977; Calne *et al.*, 1989). In 1999. Rapamycin was approved as an immunosuppressive drug (Trotter, 2003; Zhang *et al.*, 2007).

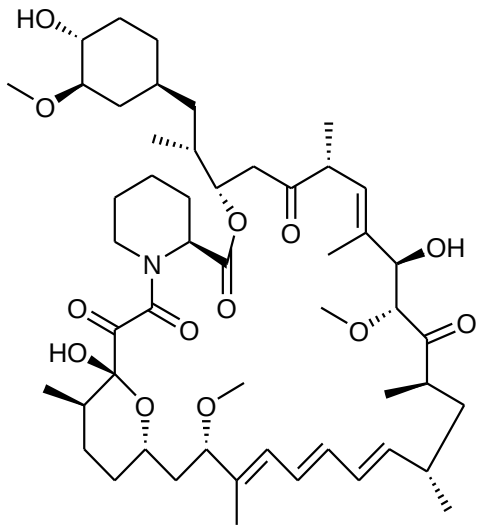


Figure 1.10: Structure of rapamycin.

1.7.1 Rapamycin as an immunosuppressant in liver transplant patients

Rapamycin is presently employed as a potential immunosuppressant in patients with HCC or metastatic cancer treated by liver resection or transplantation (van Veelen *et al.*, 2011; Shirouzu *et al*., 2009; Ashworth and Wu, 2014; Cholongitas *et al*., 2014). The efficiency of rapamycin as an immunosuppressant agent in liver transplantation is alike to that of the commonly used calcineurin inhibitors (e.g. cyclosporin A and tacrolimus) (Trotter, 2003; Cholongitas *et al*., 2014). Pre- and post-treatment with rapamycin can attenuate hepatic ischemia reperfusion injury in rats (Esposito *et al.*, 2010; Liu *et al.*, 2010). It has been reported that rapamycin can also attenuate graft injury in a cirrhotic liver transplantation in rat liver (Man *et al.*, 2006). Rapamycin inhibits cell cycle progression of T and B cells by inhibiting mTOR. This mechanism also plays a role in non-immune cells such as fibroblast, endothelial cells, hepatocytes and smooth muscle cells (Neuhaus *et al*., 2001; Semela *et al*., 2007; Mehrabi *et al*., 2006). Treatment with rapamycin can also reduce hepatic stellate cell proliferation and limit liver fibrosis (Zhu *et al.*, 1999). Therefore, it is beneficial to use rapamycin in the liver transplant settings (Neef *et al.*, 2006). There are a small number of surgical impediments associated and an advanced recurrence free survival rate over 5 years (Monaco, 2009; Geissier & Schlitt, 2009; Vivarelli *et al.*, 2010; Schnitzbauer *et al.*, 2010). Thereby, rapamycin is of particular growing interest as an immunosuppressant for patients undergoing liver resection or transplantation for treatment of HCC.

Nevertheless, a uniform immunosuppression regimen of post-transplantation settings has not yet been determined.

1.7.2 Rapamycin as an anticancer drug to treat HCC patients

Rapamycin inhibits cell proliferation and can effectively reduce the development of cancer or tumor cells and angiogenesis (Yuan *et al.*, 2009; Shirouzu *et al.*, 2010; Heuer *et al*., 2009). The anticancer property of rapamycin has been shown in clinical trials for the treatment of different cancers (Toso *et al.*, 2010; Shirouzu *et al.*, 2010; Deng *et al.*, 2015).

Several studies provide evidence that rapamycin is well tolerated in HCC patients and has unique anticancer properties (Shirouzu *et al*., 2010; Toso *et al*., 2010). At this moment, rapamycin is used as an aid for HCC, cholangiocarcinoma and neuroendocrine tumors metastases (Mártinez *et al*., 2010). Rapamycin treatment is also effective to reduce cell motility in breast rhabdomyosarcoma, prostate and cervical adenocarcinoma (Liu *et al*., 2006). Currently, rapamycin as a chemotherapeutic agent in advanced HCC is at an early stage of clinical development (Germano and Daniele, 2014).

Tumor regrowth is a common recurrence following liver surgery for the treatment of HCC patients (Shah *et al.*, 2007; Toso *et al*., 2010). At present, a principal objective of research is the impact of immunosuppressive drugs upon tumor recurrence (Heuer *et al*., 2009). The patients who underwent liver resection or transplantation and received rapamycin had lower recurrence rate of HCC or tumor compared to other currently used immunosuppressive drugs (Germano and Daniele, 2014; Toso *et al*., 2010).

1.7.3 Associated problems of rapamycin using

The major side effects associated with rapamycin usage are cytopenia, dislypidemia, anemia, hyperlipidemia, leukopenia, thrombocytopenia, induction in hepatic artery thrombosis and impaired wound healing, although incident is dose and related to length of drug treatment (Trotter, 2003; Weinreich *et al.*, 2011; Mehrabi *et al.*, 2006; Everson, 2006; Shenoy *et al.*, 2007; Alamo *et al.*, 2009; Arias-Diaz *et al.*, 2009; Shen *et al.*, 2009). Less nephrotoxicity has been shown in patients treated with rapamycin compared to calcineurin inhibitors (Trotter, 2003; Chang *et al.*, 2000; Esposito *et al.*,

2011) but this effect is not evidently established, since other investigations are reported that there is no difference (Campbell *et al.*, 2007).

Several studies have identified side effects of rapamycin in liver transplantation (Antolin *et al.*, 2011; Trotter, 2003). Thus, the optimal role of rapamycin as immunosuppressant has not yet been established, and it remains a matter of ongoing clinical trials.

1.7.4 Effects of rapamycin on HO-1 and Prx-1 expression

Rapamycin can increase HO-1 expression in different organs (Goncalves *et al.*, 2006; Kist *et al.*, 2012; Esposito *et al.*, 2010; Visner *et al.*, 2002). As discussed above, HO-1 and Prx-1 are anti-oxidants and can protect liver from oxidative stress-induced liver damage during ischemia reperfusion. Moreover, HO-1 expression can enlarge intracellular cytoprotective mechanisms against different types of insults involving innate and acquired immunity (Wang *et al.*, 2014; Glanemann *et al.*, 2005). Therefore, it has been suggested that induction of HO-1 and Prx-1 expression by rapamycin would be beneficial for liver during ischemia reperfusion injury which as shown in Fig 1.11. Moreover, rapamycin is already in clinical use as an immunosuppressant for the treatment of HCC patient or post-transplantation settings (Kaibori *et al.*, 2015; Toso *et al.*, 2010; Deng *et al.*, 2015). However, it is not clear whether rapamycin can increase HO-1 and Prx-1 expression in HCC cells. Pre-treatment with rapamycin for 24 hours *in vivo* had only a little effect on HO-1 mRNA expression (Kist *et al.*, 2012). However, the effect of rapamycin on HO-1 and Prx-1 expression in liver following ischemia reperfusion injury remains unclear. The optimal dose of rapamyin to induce HO-1 and Prx-1 expression for the treatment of HCC patients is also not well established.

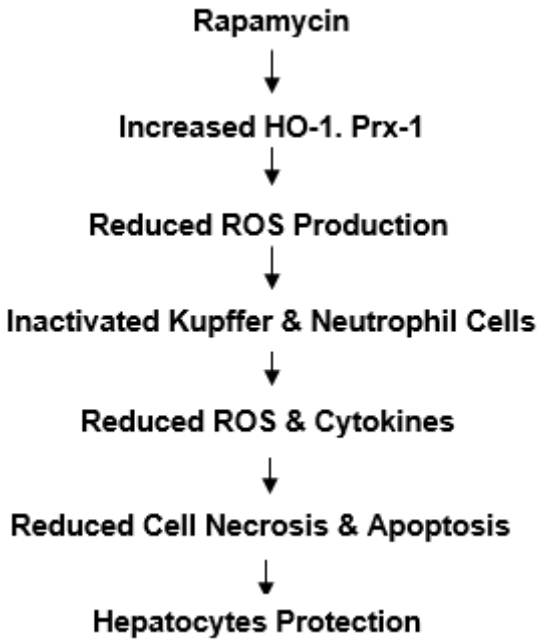


Figure 1.11: Proposed signaling pathway of protection liver from ROS induced damage by rapamycin. Rapamycin can induce HO-1 and Prx-1 expression resulting reduced ROS production. This reduced ROS can inactivate kupffer and neutrophil cells which lead to reduce ROS and cytokine induced necrosis and apoptosis. This figure has been compiled from Kaibori *et al.,* 2015; Selzner *et al*., 2003.

1.7.5 Effects of rapamycin on bile flow

It has been reported that pre-treatment with rapamycin caused a little increase in HO-1 expression but inhibited a decrease in 20% of basal bile flow and a decrease in 50% of bile flow recovery after ischemia (Kist *et al.,* 2012). As clarified above, bile is produced by secretion of bile acids and other bile essentials from hepatocytes and cholangiocytes, supported by various bile acid transporters (Arrese & Trauner, 2003; Boyer, 2003). There is no direct evidence regarding the mechanism of reduced bile flow by rapamycin. However, different mechanisms by which rapamycin might inhibit bile flow indirectly can be recognised where bile acid transporters involve. These likely mechanisms are described below:

Rapamycin may decrease bile acid synthesis

It has been reported that rapamycin is a competitive inhibitor of CYP27A1 activity (Gueguen *et al.,* 2007), a mitochondrial cytochrome P450 enzyme, which is expressed in the liver and in many extra hepatic tissues (Rennert *et al.,* 1990; Bjorkhem *et al.,* 1994). However, CYP27A1 is regulated by two other enzymes, Cyp7a1 and Cyp8b1,

key enzymes involved in bile acid synthesis. It is unknown whether rapamycin inhibits Cyp7a1 and Cyp8b1, however taurine & glycine conjugated bile acids are produced by the regulation of these two enzymes (de Aguiar Vallim *et al.*, 2013; Bodman *et al.*, 2013).

Rapamycin may decrease cholangiocyte function

Blocking of STAT3 by rapamycin was associated with impaired cholangiocyte regeneration in cold ischemia reperfusion (Chen *et al.*, 2010). An *in vivo* study in pigs supports the hypothesis that rapamycin impairs cholangiocyte regeneration (Kahn *et al.*, 2005). Perhaps this mechanism provides an explanation as to why decreased bile flow was found in recovery by rapamycin after ischemia reperfusion injury and not in baseline bile flow. Inhibition of STAT3 by rapamycin might outbalance its induction by ROS, hereby aggravating cholangiocyte dysfunction during ischemia reperfusion.

Rapamycin induces expression of antioxidant HO-1 and Prx-1 and this in turn may inhibit bile acid transporter

Nrf2 is a key regulator of the expression of antioxidant enzymes (HO-1 and prx-1) and several bile acid transporters (Weerachayaphorn *et al.*, 2009; 2012; 2014; Zollner *et al.*, 2010). Rapamycin may activate the same pathway to express these antioxidant enzymes and bile acid transporters; hence, it is hypothesised that Nrf2 activation by rapamycin changes bile acid transporter expression, and thereby, bile flow.

Inhibition of bile flow is not due to a direct effect of rapamycin on bile acid transporters

Intracellular accumulation of bile salts can lead to reduced bile flow, hepatocyte apoptosis and progressive liver damage, regulatory mechanisms that can co-ordinate the expression of genes encoding bile acid transport proteins and enzymes (Weerachayaphorn *et al.*, 2014; Chawla *et al.*, 2001).

Rapamycin may change bile composition

Rapamycin may predispose to reduce bile flow due to change in bile composition (e.g. more sludging, possibly altered bacterial flora with increased biofilm formation) in the post-operative inflammatory milieu (Tabibian *et al.*, 2013; Aranha *et al.*, 2008).

Rapamycin may alter bile acid transporter expression

Rapamycin decreased Oatp1 and Mrp2 expression in rat liver (Bramow *et al.*, 2001). Rapamycin also inhibited Ntcp and Oatps expression *in vitro* (Picard *et al*., 2011; Oswald *et al*., 2010). In a cholestasis animal model, rapamycin inhibited bile acid transporter expression (Bramow *et al.*, 2001). Thereby, it is postulated that rapamycin may change bile acid transporter expression, thereby, directly inhibiting bile production. However, it is still unclear which bile acid transporters are responsible for reducing bile flow during ischemia reperfusion injury treated by rapamycin in normal liver or HCC liver and which mechanisms are involved.

1.7.6 Intracellular signalling pathways regulated by rapamycin in hepatocytes

Under normal physiological conditions, PI3K is activated by insulin receptor substrate 1 (IRS1). PI3K phosphorylates phosphotidylinositol (4,5)-biphosphate (PIP-2), which is converted to phosphatidylinositol (3,4,5)-triphosphate that activates the phosphorylation of serine/threonine protein kinase (PKB/AKT), leads to activate mTORC1. Then activated mTORC1 leads to phosphorylate its downstream target 70S ribosomal protein S6 kinase (S6K1) which is presumed to extend the G_1-phase (cell proliferation) by reduction of ribosomal protein synthesis and interruption of transcriptional process. Concomitantly, phosphorylation of S6K1 by mTORC1 generates a negative feedback loop that attenuates phosphorylated S6K1 through PI3K signaling, leading to mTORC1 inhibition (Fig 1.13) (Ashwarth and Wu, 2014).

mTORC1 activates its downstream target 70S ribosomal protein S6 kinase (S6K1) by phosphorylation and proliferates cell growth (Sehgal, 2003). Rapamycin has been shown to inhibit mTORC1 by dephosphorylation, thereby disrupting S6 kinase 1 and inhibiting cell proliferation (Fig. 1.12).

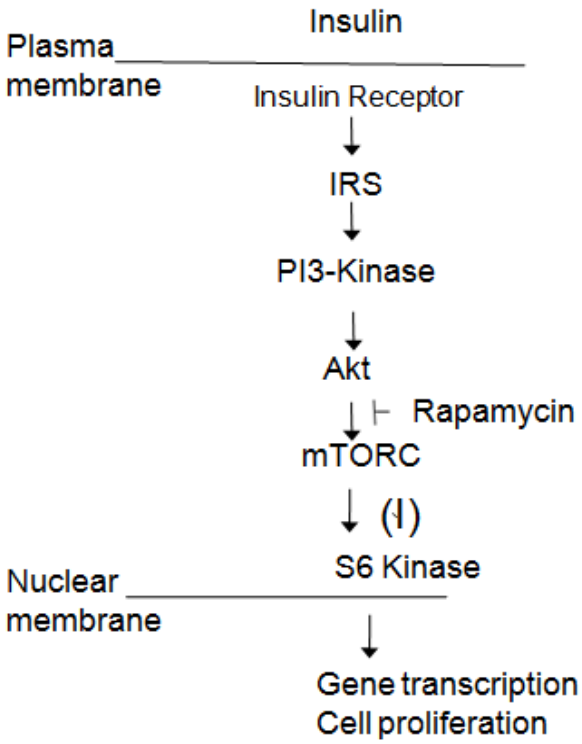


Figure 1.12: Proposed intracellular signaling pathways for regulation of cell growth caused by rapamycin. Rapamycin inhibits mTORC1 by dephosphorylation, thereby disrupting S6 kinase 1 and inhibit cell proliferation. The symbols represent PI3K- Phosphoinositadyl 3 kinase; mTORC1- Mammalian Target of Rapamycin Subunit C1. This figure has been compiled from Kist *et al.*, 2012.

It has been well established that Nuclear factor-E2-related factor (Nrf2) is the chief transcription factor that controls expression of antioxidant enzymes (Prestera *et al.*, 1995) and several bile acid transporters including Mrps, Bsep, Ntcp (Weerachayaphorn *et al.*, 2012) and some are engaged in cellular defense against oxidative stress (Zollner *et al.*, 2010). ROS activates Nrf-2 which translocates to the nucleus (Soriano *et al.*, 2009), thereby activating antioxidant-responsive element (ARE) (Zoncu *et al.*, 2011; Visner *et al.*, 2003; Mann *et al.*, 2007) (Fig 1.13). ROS activates PKC that leads to activate MAPK cascade to dissociate Keap1 from Nrf2, which allows translocation Nrf2 to the nucleus, leads to bind transcription factor ARE (Kim *et al.*, 2014; Hu *et al.*, 2013) (Fig. 1.13).

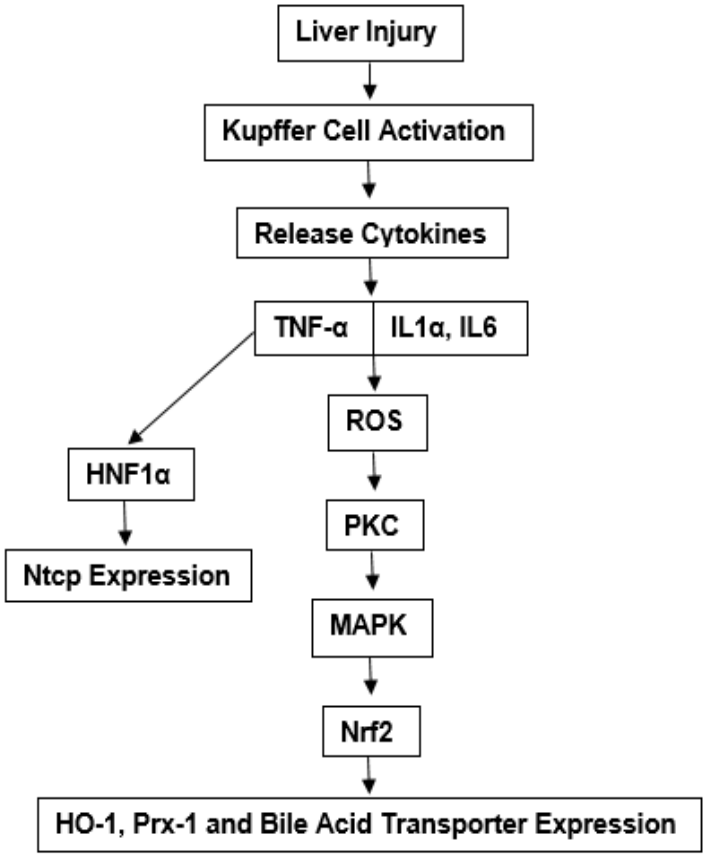


Figure 1.13: Proposed intracellular pathway to induce antioxidant enzyme and bile acid transporter expression by liver injury. ROS activates PKC that leads to activate MAPK cascade to dissociate Keap1 from Nrf2, which allows translocation Nrf2 to the nucleus, leads to bind transcription factor ARE. This figure has been compiled from Kist *et al.*, 2012; Zoncu *et al.*, 2011, Kim *et al.*, 2014.

It is presumed that rapamycin has been shown to inhibit mTORC1 by dephosphorylation, thereby disrupting S6 kinase 1 mediated feedback inhibition of PI3-K (Kist *et al.*, 2012; Ashwarth and Wu, 2014) (Fig 1.14). This leads to increased activity of Akt phosphorylation that activates Akt to dissociate Keap1 from the Keap1-Nrf2 complex. Nrf2 then translocates to the nucleus and binding to ARE in the promoter of the antioxidant (e.g. HO-1, Prx-1) (Kist *et al.*, 2012) and bile acid transporter (e.g. BSEP, MRPs, NTCP) genes and activating transcription (Weerachayaphorn *et al.*, 2009; 2012) (Fig. 1.14).

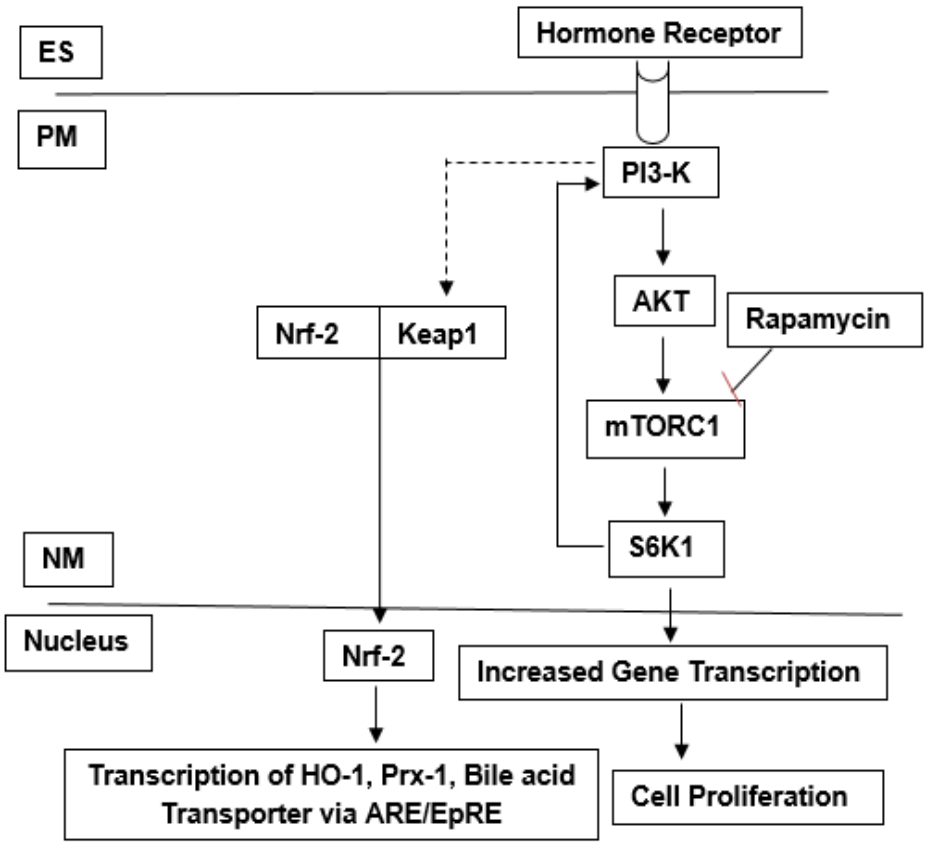


Figure 1.14: Proposed signaling pathways for regulation by rapamycin of HO-1 and BA transporters expression. Rapamycin inhibits mTORC1 by dephosphorylation, thereby disrupting S6 kinase 1 mediated feedback inhibition of PI3-K. This leads to increased activity of Akt phosphorylation that activates Akt to dissociate Keap1 from the Keap1-Nrf2 complex. Nrf2 then translocates to the nucleus and binding to ARE in the promoter of the antioxidant and bile acid transporter genes and activating transcription. This figure has been compiled from Kist *et al*., 2012.

As described above, Nrf2 is a potential regulator of HO-1 and Prx-1 expression (Prestera *et al.*, 1995). However, it is documented that PI3-K/PKB pathway can regulate Ntcp expression (Webster & Anwer, 1999) and Nrf2 is a key controller of bile acid transporter expression (e.g. Bsep, Mrp2, Ntcp) (Weerachayaphorn *et al.*, 2009; 2012, 2014). Thus, it is presumed that this same pathway may be involved in the expression of antioxidant (e.g. HO-1 and Prx-1) and bile acid transporters (e.g. Ntcp, Bsep).

1.8 Oltipraz as a potential anticancer agent

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] has been widely studied as a cancer chemoprotective agent (Bolton *et al.*, 1993; Jacobson *et al.*, 1997; Wang *et al.*, 1999., Kang *et al.*, 2012). It is presumed that oltipraz acts as a chemoprotective agent

against aflatoxin B1 induced hepatocarcinogenesis (Langouët *et al.*, 1995). In China, oltipraz is currently in clinical trials as chemoprotective agent for the prevention of hepatocellular carcinoma (HCC) (Kensler *et al.*, 1998; Ko *et al.*, 2006). It has an inhibitory effect on the growth of pulmonary adenomas and forestomach cancers (Brooks *et al.*, 2009; Wattenberg *et al.*, 1986). Oltipraz has a therapeutic effect on cirrhotic liver (Kang *et al*, 2002; Cho *et al.*, 2006).

1.8.1 Properties of oltipraz

Oltipraz acts as a potent activator of Nrf2 that leads to upregulated antioxidant enzymes, phase II-detoxifying enzymes, canalicular bile acid transporters (e.g. Mrp2) and drug metabolites in liver (Helzlsouer and Kensler, 1993; Johnson *et al.*, 2002; Kensler *et al.*, 2003; Weerachayaphorn *et al.*, 2009). Synthetic oltipraz was developed for the treatment of schistosomiasis (Bae *et al.*, 2005). It is found among members of the *cruciferae* plants (Kensler and Helzlsouer, 1995). Several studies reported that oltipraz stimulates regeneration of damaged liver, and exhibits beneficial effects such as cell differentiation, antifibrosis and cytoprotection (Cho *et al.*, 2009; Huh *et al.*, 2004; Matsuda *et al.*, 1997; Ueki *et al.*, 1999).

1.8.2 Metabolism of oltipraz

Derivative of oltipraz

In mammals, oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione] has four distinct oxidized derivatives (Fig. 1.15). These four derivatives are M1: 4-methyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiol-3-one; M2: 7-methyl-6,8-bis-(methylthio)-pyrrolo-[1,2-α]-pyrazine; M3: 7-methyl-6,8-bis-(methylthio)*H*-pyrrolo [1,2-α]-pyrazine; M4: 7-methyl-6,8-bis(methylsulfinyl)*H*-pyrrolo [1,2-α]-pyrazine (Ko *et al.*, 2006).

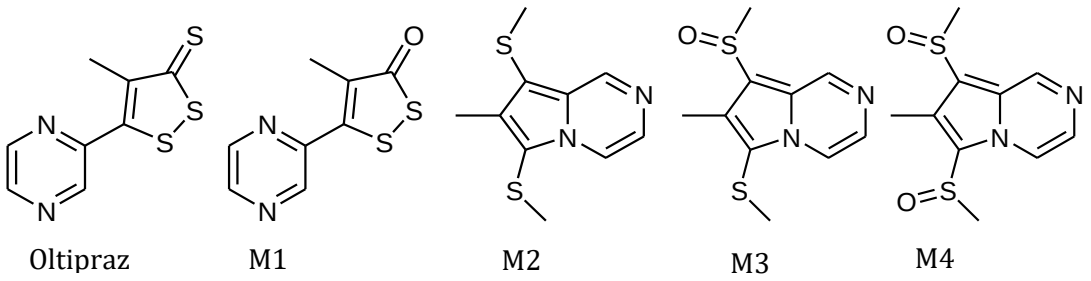


Figure 1.15: Struture of oltipraz and its derivatives. M1: 4-methyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiol-3-one; M2: 7-methyl-6,8-bis-(methylthio) pyrrolo-[1,2-α]-pyrazine; M3: 7-methyl-6,8-bis-(methylthio) *H*-pyrrolo [1,2-α]-pyrazine; M4: 7-methyl-6,8-bis(methylsulfinyl)*H*-pyrrolo [1,2-α]-pyrazine.

Metabolism

Oltipraz and its derivatives are metabolised by different pathways and these four derivatives have distinguished properties (Kang *et al.*, 2012; Ko *et al.*, 2006). Firstly, oxidative desulfuration of the thione group of oltipraz (oltipraz, [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione]) yields 4-methyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiol-3-one (M1), which has no record to be metabolized again. Secondly, desulfuration, methylation, and molecular rearrangement yields 7-methyl-6,8-bis(methylthio)pyrrolo [1,2-α]-pyrazine (M2), which can be further metabolized to other oxidized forms, 7-methyl-6,8-bis(methylthio)*H*-pyrrolo [1,2-α]-pyrazine (M3) and 7-methyl-6,8-bis(methylsulfinyl)*H*-pyrrolo [1,2-α]-pyrazine (M4) (Bieder *et al.*, 1982; O'Dwyer *et al.*, 1997; Kang *et al.*, 2012).

1.8.3 Anticancer actions of oltipraz

Oltipraz has been shown to inhibit the development and progression of a variety of carcinogen-induced rodent tumors of multiple organ sites, including the breast, bladder, colon, stomach, liver, fore-stomach, tracheal, lymphnodes, lung, pancreas and skin (Weerachayaphorn *et al.*, 2014; Clapper, 1998). Oltipraz is effective in resolving accumulated fibres and regenerating cirrhotic liver in animal models. It can reduce the number and volume of cirrhotic nodules and can eliminate accumulated extra cellular matrix (ECM) (Kang *et al.*, 2002 a. b). Moreover, oltipraz can effectively induce antioxidant enzymes. Therefore, it is hypothesised that induction of protective enzyme gene expression by using oltipraz may be a feasible approach in preventing cancer (Kang *et al.*, 2002). Using oltipraz chronically in HCC patients may cause liver damage by a cholestatic effect but this possibility has not been clinically examined.

1.8.4 Intracellular signaling pathways

Oltipraz is a potent activator of the transcriptional factors Nrf2 and C/EBPβ (CCAAT/enhancer binding protein β) (Kang *et al*., 2002.a). After entering the cell cytoplasm, oltipraz is oxidized and produces four derivatives M1, M2, M3 and M4 (Fig. 1.16). The oltipraz derivatives M1 and M2 directly promote nuclear translocation of transcriptional factor C/EBPβ (CCAAT/enhancer binding protein β) and activate C/EBPβ binding to the promoter of target gene, that may lead to the expression of phase II gene (e.g. HO-1, GSTs, γ-glutamylcysteine, quinine reductase) (Kang *et al.*, 2003; Ko *et al.*, 2006). The oltipraz derivative M2 also can activate another transcriptional factor Nrf2 by dissociating Keap1 blocking PI3K-p70S6 kinase pathway (Fig. 1.16) (Cho *et al.*, 2009). This dissociated Nrf2 translocates to the nucleus and promotes Nrf2 binding to the promoter region of target gene EpRE/ARE which may lead to the expression of phase II genes including HO-1, Prx-1 and some bile acid transporters (Bsep, Mrp2) (Ramos-Gomez *et al.*, 2001; Ko *et al.*, 2006). However, oltipraz can block the PI3K pathway by inhibition of PI3K or p70S6 kinase (Cho *et al.*, 2009). Oltipraz increases liver regeneration after PH (partial hepatectomy) through the stimulation of C/EBPβ and increased cyclin E expression, that are regulated by the PI3K-p70S6 kinase pathway (Cho *et al.*, 2009). To date, there are no reports that M3 and M4 can activate any transcriptional factor (Ko *et al.*, 2006).

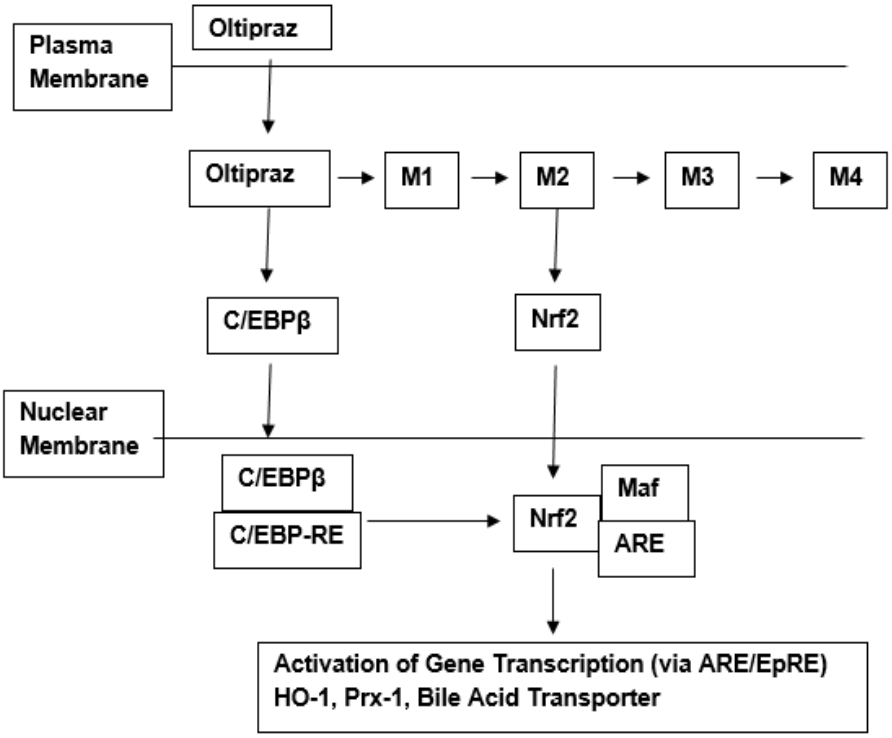


Figure 1.16: Proposed intracellular mechanism of antioxidant and bile acid transporter expression by oltipraz. Oltipraz is oxidized to M1 or M2. M2 is then again biotransformed to M3 or M4. M1 promotes C/EBPβ activation, and M2 activates both Nrf2 and C/EBPβ for the gene induction. This figure has been compiled from Ko *et al.*, 2006.

Oltipraz can also proliferate liver cells via cyclin E by activating directly transcription factor C/EBPβ and (Cho *et al.*, 2009; Ko *et al.*, 2006) (Fig. 1.16). Oltipraz can bind to platelet-derived-growth factor-beta receptor (PDGFβR) that can activate Ras and subsequently phosphorylate mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK). Phosphorylated MAPK activates its downstream target the phosphatidylinositol 3-kinase (PI3K)/Akt/70S ribosomal protein S6 kinase 1 (p70S6K1) signal pathway (Weerachayaphorn *et al.*, 2014). This activated p70S6K1 stimulates transcription factor CCAAT/enhancer binding protein β (C/EBPβ) which can activate cyclin E that enhances cell proliferation (Cho *et al.*, 2009).

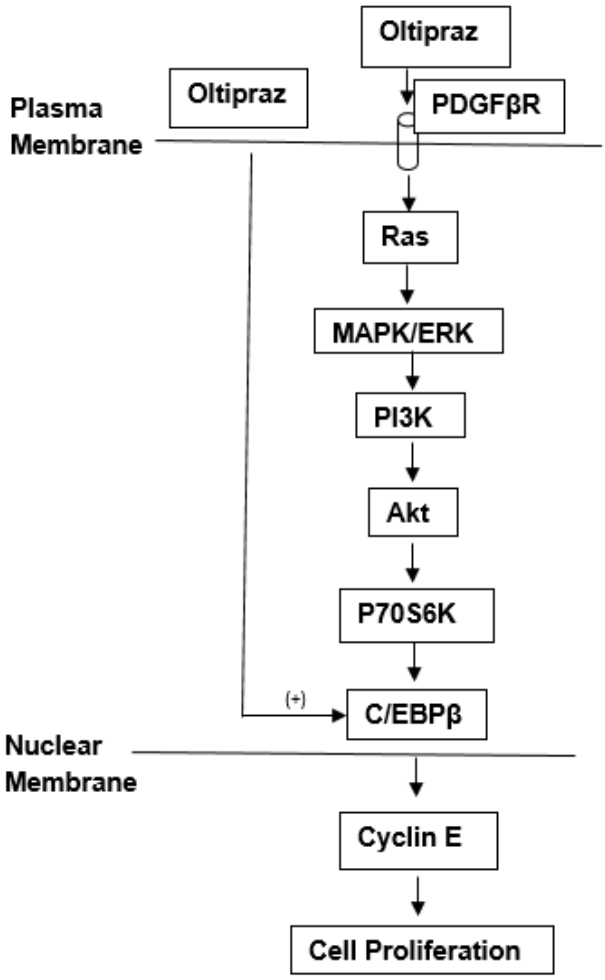


Figure 1.17: Proposed intracellular signaling pathways for regulation of cell growth caused by oltipraz. This figure has been compiled from Cho *et al.*, 2009.

1.8.5 The possibility of redox balance by oltipraz during ischemia reperfusion injury

As described above, oltipraz can regulate antioxidant gene expression by inducing phase 2 enzymes (Kang *et al.*, 2003) and/or the manganese superoxide dismutase gene (an antioxidant enzyme in the mitochondrial matrix that protects cells from oxidative damage) (Antras-Ferry *et al.*, 1997). The oxidized form of oltipraz M1 and M2 can induce down-regulation of ROS production by activating AMPK (Shin and Kim, 2009), eliciting mitochondrial dysfunction (Kwon *et al.*, 2009). Thus, oltipraz may reduce hepatic ischemia reperfusion injury by activating AMPK which can prevent ROS-induced apoptosis and necrosis (Peralta *et al.*, 2001). Moreover, pyrrolopyrazine thione (PPD), a metabolic intermediate of the conversion to M2 by oltipraz, is capable of mediating the activity of phase 2 enzymes, interacting with GSH and thereby,

producing oxygen free radicals (Velayutham *et al.*, 2005). It is also noticeable that PPD can downregulate the antioxidant activity of *cyt c* and thus, induces ROS levels in mitochondria (Velayutham *et al*, 2007) which can indirectly enhance phase 2 enzymes via mitochondrial ROS generation and Nrf2 activation. Thus, oltipraz may act as a cytoprotective agent during ischemia reperfusion injury.

1.9 H4IIE cell as a cellular model of HCC

For the increased number of HCC patients, there is a strong consensus to develop relevant cell lines, animal models, and biomarkers which will facilitate precise translation from preclinical research to clinical practice for the development of treatments for HCC (Dalton and Friend, 2006). *In vitro* examination of HCC cell lines is, in general, an early step of anticancer drug development that involves evaluating cell proliferation, viability, apoptosis and clonogenicity (Bagi and Andresen, 2010; Tomuleasa *et al.*, 2010). The major portion of this investigation depends on the effect of rapamycin and oltipraz for the treatment of advanced HCC patients. Therefore, the H4IIE cell line was selected as a model of HCC cells in this study.

The H4IIE cell line, derived from the Reuber hepatoma H-35 (Reuber, 1961) by Pitot and co-workers (Pitot *et al.*, 1964), is a continuous cell line and is widely used for different purposes because of its unique characteristics (Tillitt *et al.*, 1991; White *et al.*, 2004; Willett *et al.*, 1996; Wätjen *et al.*, 2005; Zhou *et al.*, 2014; Yasui *et al.*, 2010; Niering *et al.*, 2005; Lejeune *et al.*, 2002; Kaisarevic *et al.*, 2015; Dutta *et al.*, 2011). H4IIE cell lines are fast dividing cells that share same basic molecular and functional characteristics including expression of bile acid transporters, enhanced DNA repair and capability to adapt and balance their own differentiation and self renewal capacity along with genetic constraints and environmental stimuli (Zhu *et al.*, 2007; Faivre *et al.*, 2007, Tomuleasa *et al.*, 2010; Dalerba *et al.*, 2007; Clarke and Fuller, 2006; Wege and Brummendorf, 2007). Apart from excellent growth characteristics and low basal cytochrome P4501A1 activity, the H4IIE cells have an inducible AHH (aryl hydrocarbon hydroxylase) enzyme activity that enables this cell line the ability to be used for xenobiotic testing (Tillitt *et al.*, 1991).

However, no cell lines are very sensitive or resistant to imposed treatments (Yang *et al.*, 1992). Although cell culture provides important information about drug efficacy

and mechanisms of action, *in vitro* systems lack the power to recapitulate the complex relationship between the tumor and its microenvironment, including interactions between tumor cells and the organ where the tumor resides, local blood supply and angiogenesis, and the influence of hormones, growth factors and cytokines on tumor growth and survival (Bagi and Andresen, 2010).

1.10 Summary of unresolved questions which have led to the aims of this project

In the published work of others including Kist *et al*., 2012 there is some evidence that rapamycin can induce HO-1 and Prx-1, and as discussed this might be beneficial in reducing ischemia reperfusion injury. However, the results of Kist *et al*. are limited and are unconvincing. Therefore, since there is a possibility that pre-treatment with rapamycin can protect against ischemia reperfusion injury, it is worthwhile to investigate systematically the actions of rapamycin in inducing HO-1 and Prx-1 especially the onset time and degree of induction, and dose needed.

As discussed above, action of rapamycin on liver leads to reduction in basal bile flow and in bile flow recovery after ischemia reperfusion injury. Such a reduction can indicate poor liver recovery from surgery and is detrimental. Therefore, as rapamycin is used in a number of ways to treat liver patients, it is important to clearly define its effects on bile flow and to elucidate the mechanisms of action.

It is thought that changes in mRNA expression i.e. transcription of bile acid transporters are the most likely explanation for the Alwine Kist observations (pretreatment of rapamycin reduced bile flow recovery after warm ischemia reperfusion), because bile acid transporters have a direct effect on bile acid transport compared to indirect other mechanisms.

As described above in untreated HCC patients, there is a significant proportion of liver that is tumor and properties of the tumor cells are very different from normal cells. Therefore, it is important to know whether the actions of rapamycin on HO-1 induction and on bile flow are the same or different in liver tumor tissue. This is particularly important for patients receiving rapamycin treatment over the long term.

Finally, there is little doubt that the induction of antioxidant enzymes before liver surgery could significantly reduce ischemia reperfusion injury. There are many agents that might achieve this but apart from rapamycin, none can be readily applied clinically. As discussed oltipraz can, among other things, activate Nrf2 and hence, induce antioxidant enzyme expression. Therefore, it is a good potential candidate. However, the clinical trials for oltipraz have been in progress for some time suggesting there may be some problems with use of this drug. Therefore, it is important to determine the conditions under which oltipraz can induce antioxidant expression, and to determine whether there are side effects, particularly on bile flow.

These considerations and questions have led to the aims that are stated in the next section.

1.11 Aims

The specific aims of this investigation are:

1. To characterize the abilities of rapamycin to induce the expression of heme oxygenase 1 and peroxiredoxin 1 in normal hepatocytes.
2. To determine the effects of rapamycin on expression of bile acid transporters in normal hepatocytes.
3. To characterize the abilities of rapamycin and oltipraz to induce expression of heme oxygenase 1 and peroxiredoxin 1 in transformed liver cells.
4. To determine the effects of rapamycin and oltipraz on expression of bile acid transporters in transformed liver cells.

To achieve aim one (1) and two (2), rat liver was subjected to rapamycin pre-treatment *in vivo*, and isolated cultured rat hepatocytes were incubated with rapamycin, respectively. The rat liver *in vivo* was subjected to ischemia (~60 min) and reperfusion (~90 min) by continuous clamping protocol done by Alwine Kist and described in the Materials and Methods chapter (Fig 2.1). This technique was shown to cause ischemia reperfusion injury in the superior lobes without affecting the inferior lobes (Nieuwenhuijs *et al.*, 2007; Wilson *et al.*, 2011). Therefore, tissue from the inferior lobe was used as the control in these experiments. The isolated rat liver cells were cultured and used for investigating the effect of different doses of rapamyin. Cultured rat hepatocytes allow the direct testing of rapamycin using different doses and time courses. RT-qPCR was used to investigate mRNA expression because changes in

mRNA levels strongly correlate with changes in protein level (Vogel and Marcotte, 2012). In addition, this method is highly sensitive and has higher throughput to measure primarily the drug activity on gene expression (Grube *et al*., 2015). However, in many cases, the mRNA level of a certain gene is not positively correlated with its protein level because regulation of gene expression is tightly controlled at the levels upstream of translation and multiple post-translational modifications can profoundly affect the enzymatic activities and interaction modes of many proteins. The data of HO-1 and Prx-1 expression will be presented in Chapter 3, and then followed by bile acid transporter expressions in Chapter 4.

To achieve aim three (3) and four (4) H4IIE cells were used as a model of HCC rat liver cells. For these studies, H4IIE cells without treatment were used as control. The relative gene expression of HO-1, Prx-1 and bile acid transporters were measured by using RT-qPCR. The data of HO-1 and Prx-1 expression will be presented in Chapter 5, and then followed by bile acid transporter expression in Chapter 6.

CHAPTER II: MATERIALS AND METHODS

2.1 MATERIALS

The rat H4IIE liver (CRL-1548) cell line was obtained from The American Type Culture Collection (ATCC, Rockville, MD, USA); collagenase (Type IV) was obtained from Worthington Biochemical Corporation; Dulbecco's Modified Eagles Medium (DMEM), agarose, EDTA, glucose, glycine, phenol, sodium bicarbonate, triton X-100, trizma base, TBE (10X) buffer were from Sigma-Aldrich (NSW, Australia); foetal bovine serum (FBS), antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/mL), DNase enzyme, RNase Out ribonuclease inhibitor, M-MLV reverse transcriptase, dNTP set, accuPrime taq DNA polymerase high fidelity, TRIzol reagent and UltraPure distilled water were from Invitrogen (Mt Waverley, Victoria Australia); All primers and probes were from GeneWorks (Adelaide, Australia); rapamycin was from LC Laboratories (Woburn, MA, USA) and oltipraz (LKT Lab.Inc.) were used.

2.2 METHODS

2.2.1 Culture of H4IIE rat liver cells

Subculture

H4IIE rat liver cells (Darlington, 1987) were cultured in 75cm^2 sterile flasks in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (0.1 mg/mL) and 10 mM HEPES in 5% (v/v) CO_2 (pH 7.4) at 37^{o}C (Claire *et al.*, 2015). Once a week the cells were subcultured to another 75cm^2 flask. Subculture was performed under sterile conditions by removing culture medium, washing cells 3 times with phosphate buffered saline (PBS) (The PBS solution contained mM: NaCl, 136; KCl, 4.7; KH_2PO_4, 1.3; Na_2HPO_4, 3.2; adjusted to pH 7.4 with NaOH) followed by addition of 1.0ml of the trypsin (0.25% w/v) plus 0.5ml EDTA (1mM), and left for 2-4 min at 37^{o}C to detach the cells. Once the cells were de-attached, 9.0ml of new DMEM was added and the cells were resuspended by pipetting up and down. Finally, 2ml of the suspension was transferred to another flask in which 8.0ml of fresh DMEM was added before. After 48

h the medium was replaced by fresh DMEM (DMEM was used for no longer than 1 month and was stored at 4^{o}C) in the same flask. The cells were used for 25-30 passages in order to preserve the morphological /functional characteristics of the cellular line.

Incubations for PCR

For every treatment, cells were split into 4 to 8 groups according to experimental design and all the groups had three replicates. Drug treatment groups were compared with the control and vehicle group. The H4IIE cells were grown in sterile T-75 tissue culture flasks. The day before the experiments, H4IIE cells were split and a number of 2.5 x 10^6 cells were cultured into T-25 flask. The prepared doses were administered into 1-day old T-25 cell culture flasks and doses were prepared just before using. After 24 h, fresh culture medium was replaced and drugs (rapamycin and oltipraz) were added. Flasks were incubated for 36 hours in 5% (v/v) CO_2 at 37^{o}C. H4IIE cells were then harvested by collagenase and washed three times with 5 ml PBS. Then 1 ml Trizol was added and kept at -80^{o}C before using for the next step.

2.2.2 Differentiation of H4IIE rat liver cells

H4IIE rat liver cells were cultured in T-25 sterile flasks in DMEM supplemented with fetal bovine serum (10% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), insulin (100 nM) and dexamethasone (100 nM) for 5 to 10 days in 5% (v/v) CO_2 (pH 7.4) at 37°C until they showed the signs of differentiation as described previously (Aromataris, Roberts, Barritt, & Rychkov, 2006). Qualitative observation mainly in size and shape was checked by inverted Olympus microscope (CKX41, Tokyo, Japan). When cells were coarse, glittered, round and healthy than control cells, cells were then judged to be differentiated.

2.2.3 Isolation and culture of rat hepatocytes

Animals were given appropriate care and the experimental protocols were conducted according to the criteria outlined in the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes” (National Health and Medical Research Council of Australia). The Flinders University Animal Welfare Committee approval number is 165/02 (i). Animals were obtained from the animal house of Flinders Medical

Centre and were housed at 60 % humidity, 22°C and free access to food and water with a 12-hour light/dark cycle.

Isolation

For the preparation of isolated primary hepatocytes, Zucker rats were anaesthetised in the fume hood with fluothane and then injected immediately with a combination of Ketamine (100mg/kg) and Xylazine (8mg/kg). Body temperature was maintained between 36.5°C and 37.5°C using a heating pad and a heating lamp. Warm saline solution (0.9%) was provided in the peritoneal cavity through the process to balance for the intra operative fluid loss and to keep the organs moistened to avoid adhesions. After cannulation of the portal vein, the liver was perfused *in situ* for 15 minutes in the presence of collagenase, and hepatocytes isolated as previously described (Berry *et al.* 1991). At the end of the digestion period, the liver was carefully cut from the rats and chopped up using scissors on ice. After a 10-minute incubation in a water bath, the cells were separated from the liver pieces. 100ml wash buffer was added to the chopped liver, filtered the mixture and centrifuged for 1.25 minutes at 500 rpm. The anaesthetized rats were euthanized and the chest was cut and the aorta was cannulated. Jin Hua isolated rat hepatocytes by following this method and provided for these experiments.

Culture

Isolated hepatocytes were resuspended in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and plated on T-25 flask and incubated at 37ºC in 5% (v/v) CO_2 in air for 4 hours. Culture medium was replaced after 4-hour incubation when cells were attached at the bottom of the flask with DMEM supplemented with penicillin (100 units' ml^{-1}), streptomycin (100 μg/ml), 10% (v/v) fetal bovine serum (FBS), and cells were incubated further for 24 hours before dose administration. For every treatment, hepatocytes were split into 4 groups according to experimental design and all the groups had three replicas. Drug treatment groups were compared with the control and vehicle group. The isolated hepatocytes were cultured into T-25 tissue culture flasks for the treatment. The prepared doses were administered into 6-hour cultured T-25 flasks and doses were prepared just before using. After a 36-hour incubation, hepatocytes were harvested by collagenase and washed three times with PBS. Then 1 ml Trizol was added and kept at -80ºC before using for next step.

2.2.4 Induction of hepatic ischemia reperfusion injury

Rat livers, subject to ischemia and reperfusion as described by Kist *et al.* (Kist *et al.*, 2012) preserved by Alwine Kist in -80^{0}C freezer, were used for this investigation. According to Kist report, male Sprague Dawley rats (250-400 gm) were randomly allocated in two groups: rapamycin group and vehicle group. Two groups were compared to investigate the effectiveness of preconditioning with rapamycin. Rats were subjected to a segmental ischemia (60~70%) of the liver by clamping the hepatic artery and portal vein to the bilateral median and left lateral (superior) lobes (red colour), while the caudate (inferior) lobes and right lateral lobes stayed normal blood flow (blue colour) (Fig 2.1). Following midline laparotomy, cannulation was done in the common bile duct through silicon cannula (ID 0.5mm, OD 1.0mm) and bile was collected from the superior lobes only by introducing cannular proximal to the site of the bile duct bifurcation. Clamping of the blood vessels was continued for 60 minutes subsequent to a 20-minute equilibration period. The hepatic artery and portal vein were separated and then clamped over the branches using a microvascular clamp (micro serrefine 10mm length x 2mm wide, Fine Science Tools, Foster City, CA, USA) leading to the inferior lobes. The liver was inspected before reperfusion. If collateral vessels remain it directs incomplete ischemia. Following 60 minutes of clamping, reperfusion was started by exclusion of the clamp and the bile flow recovery was calculated for 90 minutes' reperfusion period. The liver was then flushed by the portal vein to eliminate all the blood cells, with 10 ml of warm saline solution (0.9%). The rat was then euthanized by heart exsanguinations. The inferior lobes and superior lobes were separately weighed after harvesting of the liver, and the liver samples were randomly collected at about 50 mg for RNA isolation and preserved in liquid nitrogen.

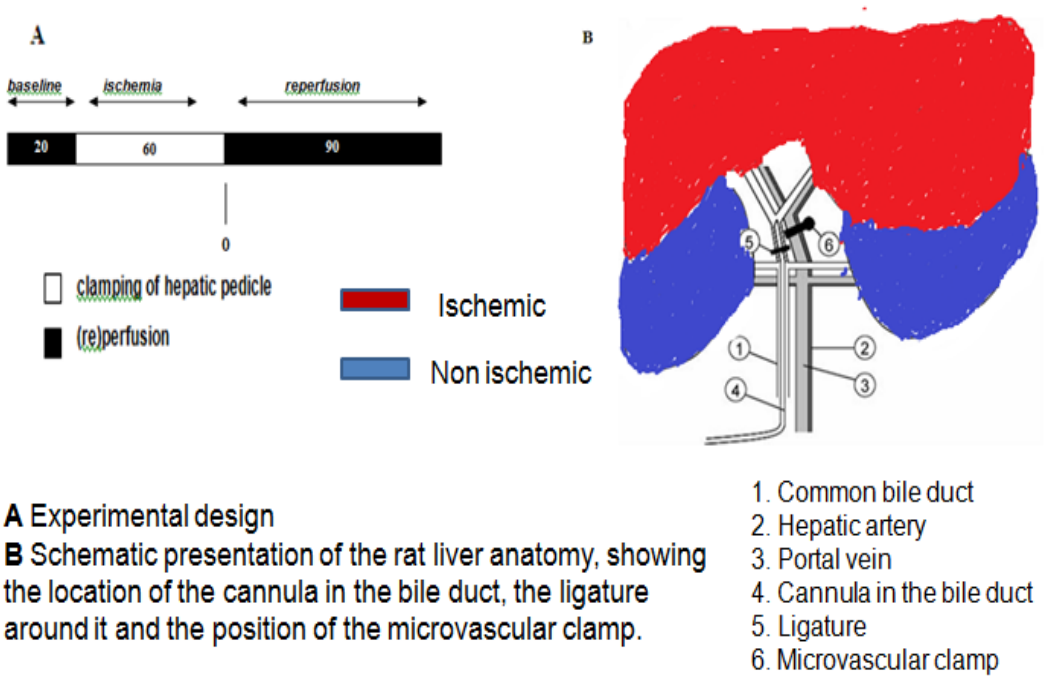


Figure 2.1: Schematic representation of the rat liver *in vivo* subjected to ischemia and reperfusion by continuous clamping protocol. This figure has been adapted from Alwine Kist report.

2.2.5 Preparation of solution

The rapamycin solution comprised 50 mg rapamycin dissolved in 2 ml 100% sterile ethanol. This formed the stock solution concentration of 25 mg/ml, which was stored at -20^{o}C by aliquoting, each aliquot contained 20 µl. Before administration, a working solution was made out of the stock solution. For preparation of the working solution, 2 µl rapamycin (25 mg/ml) was added in 198µl sterile ethanol and made up 100X diluted solution which finally gave 0.273 mM rapamycin. From this working solution, 0.01, 0.05, 0.1, 0.5, 1.0 and 5µM doses were prepared. For 10µM CoPP working solution preparation, a solution (0.1 mM NaOH plus 0.9% NaCl) was prepared and 10mg CoPP was added in the 10ml previously prepared NaOH and NaCl solution which gave finally 1.61 mM concentration. After mixing properly, the pH was adjusted immediately before administration and used without freezing. For 8 ml fresh medium, 50µl gave 10µM concentration of CoPP. Fresh solution was prepared each time. When oltipraz working solution was prepared, 1ml DMSO was added in 10mg oltipraz, stirred properly for 10 minutes, final concentration was 44.2 mM. From this working solution, 12.5, 25, 50, 75, 100, 150µM doses were prepared.

2.2.6 Measurement of cell viability

The number of viable cells was determined by using trypan blue and a haemocytometer (Bright Line, Hausser Scientific, Horsham, PA, USA) (Berry *et al.* 1991). Cells were harvested with PBS-EDTA solution (0.1µM EDTA in PBS) and assayed for cell viability using 1:1 trypan blue staining, with 50 µl of cell suspension and 50 µl of 0.4% (w/v) trypan blue solution. Two drops of cell suspension with 0.4% w/v trypan blue (1:1) were added on the surface of haemocytometer. Then the haemocytometer was placed on microscope stage and the number of unstained (viable) cells and stained (non-viable) cells were counted, as per the methods described by Abcam (2014). Cells were calculated as a percentage of viable cells in the following way:

$$\% \text{ viable cells} = \frac{\text{no of unstained cells}}{\text{total number of cells}} \times 100$$

2.2.7 Molecular Biology

RNA Isolation

H4IIE cells (2.5×10^6) collected from T-25 flasks, isolated cultured rat hepatocytes and excised rat liver were lysed in 1 ml TRYzol reagent (Elliott *et al.*, 1997). Total RNA was purified following the manufacturer's (Invitrogen) protocol which is briefly described. After lysis in 1 ml TRYzol reagent, tubes were centrifuged at 12,000g (12500 rpm) for 10 minutes at 4ºC (Elliott *et al.*, 1997). Supernatant was transferred to a fresh 1.7 ml plastic Eppendorf tube, 200 µl chloroform was added into the supernatant. Tubes were vigorously shaken by hand for 15 seconds, then incubated at room temperature for 3 minutes, centrifuged at 12,000g for 15 minutes at 4ºC. Following centrifugation, three layers were formed, the top aqueous phase containing RNA, the middle interphase, and the bottom red, chloroform phase containing DNA and proteins. The supernatant was transferred (~500-600 µl) to a fresh 1.7 ml eppendorf tube, 600 µl of isopropyl alcohol was added and tubes were vigorously shaken by hand for 10 seconds. Then tubes were incubated at room temperature for 10 minutes followed by centrifugation at 12,000g at 4ºC for 10 minutes. The supernatant was decanted and the RNA precipitate was washed with 1 ml 75% (v/v) ethanol (pre-chilled at -20ºC for 1 hour) by briefly vortexing. The tubes were centrifuged at 7,500g (9500 rpm) for 5 minutes at 4ºC. This step was repeated twice for RNA purification. The RNA pellets

were dried in air at room temperature for 3-5 minutes and dissolved in 80 µl RNase free H_2O at 55-60^oC for 10 minutes. The samples were aliquotted into 20µl volumes and stored at -80^oC for later determining RNA concentration and cDNA synthesis.

RNA Quantification

RNA quantification was conducted through the NanoDrop 8000 (Thermo Scientific) by loading 2µl of each sample for each detection (Elliott *et al.*, 1997). The absorbance of the diluted RNA sample was calculated by dividing the UV absorbance at 260 and 280 nm. The nucleic acid concentration was calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration through the machine's own software. The sample was considered pure if the 260 to 280 absorbance ratio was more than 1.8.

$A = \varepsilon Cl$

A = Absorbance at particular wavelength.

C = Concentration of nucleic acid.

l = Path of space cuvette (usually 1 cm).

ε= extinction coefficient (e for RNA is 0.025 $(mg/ml)^{-1}cm^{-1}$

Evaluation of RNA Quality

Maintaining RNA quality and quantity after subsequent RNA isolation is important for the accuracy of real time PCR results. This procedure was done by gel electrophoresis (Magdeldin, 2012; Elliott *et al.*, 1997). The method for evaluating RNA is briefly described below.

The gel container was taped and the desired comb was inserted. 0.5 g (1 % w/v). Agarose was weighed out for a 50 ml 1xTBE (Tris-Borate EDTA) buffer. The suspension was then heated in the microwave until it was clear. The solution was left to cool and 2 µl of GelRed^{TM} was added. The gel was poured into the container without producing air bubbles. Then the container was kept in open air for approximately 30 minutes until it obtained a light grey color. The RNA sample was prepared by mixing 100-500 ng of RNA, 2 µl 6 x gel loading dye and appropriate volume of 1 x TBE buffer to make up total volume of 12 µl. RNA samples were then loaded into the gel and run for 1 hour at 110 V in 1 x TBE buffer before analysis. By running samples on a gel with electrophoresis, the isolated RNA can be checked by ImageMaster VDS-CL

(Amersham Pharmacia Biotech, now GE Healthcare Life Sciences) under the UV light following manufacturer's instructions. The quality of the RNA was ensured after gel electrophoresis, 2 bands showed, similar to the image below (Fig 2.2). The upper one (28s, ribosomal RNA) is usually 2 times thicker than the lower one (18S ribosomal RNA). The absence of bands indicated that the RNA was degraded.

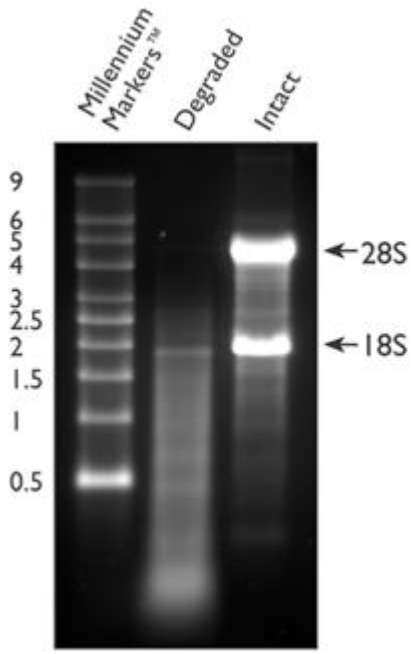


Figure 2.2: Image of RNA band after gel electrophoresis.

DNAse Treatment

The existence of genomic DNA in RNA preparations is a recurrent cause of false positive results in RT-PCR-based assays aimed at gene expression analysis due to PCR sensitiveness. This occurrence cannot be ignored when specific measures in the assay design are applied such as intron-spanning primers design. This situation can be exacerbated when investigated genes present as pseudogenes in the DNA. Therefore, removal of DNA present in the RNA solution by DNase digestion is an essential step. The method is briefly described as in Dotti and Bonin, 2011; Ivarsson and Weijdegård, 1998.

1 µl 10x DNase1 reaction buffer (invitrogen) and 1 µl DNase1 enzyme was added in the 1 µg RNA in 8 µl water, gently homogenized and incubated at room temperature

for 15 minutes. Then 1µl DNase stop solution was added in the mixture, homogenized and heated at 70^{o}C for 10 minutes in order to inactivate DNase1 activity for reverse transcription step. After completing this step, the samples were kept immediately in ice and stored at -80^{o}C for next steps.

cDNA Synthesis

Reverse transcription was carried out on RNA treated with DNase to remove DNA using M-MLV Reverse Transcriptase following the manufacturer's (Invitrogen) protocol which is briefly described below. 1 µl oligo DT (0.5 mg/ml) was added in the 1.0 µg RNA in 11 µl DNase treated mixture, gently homogenised and heated at70^{o}C for 10 minutes to denature RNA and bind oligo DT at the end of RNA. Following heating, the samples were kept in ice immediately for further twisting. 9 µl reverse transcriptase mixture ([0.5 µl of RNAse out, 0.5 µl NFW (nuclease free water), 4 µl 5x M-MLV first strand buffer, 2 µl 0.1 M DTT (dl-dithiotheritol), 1 µl 20mM dNTP's (deoxyribonucleotide triphosphate), 1 µl M-MLV Reverse Transcriptase (200U/µl)]) was added in the 12 µl RNA solution, gently homogenised and incubated at 37^{o}C for 50 minutes. After completion of incubation, the samples were heated to 70^{o}C for 15 minutes for inactivation of M-MLV reverse transcriptase. The samples were cooled in ice without delay and stored at -80^{o}C for subsequent steps.

Conventional Polymerase Chain Reaction (PCR)

The conventional PCR was conducted to confirm for each batch cDNA that cDNA was correctly synthesized during the Reverse Transcription step. Besides this, conventional PCR was also used for correct primer function. PCR is a technique in Molecular Biology where a single copy of DNA is amplified across several orders of magnitude, producing thousands to millions of copies. PCR, developed by Kary Mullis in 1983, is now extensively used in Medical and Biological Sciences for different purposes (Bartlett and Stirling, 2003; Saiki *et al.*, 1985). In principle, PCR consists of three steps (denaturation, annealing and extension/elongation) and 20-40 repeated cycles. In denaturation step, double-stranded cDNA becomes single stranded by the reaction temperature 94 to 98°C for 10-20 seconds. The reaction temperature is then lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded

DNA template. The elongation step is performed at a temperature of 75–80 °C, that depends on taq polymerase activity, for 40-60 seconds (Lawyer *et al.*, 1993).

PCR master mixture was prepared by 5 µl 10 x PCR reaction buffer, 2 µl 5mM dNTPS, 0.25 µl 5U/µl Taq DNA polymerase, 0.9 µl 50µM forward/reverse primer (Table-2.1), 37.85 µl nuclease free water (NFW) for each sample. After PCR master mixture preparation, 45 µl mixture was poured into a PCR tube, 5 µl 10 x diluted cDNA (~0.1 µg) was added which was 50 µl in total volume. Subsequently gentle homogenization, the PCR machine was carried out at the following condition: 1 cycle at 95°C for 10 minutes followed by 35 cycles of 95°C for 20 seconds, 60°C for 60 seconds with a final elongation at 72°C for 60 seconds (Lawyer *et al.*, 1993).

DNA Electrophoresis

This method is briefly described below according to Dotti and Bonin, 2011; Ivarsson and Weijdegård, 1998.

2% (w/v) TBE agarose gel was prepared by dissolving appropriate amount of agarose (1.0 g) into 50 ml of 1 x TBE buffer. DNA sample was prepared by mixing 10 µl 10 x diluted DNA (0.1 µg), 2 µl 6 x gel loading dye and appropriate volume of 1 x TBE buffer to make up total volume of 12 µl. After preparations, DNA mixtures were loaded into the gel and run for 1 hr at 110 V in 1 x TBE buffer. The gel was staining in 3 x GelRed stain (Biotium INC., CA USA) following completion of running for 25-30 minutes before analysis by ImageMaster VDS-CL (Amersham Pharmacia Biotech, now GE Healthcare Life Sciences) under the UV light. Then the image was saved and printed for analysis.

Real Time Q-RT-PCR

Q-RT-PCR was conducted using the Rotor-Gene 3000 (Corbett Life Science, Sydney, NSW, AU), which was a combination of thermocycler and fluorescent detector. Quantitative assessment of tested mRNA expression was performed using β-actin as the housekeeping gene. The nucleotide sequences of each primer (GeneWorks, Hindmarsh, SA, AU) and fluorescent probes (GeneWorks, Australia) were chosen from the published paper which as indicated in table 2.1 The probes were 5’ labelled by a 6-

carboxy-fluorescein reporter (FAM) and 3' labelled by a black-hole quencher 1 (BHQ) or TMRA (Table 2.1). The Efficiency of primers was calculated before conducting qRT-PCR. How the efficiency of primers measured was described later. Both pairs of primers (β-actin and tested genes) had a mean efficiency of 87%. This method is briefly described below according Wilson *et al.*, 2011.

PCR reaction mixture was prepared with 0.1 µl Taq polymerase (5 U/µl), 5 µl of 10 x buffer, 0.8 µl of dNTPs (5 mM), 0.36 µl of primers (sense/antisense), 5 µl of cDNA 10 x or 25 x diluted (0.025 or 0.1 µg), 0.8 µl of probe (5 µM) and appropriate volume of nuclease free water to a final volume of 20 µl. After a denaturing step of 10 minutes at 95°C, 40~45 cycles were performed: 95°C for 15 seconds and 60°C for 60~75 seconds, and ending with 60 seconds at 72°C. Each sample was tested in triplicate and each run contained a positive control (RNA sample from previous successful PCR), a negative control (nuclease free water).

Relative quantification of the tested gene mRNA was performed using the comparative cycle threshold (Ct) method. According to Zhang *et al.*, 2013, results of the Q-RT-PCR data were represented as Ct values, where Ct indicates the fractional cycle number at which the amount of amplified target reaches to a fixed threshold. For the RNA internal control, the Ct value derived from β-actin mRNA expression was used. ΔCt is the difference in number of Q-RT-PCR cycles to reach the threshold for tested gene and β-actin in one tested sample. ΔΔCt represents the difference in ΔCt derived from the tested sample and the Ct of the reference group (control). The relative tested mRNA expression, compared with the control as reference group, was determined by raising 2 to the power of the negative value of ΔΔCt for each sample (Zhang *et al.*, 2013).

This method was used to quantify the amount of cDNA. It was a relative method where PCR products were compared with a reference sample (e.g. from an untreated sample). For this sample, the formula $2^{-\Delta\Delta Ct}$ was put at 1.

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

$\Delta\Delta Ct = \Delta Ct_{\text{Gene of interest}} - \Delta Ct_{\text{Reference}}$

Fold induction = $2^{-\Delta\Delta Ct}$

In this formula, the gene of interest (HO-1, Prx-1, Mrp-2, Bsep, Ntcp, Oatp1, Oatp2) and housekeeping gene (β-actin) were correlated to the reference sample. The ΔΔCt value of this reference was 0, so that $2^{-\Delta\Delta Ct} = 1$.

Primers

Table-2.1: List of sense and antisense primers and probes used in this study

Name	Forward	Reverse	Probe	Expected base pair
1. B-Actin	TGCCCATCTATGAGGGTTACG	CGCTCGGTCAGGATCTTCA	FAM-CTGGCCGGGACCTGACAGACTACCTC-BHQ	100
2. HO-1	CACAGGGTGACAGAAGAGGCTAA	CTGGTCTTTGTGTTCCTCTGTCAG	**FAM**-CAGCTCCTCAAACAGCTCAATGTTGAGC- **BHQ**	160
3. Prx-1	TGTGGATTCTCACTTCTGTCATCTG	TGCGCTTGGGATCTGATACC	**FAM**-CAAGGAGGATTGGGACCCATGAACATTCCC-**BHQ**	101
4. Ntcp	ATGACCACCTGCTCCAGCTT	GCCTTTGTAGGGCACCTTGT	**FAM**-CCTTGGGCATGATGCCTCTCCTC - **TAMRA**	111 (100-350)
5. Oatp1	ACCTGGAACAGCAGTATGGAAAA	ACCGATAGGCAAAATGCTAGGTAT	**FAM**-TACTGCAGAGGCAATATTCCTCATAGGTGTTTATAG	163
6. Oatp2	TGTGATGACCGTTGATAATTTTCCA	TTCTCCACATATAGTTGGTGCTGAA	**FAM**–TGGCAGGCTTAACAACCTCTTATGAAGGG	81<
7. Oatp9	ACGACTTTGCCCACCATAGC	CCACGTAAAGGCGTAGCATGA	**FAM**–CCCTCTACCTGGGAATCCTGTTTGCAAT	117 (100-350)

8. Mrp2	TAGTCTTCGCCG TACACTGAGC	ACATTCACATTTT TAATCTTCAAGG AGTT	**FAM**– CAGCTCTAGGTC CAGCAGCCGAC GT- **BHQ**	93< (100-350)
9. Bsep	CCAAGCTGCCAAG GATGCTA	CCTTCTCCAACA AGGGTGTCA	**FAM-** CATTATGGCCCT GCCGCAGCA- **TAMRA**	78< (100-350)
10. Mdr1a	GCAGGTTGG CTGGACAGATT	GGAGCGCAA TTCCATGGATA	**FAM-** CCGCCAGAGTTC CCAGCAGCATG- **TAMRA**	70< (100-350)
11. Mdr1b	AAACATGGCACGT AACCAAAGTT	AAAATGTGGCCC TGTTTAATGATT	**FAM-** CACTGTTAAAGG TAATTTCATCAA GACGAGAAGCC TTC-**TAMRA**	
12. Mdr2	AGTTCACGGGCGC ATCAA	AAAAGACACTGG TGGCACGTT	**FAM-** CATCAAGTTCAT TGGTTTCCACAT CCAGC-**TAMRA**	75< (100-350)
13. Bcrp	CAGGTAGGCAATT GTGAGGAAGA	AATCAGGGCATC GATCTGTCA	FAM- CATGCAAGCCA GGGCCACATGA- TAMRA	93< (100-350)

List of published papers from where the sequence of primers and probes were obtained. 1, 3 - Wilson *et al*., 2011; 2-Kist *et al*., 2012; 4-Sturm *et al.*, 2005; 5, 6, 7, 8-St-Pierre *et al.*, 2004; 9, 10, 11, 12, 13-Ros *et al.*, 2003.

Determination of qPCR amplification efficiency of target primer

For determination of the qPCR amplification efficiency of each primer, a standard curve (log amount of cDNA versus mean Ct value) for each primer was generated (Zhang *et al.*, 2013). A pool of series dilutions (6.25x, 12.5x, 25x, 50x, 100x, 200x, 400x) were prepared based on the total RNA concentration of control. The qPCR was performed by mixing 5 µl of cDNA (25x dilute), 0.8 µl Probe (5 µM), 0.36 µl For/Rev primer (50 µM), 2 µl $MgCl_2$ (50 mM), 0.8 µl dNTPs (5 mM), 0.1 µl Taq (5 U/ µl), 2 µl 10xBuffer, and appropriate volume of UltraPure distilled water to a final volume of 20 µl and running on Rotor Gene 3000 (Corbett) real time PCR machine. The qPCR condition 1 cycle at 95^{o}C for 10 minutes (initial denaturing), 2-40 cycle at 95^{o}C for 15 seconds

(denature) and 60^{o}C for 1 minute (annealing and extension), 41 cycle at 72^{o}C for 1 minute (ensure finalizing of reaction). The condition for melting curve: ramping from 72^{o}C to 95^{o}C, hold 45 seconds on the 1st step, hold 5 seconds on next steps, Melt A (FAM). Expressed gene products were amplified and quantitated using Taqman Probe Technology (fluorescent labelled probe with quencher attached). Data was analysed using the Comparative CT method ($2^{-\Delta\Delta CT}$) with β-actin as the housekeeping/control gene for comparison of HO-1, Mrp-2 and Ntcp levels between differentiated and undifferentiated H4IIE rat hepatocytes.

2.2.8 Statistical Analysis

Statistical analysis was performed using Prism v5.0 (GraphPad Software). Results are expressed as mean ± SEM (standard error of mean). Student's t-test or ANOVA followed by Post Hoc Tukey's test was used for analyzing RT-PCR data. Means of two groups were compared using Student's t-test and when the treatment groups were more than two, ANOVA was used for comparison the mean value. To determine the correlation between variables and expression of mRNA, a linear regression line was carried out. Statistical significance was considered when P values were less than 0.05.

CHAPTER III: EFFECTS OF RAPAMYCIN ON THE EXPRESSION OF HEME OXYGENASE 1 AND PEROXIREDOXIN 1 IN RAT LIVER *IN VIVO* AND IN ISOLATED CULTURED RAT HEPATOCYTES

3.1 INTRODUCTION

As discussed in the General Introduction, advanced hepatocellular carcinoma (HCC) and metastatic cancer of the liver can only be effectively treated by surgical liver resection or liver transplantation. However, these surgical procedures often result in reduced liver function through ischemia reperfusion injury leading to oxidative stress and patient morbidity death (Kaibori *et al.*, 2014; Germano & Daniele; 2014; Ashworth & Wu, 2014). Ischemia reperfusion injury has been shown to enhance HO-1 and Prx-1 expression in the liver (Wang *et al.*, 2014; Wilson *et al.*, 2011). HO-1 inhibition has been found to increase injury in liver ischemia reperfusion (Wang *et al.*, 2014; Morse *et al.*, 2009; Ferenbach *et al.*, 2010; Devey *et al.*, 2009; Ke *et al.*, 2009). These data suggest that induction of HO-1 and Prx-1 may be protective in ischemia reperfusion injury.

Rapamycin has also been reported to increase HO-1 expression in kidney, lung and heart (Zhou *et al.*, 2006; Esposito *et al.*, 2010; Visner *et al.*, 2003). However, the effect of rapamycin on HO-1 and Prx-1 expression in liver following ischemia reperfusion injury is unclear. In the published work of others including Kist *et al*., there is some evidence that rapamycin can increase HO-1 and Prx-1 in liver and as discussed this might be beneficial in reducing ischemia reperfusion injury. However, the results of Kist *et al*. are limited in extent and incomplete and unconvincing. Therefore, since there is a chance pre-treatment with rapamycin can protect against ischemia reperfusion injury, it is worthwhile to investigate systematically the actions of rapamycin to induce HO-1 and Prx-1, particularly time of onset, degree of induction, and dose needed.

Therefore, the aims of these experiments described in this chapter are:

i. To confirm that rapamycin induces HO-1 and Prx-1 mRNA expression in rat liver.
ii. To determine whether rapamycin acts directly on hepatocytes and induces HO-1 and Prx-1 mRNA expression in hepatocytes, and
iii. To determine the doses of rapamycin needed to achieve these aims.

To achieve this goal (i) rat liver subjected to rapamycin pre-treatment *in vivo*, and (ii) isolated cultured rat hepatocytes incubated with rapamycin were used. The rat liver *in vivo* subjected to ischemia (~60 min) and reperfusion (~90 min) by continuous clamping protocol done by Alwine Kist (Fig. 2.1) and isolation of rat hepatocytes were described in details as in Materials and Methods Chapter. The data of HO-1 expression will be presented first, and then followed by Prx-1 expression.

3.2 RESULTS

3.2.1 Effects of rapamycin on HO-1 and Prx-1 mRNA expression in rat liver

To understand the effect of rapamycin on HO-1 and Prx-1 expression in the presence and absence of ischemia reperfusion injury, rat liver *in vivo* after pre-treatment of rapamycin for 24 hours was used. Results are shown in Fig. 3.1.

1. Ischemia reperfusion group

In the absence of pre-treatment with rapamycin, ischemia reperfusion caused a 4-fold increase in HO-1 (A) and Prx-1 (B) mRNA expression (Fig. 3.1, non-ischemia reperfusion vehicle compared with ischemia reperfusion vehicle).

2. Rapamycin non-ischemia reperfusion group

In the absence of ischemia reperfusion, rapamycin caused a 5-fold increase in HO-1 (A) and 7-fold increase in Prx-1 (B) mRNA expression (Fig. 3.1, non-ischemia reperfusion vehicle compared with non ischemia reperfusion rapamycin group). The induction caused by rapamycin of HO-1 and Prx-1 mRNA expression is comparable in

magnitude with that induced by ischemia reperfusion (in absence of rapamyin pre-treatment).

3. Rapamycin ischemia reperfusion group

In the presence of ischemia reperfusion, the induction of HO-1 (A) and Prx-1 (B) mRNA caused by rapamycin was not further enhanced by ischemia reperfusion compared to ischemia reperfusion vehicle (Fig. 3.1).

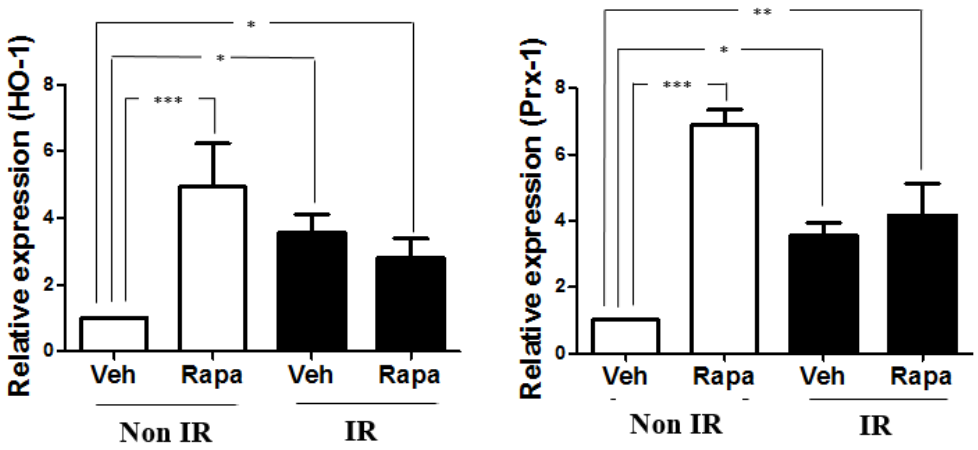


Figure 3.1: Rapamycin induces HO-1 (A) and Prx-1 (B) mRNA expression in rat liver *in vivo*. Relative fold changed (normalized to β-actin expression) in the mRNA expression of HO-1 and Prx-1 pre-treatment by rapamycin for 24 hours in liver compared to livers pre-treated with vehicle. The mean of the vehicle inferior lobes was taken as a reference group. The results are expressed as mean ± SEM (n = 9, 3 individual separate experiments). To compare the relative HO-1 mRNA expression within the groups between the inferior and the superior lobes, the comparison was made using ANOVA with Post Hoc Tukey's test. Degrees of significance for comparison of rapamycin with vehicle are *p<0.05, **p<0.01 and *** P<0.001.

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3.2.2 Effects of rapamycin on HO-1 and Prx-1 mRNA expression in isolated cultured rat hepatocytes

If the increase in HO-1 and Prx-1 expression induced by rapamycin in the intact rat is due to the direct action of rapamycin on hepatocytes, the effects of rapamycin on HO-1 and Prx-1 expression in cultured hepatocytes were investigated. Previous experiments were done with isolated hepatocytes and HO-1 induction was achieved with the doses ranging 0.1-0.55 µM rapamycin for 36 hours (Kist *et al.*, 2012). Therefore, in this present experiment concentrations of rapamycin at 0.1 and 0.5 µM for 36 hours were

initially employed. After RNA extraction, cDNA was synthesised and RT-qPCR was conducted. In the case of HO-1 mRNA expression, rapamycin caused a 3-fold increase at 0.1 µM and a 2-fold increase at 0.5 µM as shown in Fig. 3.2 (A). The Prx-1 mRNA expression was nearly 3- fold at 0.1µM and 2-fold at 0.5µM of rapamycin compared with that of vehicle as shown in Fig. 3.2 (B).

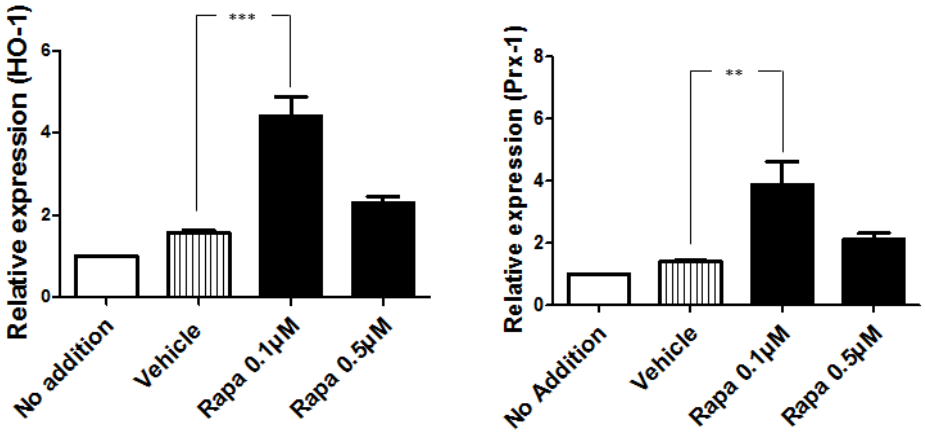

Figure 3.2: Rapamycin induces HO-1 (A) and Prx-1 (B) mRNA expression in isolated cultured rat hepatocytes for 36 hours. Relative fold changed (normalized to β-actin expression) in the mRNA expression of HO-1 and Prx-1 treated by rapamycin compared with vehicle. Results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance were determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with control (**p<0.01 and *** P<0.001).

3.2.3 Dose-response curve for the action of rapamycin on the expression of mRNA encoding HO-1 and Prx-1

To determine the optimum dose of rapamycin for HO-1 and Prx-1 expression further experiments were conducted with varying concentrations (0.0, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 µM) in isolated cultured rat hepatocytes and incubated for 36 hours. Results are shown in Fig. 3.3. As can be seen from Fig. 3.3, the results show a biphasic dose response curve. In the case of HO-1 (A), the rapamycin concentrations which gave half maximal (S0.5 value) induction and inhibition were 0.05 and 0.5 µM respectively. In the case of Prx-1 (B), the rapamycin concentrations which gave half maximal (S0.5 value) induction and inhibition were 0.02 and 0.4 µM respectively.

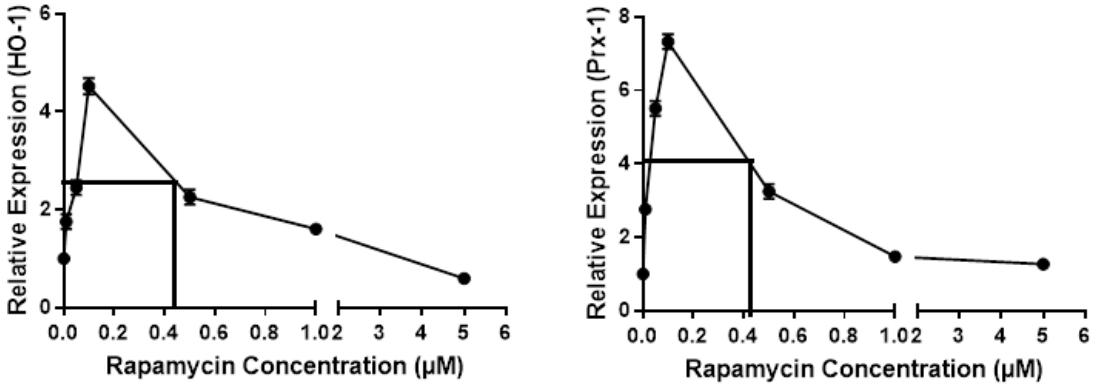

Figure 3.3: Dose-response biphasic curve for the induction and inhibition by rapamycin on the expression of mRNA encoding HO-1 and Prx-1 in isolated cultured rat hepatocytes. This line was achieved by fitting with linear regression equation (variable slope). Relative fold changed (normalized to β-actin expression) in the mRNA expression of HO-1 and Prx-1 treated with rapamycin compared with hepatocytes (no addition). Results are the mean ± SEM (n = 9, three individual experiments).

3.3 DISCUSSION

The aim of this investigation was to confirm the effect of rapamycin on HO-1 and Prx-1 mRNA expression in rat liver *in vivo* and in isolated cultured rat hepatocytes and to determine the doses of rapamycin to achieve this goal. Two main findings were observed in this chapter. Firstly, ischemia reperfusion caused a 4-fold increase in HO-1 and Prx-1 mRNA expression in rat liver *in vivo* (see summary Tab. 3.1). This action was unaffected by rapamycin pre-treatment. Secondly, rapamycin caused a 5-and 7-fold increase in HO-1 and Prx-1 mRNA expression respectively in rat liver *in vivo* and caused a 3-fold increase in HO-1 and Prx-1 mRNA expression in primary cultured rat hepatocytes (see summary Tab.3.1). These results indicate that rapamycin has direct action on hepatocytes. However, the observed dose response curve of HO-1 and Prx-1 was biphasic. This result suggests higher concentration of rapamycin shows decreased expression of HO-1 and Prx-1 mRNA.

Table 3.1: Summary of the effects of rapamycin on antioxidant enzymes mRNA expression in liver (ischemia reperfusion and non ischemia reperfusion liver) and isolated cultured hepatocytes.

Antioxidant enzyme	Effect of IR in liver	Effect of rapamycin in non IR liver (24 hour)	Effect of IR following rapamycin (24 hour) in liver	Effect of rapamycin in isolated cultured hepatocytes for 36 hours
HO-1	4-fold Increased	5-fold Increased	No change	3-fold Increased (Biphasic curve)
Prx-1	4-fold Increased	7-fold Increased	No change	3-fold Increased (Biphasic curve)

3.3.1 Effect of ischemia reperfusion on HO-1 and Prx-1 mRNA expression

Ischemia reperfusion itself also induced HO-1 and Prx-1 mRNA expression. Similar observations were found from other laboratories. Similar to this finding, other laboratories found an increase in HO-1 mRNA expression in liver after ischemia reperfusion injury (Wang *et al.*, 2014; Zeng *et al.*, 2010; Geuken *et al.*, 2005). In this investigation, a 4-fold increase in HO-1 mRNA expression after ischemia reperfusion injury. Compare to Kist *et al.* who found a 2-fold increase in HO-1 mRNA (Kist *et al.*, 2012). a 4-fold increase in Prx-1 mRNA expression was observed in this study. Wilson *et al.* found a 1.6-fold increase in Prx-1 protein expression (Wilson *et al.*, 2011). This result was consistent with the others although protein expression was not looked. But some references also showed that ischemia reperfusion could induce protein expression (Wang *et al.*, 2014; Zeng *et al.*, 2010; Wilson *et al.*, 2011).

Likely Mechanisms for induction of HO-1 and Prx-1 by ischemia reperfusion

The likely mechanism how ischemia reperfusion can increase HO-1 and Prx-1 expression has been described in details in General Introduction (See Fig. 1.13). Here the likely mechanism has been briefly described. Several studies have been reported that liver ischemia reperfusion injury increases the production of ROS (see Fig. 3.4) (Kageyama *et al.*, 2015; Quesnelle *et al.*, 2015; Wang *et al.*, 2014). ROS then activates Nrf-2 which translocates to the nucleus (Soriano *et al.*, 2009), thereby activating antioxidant-responsive element (ARE) (Zoncu *et al.*, 2011; Visner *et al.*, 2003; Mann *et al.*, 2007), leading to increase transcription of HO-1 and Prx-1 (Fig 3.4). ROS also

activates PKC that leads to activation of the MAPK cascade, dissociation of Keap1 from Nrf2, which allows translocation of Nrf2 to the nucleus, and binding of the transcription factor ARE (Kim *et al.*, 2014; Hu *et al.*, 2013) (Fig 3.4).

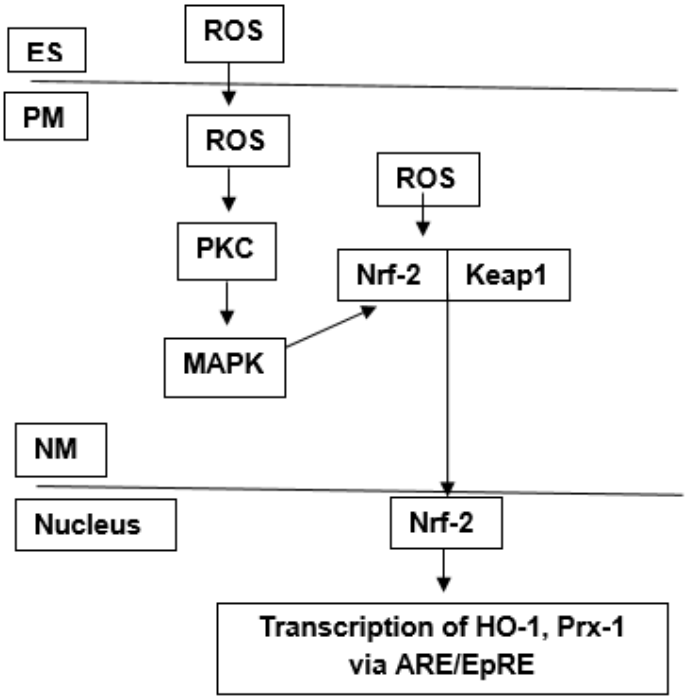

Figure 3.4: Proposed intracellular signaling pathways for regulation of HO-1 and Prx-1 expressions caused by ROS. ROS activates PKC, resulting activated MAPK, leading to dissociated Nrf2 from Keap1 and translocated Nrf2 to nucleus. Then Nrf2 binds transcription factor and starts transcription. The symbols represent ES-Extracellular Space; PM-Plasma Membrane; NM-Nuclear Membrane; ROS-Reactive Oxygen Species; PKC-Protein Kinase C; MAPK-Mitogen Activated Protein Kinase; NRF2-Nuclear Factor-Erythroid 2-Related Factor 2; Keap1- Ketch-like ECH Associated Protein 1; ARE-Antioxidant Response Element.

3.3.2 Effect of rapamycin pre-treatment in normal liver

Rapamycin pretreatment (4 mg/kg b.w. for 24 hour) increased HO-1 and Prx-1 mRNA expression in normal liver. These findings were similar to that of Zhou and colleagues who found that HO-1 mRNA expression was increased more than 3 fold by rapamycin pre-treatment (2 mg/kg) in lung (Zhou *et al.*, 2006). There was no report found on Prx-1 mRNA expression by rapamycin-pretreatment in liver.

The likely mechanisms for induction of HO 1 and Prx-1 by rapamycin have been described as in the General Introduction (see Fig. 1.14).

3.3.3 Effects of ischemia and reperfusion on the expression of HO-1 and Prx-1 following pre treatment with rapamycin

Rapamycin mediated increase in HO-1 and Prx-1 mRNA expression was not further enhanced by ischemia reperfusion in this study. This was in contrast to some investigators who found that rapamycin further enhanced HO-1 mRNA expression in ischemia reperfuson in other organ (kidney) (Goncalves *et al.*, 2006; Feitoza *et al.*, 2007). However, this was assesed at 24 hours of reperfusion, while this study looked at 60 minutes' reperfusion. Therefore, time course could be an explanation the differences in these results. Another reason could be possible that the induction of HO-1 mRNA expression by rapamycin in hepatocytes will be masked by large levels of basal HO-1 mRNA expression in other liver cells e.g. Kupffer cells when measured in whole liver (Kageyama *et al.*, 2015; Zeng *et al.*, 2010).

Likely mechanisms for induction of HO-1 and Prx-1 by rapamycin-pretreatment in ischemia reperfusion liver

The Rapamycin/Akt/PI3K/Nrf2 pathway might be involved for induction of HO-1 and Prx-1 expression by pre-treaatment with rapamycin in ischemia reperfusion liver which has been described in the General Introduction (see Fig. 1.14). However, results found in this study also suggest that ischemia reperfusion suppresses the induction of HO-1 and Prx-1 expression in raapamycin pre-treated liver. Before surgery there is no difference between superior lobe and inferior lobe. Thus, before the rat is anesthetized a 4-fold induction in HO-1 and Prx-1 mRNA expression in all lobes would be expected. This has then been reduced by the ischemia reperfusion. So, there may be more than one pathway regulating Nrf2 and or HO-1, Prx-1 expression. But, at this time it is unclear exactly which mechanisms might be involved.

3.3.4 Effect of rapamycin in cultured rat hepatocytes

In this investigation, results showed that rapamycin caused a 2-3-fold increase in HO-1 and Prx-1 expression in isolated cultured rat hepatocytes. Compared to Kist *et al.* who showed 7-8-fold increase in HO-1 mRNA expression but no increase in Prx-1 mRNA (Kist *et al.*, 2012).

Likely Mechanisms for induction of HO-1 and Prx-1 by rapamycin in cultured rat hepatocytes

It is thought that the same intracellular signaling pathway might be involved to induce HO-1 and Prx-1 mRNA expression in cultured rat hepatocytes which has been described in the General Introduction (see Fig. 1.14). Induction of HO-1 and Prx-1 by rapamycin in cultured rat hepatocytes indicates that rapamycin does have a direct effect on hepatocytes. Since hepatocytes are about 70% of all liver cells, the increases in HO-1 and Prx-1 mRNA expression in the liver *in vivo* are chiefly due to increase in hepatocytes (Kist *et al.*, 2012; Zeng *et al*., 2010). The other reason why the effect of rapamycin on HO-1 and Prx-1 induction in isolated cultured rat hepatocytes are more than that in liver could be due to rapid metabolism of rapamycin in culture (Kist *et al.*, 2012; Wacher *et al.*, 2002).

3.3.5 Dose response effects of rapamycin in isolated cultured rat hepatocytes

In this study, HO-1 and Prx-1 mRNA expression was increased at low concentrations of rapamycin whereas expression was decreased at higher concentrations of rapamycin. The induction and inhibition of dose response for HO-1 and Prx-1 in hepatoytes are very similar, essentially the same; therefore, same mechanisms may be involved for the regulation of both enzymes. The induction phase is consistent with the mechanism of Akt-Nrf2 which is described in the General Introduction (Fig. 1.14). But what will be the mechanism of inhibition is still unclear. This suggests that at higher concentrations of rapamycin, an additional pathway is activated but what this might be unknown. This may also relate to the issue above where the ischemia reperfusion itself seems to inhibit expression of HO-1 and Prx-1 mRNA. The same pathway might be involved but it is unclear what it is.

However, Akt-Nrf2 signaling pathway is the targeted pathway of this investigation and there was no investigation done to measure the expression level of Akt and Nrf2 expression through qPCR or immunoblotting assays. It was an equal important to asses Akt and Nrf2 expression level alike the HO-1 and Prx-1 expression level to complete the mechanism of action of rapamycin on liver. Experiments to measure the level of Akt and Nrf2 expreesion by qPCR or immunoblotting will be proposed as future work.

3.3.6 Limitations of using qPCR

qPCR is a highly sensitive method and also equal sensitive to errors. Any slight mistake can cause significant influence on the final results. mRNA in HO-1 and Prx-1 expression have been measuered only in this experiment and concluded to equate this result to measurement of protein expression. However, many factors can affect in the post translational modification of certain genes to express protein. Therefore, the immunoblotting assays and more preferably the enzymatic activity assays are required to assess the effects of rapamycin on the protein levels and enzymatic activities of HO-1 and Prx-1 in both normal and transformed liver cells. Unfortunately, protein expression has not been investigated due to limited time. But, to understand proper translation of this mRNA result in protein expression needs to be done in future studies. Thus, these experiments will be suggested as future work in the continuation of this project.

3.4 CONCLUSION

The results of this chapter show that rapamycin can induce HO-1 and Prx-1 mRNA in liver and hepatocytes, provide evidence that this is a direct effect on hepatocytes, and that dose response is complex. Since previous studies have shown that rapamycin can inhibit bile flow, in the next chapter we have investigated action of rapamycin on bile acid transporter expression in rat liver.

CHAPTER IV: EFFECTS OF RAPAMYCIN ON THE EXPRESSION OF BILE ACID TRANSPORTERS IN RAT LIVER *IN VIVO* AND IN ISOLATED CULTURED RAT HEPATOCYTES

4.1 INTRODUCTION

Rapamycin induced HO-1 and Prx-1 mRNA expression in liver as shown in Chapter 3. As discussed in the General Introduction, this action of rapamycin has been shown to be associated with inhibition of bile flow recovery after liver surgery (Kist *et al.*, 2012). Decreased bile flow recovery after surgery and associated ischemia reperfusion indicate poorer liver recovery after these events and may lead to poor overall recovery of the patient, therefore, is detrimental for the patient (Elferink *et al.*, 2004; Csanaky *et al.*, 2009; Klaassen and Aleksunes, 2010). Different mechanisms by which rapamycin might inhibit bile flow have been recognised where bile acid transporters involve. These mechanisms include changes in the expression of bile acid transporters and their activity (Deters *et al.*, 2001; Picard *et al.*, 2011; Oswald *et al.*, 2010), regulation the transcription of bile acid transporters by transcription factors (Alrefai *et al.*, 2005; Boyer, 2003), the degree of HO-1 expression level or anti-oxidant enzyme expression (Bramow *et al.*, 2001; Rippin *et al.*, 2001), and changes in cholangiocyte function (Kahn *et al.*, 2005). It is thought that changes in mRNA expression i.e. transcription of bile acid transporters are the most likely explanation for reduced bile flow recovery after warm ischemia reperfusion because bile acid transporters have direct effect on bile acid transport compared to indirect other mechanisms (Bramow *et al.*, 2001, Picard *et al*., 2011; Oswald *et al*., 2010). Therefore, it was hypothesised that rapamycin inhibits bile flow by inhibiting bile acid transporter expression after liver ischemia reperfusion injury. To this end, the effect of rapamycin on the expression of hepatocyte bile acid transporter expression was assessed.

Aim:

Therefore, the specific aims of these experiments described in this chapter are:

i. To assess the changes of bile acid transporter mRNA expression in rat liver.

ii. To determine whether rapamycin acts directly on hepatocytes and alters bile acid transporter mRNA expression in hepatocytes, and

iii. To determine the doses of rapamycin needed to achieve these aims.

To achieve this goal major sinusoidal uptake transporters including the Na^+-taurocholate cotransporting polypeptide (Ntcp) and the organic anion transporting polypeptides Oatp1 and Oatp2 (Fig. 4.1) (Kullak *et al.*, 2001; Reichel *et al.*, 1999) and major canalicular excrete transporters including the multidrug resistance associated protein 2 (Mrp2) and bile salt export pump (Bsep) (Fig. 4.1) (Rippin *et al.*, 2001) were tested in these experiments.

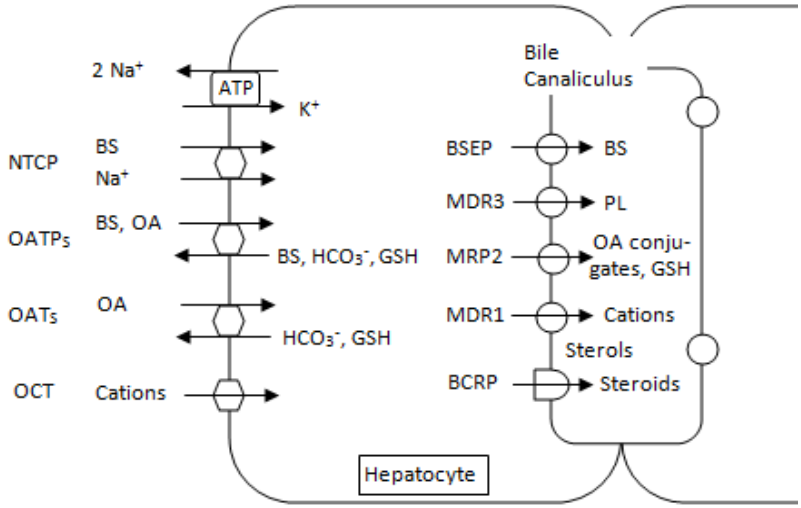

Figure 4.1: Movement of bile acid through the hepatocytes from blood to canaliculi by the direct bile acid transporters activity.

4.2 RESULTS

4.2.1 Effects of rapamycin and ischemia reperfusion on the expression of bile acid transporters in rat liver *in vivo*

The previous rat models of ischemia reperfusion (Fig 2.1) described in Chapter 2 to investigate the action of rapamycin on mRNA expression of sinusoidal transporters (Ntcp, Oatp1 and Oatp2) and canalicular transporters (Mrp2 and Bsep) in normal and ischemia reperfusion liver after rapamycin pre-treatment were used. The rat liver *in vivo* was subjected to ischemia (~60 min) and reperfusion (~90 min) by continuous clamping protocol as described by Alwine Kist (Fig. 2.1). This technique has been shown to cause ischemia reperfusion in the superior lobes without affecting the inferior lobes (Nieuwenhuijs *et al.*, 2007; Wilson *et al.*, 2011). Therefore, tissue from the inferior lobe was used as the control in this study.

4.2.1.1 Changes in expression of mRNA encoding the sinusoidal transporters Ntcp, Oatp1 and Oatp2

1. Ischemia reperfusion group (in the absence of pre-treatment with rapamycin)

Ischemia reperfusion caused a 2-fold increase in Ntcp (Fig. 4.2 A), a 2-fold decrease in Oatp1 (Fig. 4.2 B) and a 2.5-fold decrease in Oatp2 (Fig. 4.2 C) mRNA expression as indicated in Fig 4.2 (vehicle non ischemia reperfusion compared with vehicle ischemia reperfusion group).

2. Rapamycin non-ischemia reperfusion group

In the absence of ischemia reperfusion, pre-treatment with rapamycin caused a 2-fold decrease in Ntcp (Fig. 4.2 A), a 6-fold decrease in Oatp1 (Fig. 4.2 B) and a 3-fold decrease in Oatp2 (Fig. 4.2 C) mRNA expression (Fig. 4.2, vehicle non- ischemia reperfusion compared with rapamycin non ischemia reperfusion group).

3. Rapamycin ischemia reperfusion group

In the presence of pre-treatment with rapamycin, ischemia reperfusion caused a 3- fold increase in Ntcp (Fig. 4.2 A), a 3-fold decrease in Oatp1 (Fig 4.2 B) and no change in Oatp2 (Fig. 4.2 C) mRNA expression (Fig. 4.2, vehicle ischemia reperfusion compared to rapamycin ischemia reperfusion group).

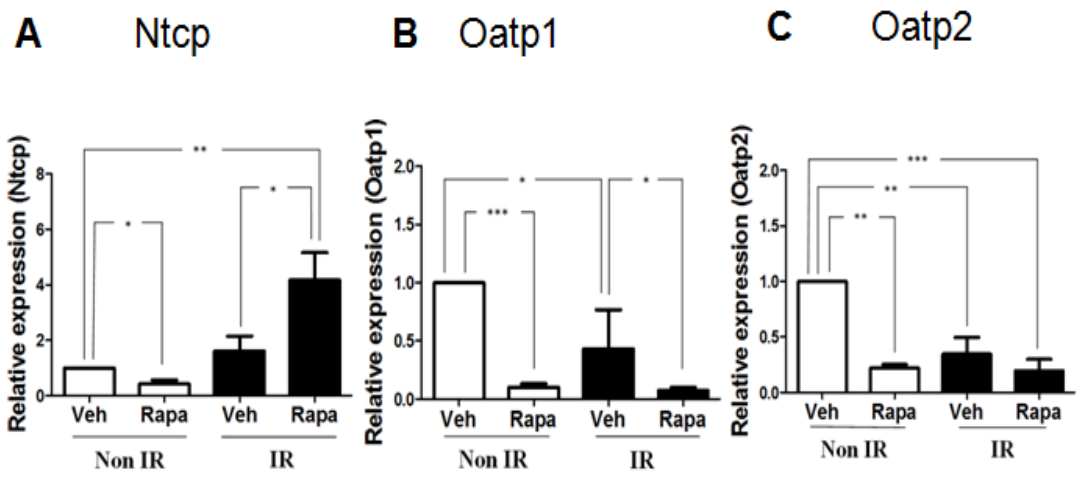

Figure 4.2: Rapamycin pre-treatment inhibits Ntcp mRNA expression (A) in non ischemia reperfusion liver but induces in ischemia reperfusion liver. However, Rapamycin pre-treatment inhibits Oatp1 (B) and Oatp2 (C) mRNA expression in non ischemia and ischemia reperfusion liver. Relative fold changed (normalized to β-actin expression) in the mRNA expression of Ntcp, Oatp1 and Oatp2 pre-treated with rapamycin for 24 hours in liver compared to livers pre-treated with vehicle. The mean of the vehicle inferior lobes was taken as a reference group. The results are expressed as mean ± SEM (n=4). To compare the relative Ntcp mRNA expression within the groups between the inferior and the superior lobes, the comparison was made using ANOVA with Post Hoc Tukey's test. Degrees of significance for comparison of rapamycin with vehicle are *p<0.05, ** P<0.01 and *** P<0.001.

4.2.1.2 Changes in expression of mRNA encoding canalicular transporters Mrp2 and Bsep

1. Ischemia reperfusion group

In the absence of pre-treatment with rapamycin, ischemia reperfusion caused a 2.5-fold increase in Mrp2 (Fig. 4.3 A) and a 2-fold decrease in Bsep (Fig. 4.3 B) mRNA expression as indicted in Fig 4.3 (vehicle non ischemia reperfusion compared with vehicle ischemia reperfusion).

2. Rapamycin non-ischemia reperfusion group

In the absence of ischemia reperfusion, pre-treatment with rapamycin caused a 7-fold increase in Mrp2 (Fig. 4.3 A) and a 2-fold decrease in Bsep (Fig. 4.3 B) mRNA expression (Fig. 4.3, vehicle non- ischemia reperfusion compared with rapamycin non ischemia reperfusion group).

3. Rapamycin ischemia reperfusion group

In the presence of pre-treatment with rapamycin, ischemia reperfusion caused a 2- fold increase in Mrp2 (Fig. 4.3 A) and no change in Bsep (Fig. 4.3 B) mRNA expression

(Fig 4.3, vehicle ischemia reperfusion compared with rapamycin ischemia reperfusion group).

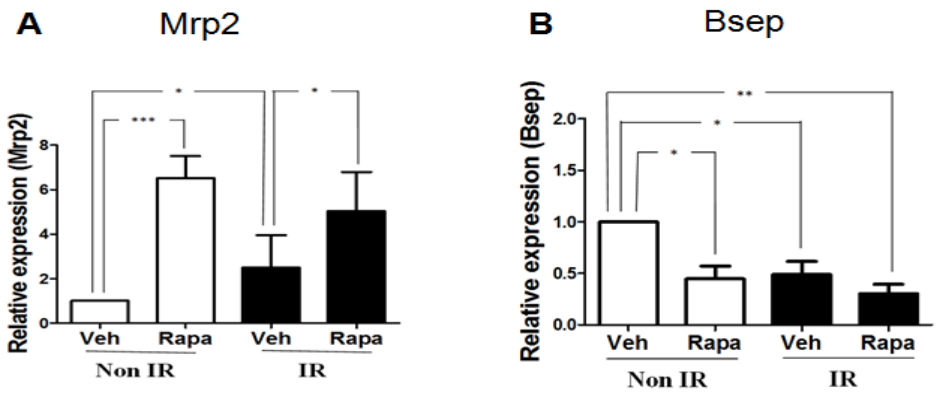

Figure 4.3: Rapamycin pre-treatment induces Mrp2 (A) mRNA expression but inhibits Bsep (B) mRNA in rat liver *in vivo*. Relative fold changed (normalized to β-actin expression) in the mRNA expression of Mrp2 and Bsep pre-treated with rapamycin for 24 hours in liver compared to livers pre-treated with vehicle. The mean of the vehicle inferior lobes was taken as a reference group. The results are expressed as mean ± SEM (n=4). To compare the relative Mrp2 mRNA expression within the groups between the inferior and the superior lobes, the comparison was made using ANOVA with Post Hoc Tukey's test. Degrees of significance for comparison of rapamycin with vehicle are *p<0.05, ** P<0.01 and *** P<0.001.

4.2.2 Effects of rapamycin on the expression of bile acid transporters in isolated cultured rat hepatocytes

In order to investigate the direct effects of rapamycin on hepatocyte expression of bile acid transporters, experiments were conducted using isolated cultured hepatocytes. This experimental system facilitates the testing of different concentrations of rapamycin and potentially the effects of different times of pre-treatment. The same concentrations of rapamycin and time period (36-hour incubation) as those experiments in Chapter 3 were selected to examine what is the effect on bile acid transporters at these selected rapamycin concentrations and time period.

4.2.2.1 Changes in expression of mRNA encoding the sinusoidal transporters Ntcp, Oatp1 and Oatp2

The results for expression of mRNA encoding Ntcp, Oatp1 and Oatp2 are shown in Fig 4.4.

Pre-incubation of the cells with 0.1 µM rapamycin for 36 hours caused a 3-fold decrease in Ntcp (Fig. 4.4 A) mRNA expression compared with that in vehicle. However, at 0.5 µM rapamycin there was no change in expression.

The results (Fig. 4.4 B) indicated that the expression of Oatp1 mRNA was decreased 5-fold and 20 fold by 0.1µM and 0.5µM rapamycin, respectively.

The expression of Oatp2 (Fig. 4.4 C) mRNA was decreased 3-fold and 5-fold by 0.1µM and 0.5µM rapamycin, respectively.

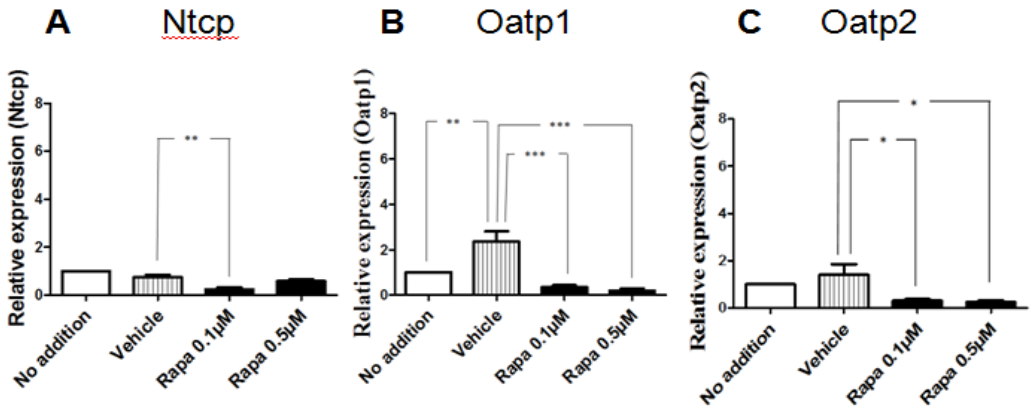

Figure 4.4: Treatment by rapamaycin inhibits the expression of Ntcp (A), Oatp1 (B) and Oatp2 (C) in isolated cultured rat hepatocytes. Relative fold changed (normalized to β-actin expression) in the mRNA expression of Ntcp, Oatp1 and Oatp2 treated with rapamycin for 36 hour compared with vehicle and no addition. The results are expressed as mean ± SEM of nine determinations from three individual experiments conducted on different days, each in triplicate. Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are *p<0.05, ** P<0.01 and *** P<0.001.

4.2.2.2 Changes in expression of mRNA encoding canalicular transporters Mrp2 and Bsep

The results for expression of mRNA encoding Mrp2 and Bsep are shown in Fig 4.5.

Pre-incubation of the cells with 0.1 µM rapamycin caused a 4-fold increase in Mrp2 (Fig. 4.5 A) mRNA expression compared with that in vehicle. However, at 0.5 µM rapamycin there was no change in expression.

Pre-incubation of the cells with 0.1 µM and 0.5 µM rapamycin caused a 3-fold and 2-fold increase in Bsep (Fig. 4.5 B) mRNA expression respectively compared with that in vehicle.

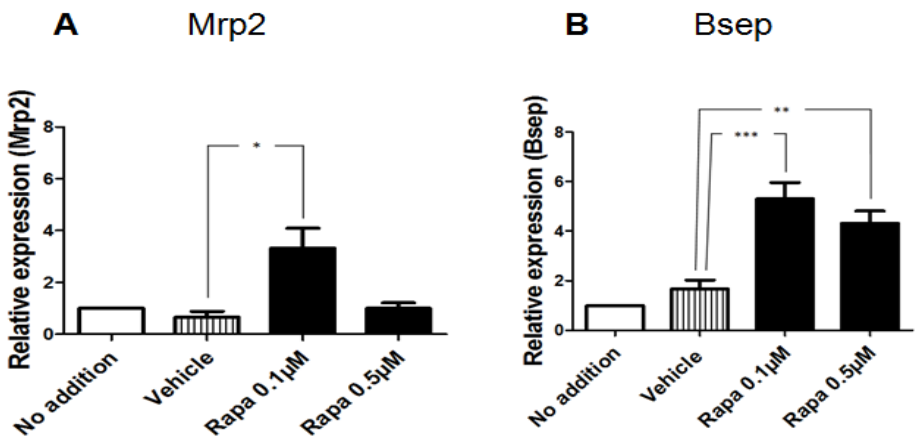

Figure 4.5: Treatment by rapamycin induces Mrp2 (A) and Bsep (B) mRNA in the isolated cultured rat hepatocytes. Relative fold changed (normalized to β-actin expression) in the mRNA expression of Mrp2 and Bsep in hepatocytes treated with rapamycin for 36 hours compared with vehicle and no addition. The results are expressed as mean ± SEM of nine determinations from three individual experiments conducted on different days, each in triplicate. Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with control are * P<0.05.

4.2.3 Dose-response curve by rapamycin on the expression of mRNA encoding Ntcp, Oatp1 and Bsep in isolated cultured rat hepatocytes

In the results above, 0.1 µM rapamycin inhibited but at 0.5 µM this inhibition was not observed in the mRNA expression of Ntcp and Bsep in isolated cultured rat hepatocytes which was unusual and unexpected. In order to understand clearly the effect of rapamycin on bile acid transporter expression further investigation was done using more concentrations of rapamycin. A dose response curve was obtained with varying concentrations (0.0, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 µM) in isolated rat hepatocytes and incubated for 36 hours. Results are shown in Fig. 4.6.

As can be seen from Fig 4.6 A, the results of Ntcp show a biphasic dose response curve. The rapamycin concentration which gave half maximal (S0.5 value) induction was 1.0 µM.

As can be seen from Fig 4.6 B, the results of Oatp1 show a monophasic dose response curve. The rapamycin concentration which gave half maximal (S0.5 value) induction was 0.05 µM.

As can be seen from Fig 4.6 C, the results of Bsep show a biphasic dose response curve. The rapamycin concentration which gave half maximal (S0.5 value) induction and inhibition was 0.05 and 1.0 µM respectively.

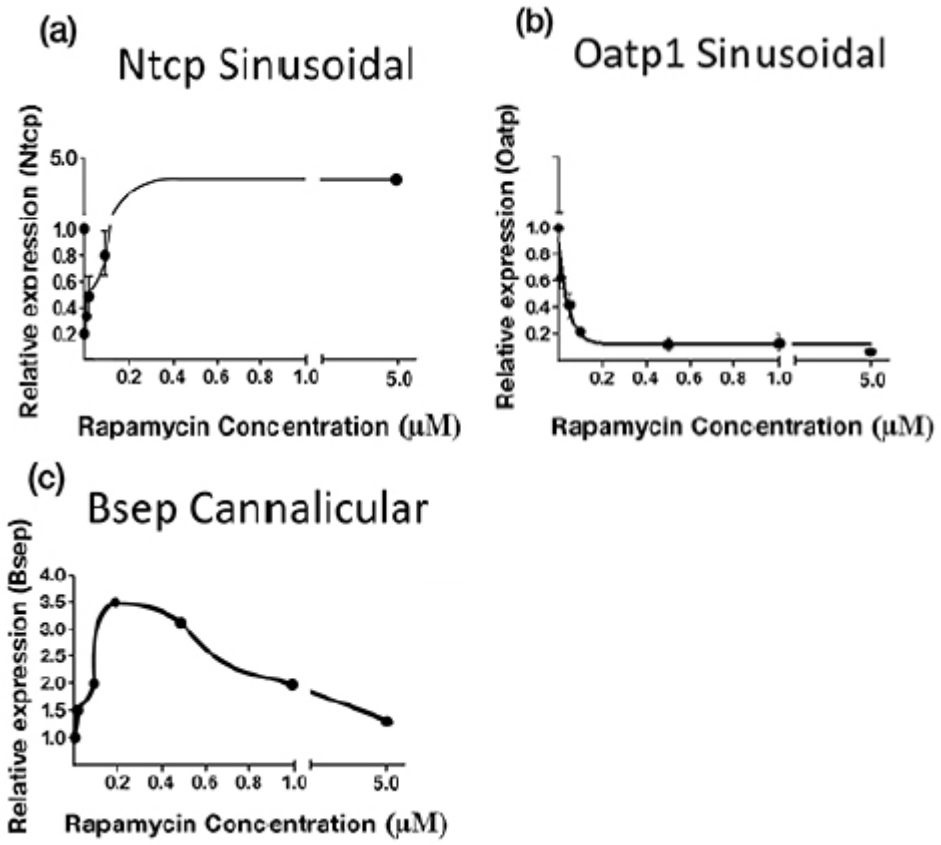

Figure 4.6: Dose-response curve by rapamycin for the induction in the expression of mRNA encoding Ntcp (A), Oatp1 (B) and Bsep (C) in isolated cultured rat hepatocytes. This line was achieved by fitting with linear regression equation (variable slope). Relative fold changed (normalized to β-actin expression) in the mRNA expression of Ntcp, Oatp1 and Oatp2 in hepatocytes treated with rapamycin compared with hepatocytes (no addition). The results are expressed as mean ± SEM of nine determinations from three individual experiments conducted on different days, each in triplicate.

4.3 DISCUSSION

The aim of this study was to investigate the effects of rapamycin on the expression of bile acid transporters in rat liver *in vivo* and in isolated cultured rat hepatocytes. The main findings were that pre-treatment of rapamycin in the absence of ischemia reperfusion caused a 3-6-fold inhibition of the expression of the sinusoidal (influx) bile acid transporters Ntcp, Oatp1 and Oatp2 mRNA (Tab. 4.1), but caused a 2-7-fold increase in the expression of the canalicular (efflux) transporters Mrp2 and Bsep mRNA (Tab. 4.1). These results indicated that rapamycin had also direct effect on bile acid

transporters expressions. The effects of ischemia reperfusion on the expression of bile acid transporters were complex. Biphasic dose response curves of Ntcp and Bsep suggested one or more intracellular signalling pathways might be involved.

Table 4.1: Summary of the effect of rapamycin on bile acid transporters mRNA expression in liver (ischemia reperfusion and non ischemia reperfusion liver) and isolated hepatocytes in culture

Transporters	Effect of IR in liver	Effect of rapamycin in non IR liver (24 hour)	Effect of IR following rapamycin (24 hour) in liver	Effect of rapamycin in isolated cultured hepatocytes for 36 hour
Sinusoidal				
Ntcp	2-fold increased	2-fold decreased	3-fold increased	3-fold decreased (Biphasic curve)
Oatp1	2-fold decreased	6-fold decreased	3-fold decreased	5-fold decreased (Monophasic curve)
Oatp2	2.5-fold decreased	3-fold decreased	No change	3-fold decreased
Canalicular				
Mrp2	2.5-fold increased	7-fold increased	2-fold increased	4-fold increased
Bsep	2-fold decreased	2-fold decreased	No change	3-fold increased (Biphasic curve)

4.3.1 Effects of ischemia reperfusion on the expression of Bile acid transporters

It was studied that mRNA expression of the sinusoidal transporters Oatp1 and Oatp2 and the mRNA expression of the canalicular transporter Bsep was significantly inhibited but the mRNA expression of the sinusoidal transporter Ntcp and the mRNA expression of the canalicular transporter Mrp2 was significantly induced in liver ischemia reperfusion injury. Similar to these findings, Tanaka *et al.* found an inhibition in Oatp1, Oatp2, and Bsep mRNA expression after liver ischemia reperfusion injury (Tanaka *et al.*, 2006; Fouassier *et al.*, 2007). In contrast to these findings, liver ischemia reperfusion injury was found to significantly decrease Ntcp and Mrp2 mRNA expression (Tanaka *et al.*, 2006; Fouassier *et al.*, 2007). However, their study was

looked at 24 hours of reperfusion, but this study was looked at 90 minutes' reperfusion. Therefore, time of reperfusion could be an explanation for the difference in the results.

Down-regulation of Mrp2, Bsep and Ntcp has been observed in various models of experimental impaired liver function such ligation of the common bile duct (Trauner *et al.*, 1997; Lee *et al.*, 2000), endotoxin induced cholestasis (Vos *et al.*, 1998; Simon *et al.*, 1996), bile fistula model (Deters *et al.*, 2002), partial hepatectomy model (Csanaky *et al.*, 2009).

Likely Mechanisms for expression of bile acid transporters by ischemia reperfusion

The actions of cytokines released from Kupffer cells and the ROS- PKC pathway in hepatocytes may account for the observed changes in expression of bile acid transporters caused by ischemia reperfusion (Fig. 4.7). Hepatic ischemia reperfusion injury causes activation of the resident macrophages within the liver (Kupffer-cell), which results in production and release of pro-inflammatory cytokines such as TNF-α, IL1β, IL6, IL-10 (Jaeschke & Farhood, 1991; Wanner *et al.*, 1996; Elferink *et al.*, 2004). These cytokines regulate the expression of some hepatic transporters (Ntcp, Oatp1, Oatp2, Bsep, Mrp2) (Geier *et al.*, 2003; Hartmann *et al.*, 2002; Tanaka *et al.*, 2006) via the transcription factors hepatocytes nuclear factor 1 (HNF1α) and/or Ntcp (Geier *et al.*, 2007; Tanaka *et al.*, 2006).

Previous studies have shown that liver ischemia reperfusion injury increases production of ROS (Kageyama *et al.*, 2015; Quesnelle *et al.*, 2015; Wang *et al.*, 2014). ROS activates PKC that leads to the activation of MAPK to dissociate Keap1 from Nrf2. This dissociation allows translocation of Nrf2 to the nucleus, leads to bind transcription factor ARE (Kim *et al.*, 2014; Hu *et al.*, 2013) (Fig. 4.7), leading to increased transcription of bile acid transporters (Fig. 4.7).

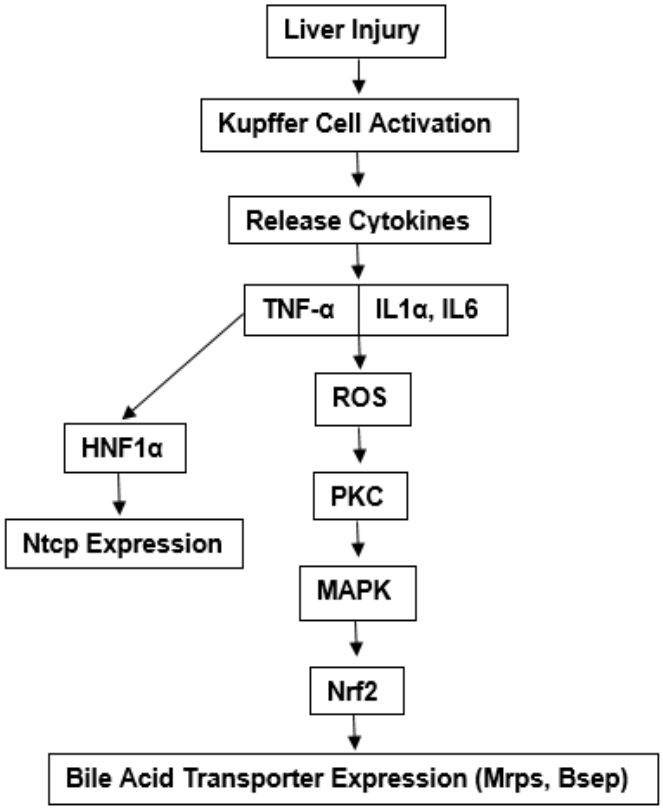

Figure 4.7: Proposed intracellular signaling pathways for regulation of bile acid transporter expressions in IR liver. Liver injury causes activation of kupffer celsl which results release of cytokines. These cytokines mediate ROS production which leads to activate PKC-MAPK pathway, resulting Nrf2 translocation and start transcription. The symbols represent ROS-Reactive Oxygen Species; PKC-Protein Kinase C; MAPK-Mitogen Activated Protein Kinase; Nrf2-Nuclear Factor-Erythroid 2-Related Factor 2.

4.3.2 Effects of rapamycin pre-treatment in normal (absence of ischemia reperfusion) liver

Rapamycin pre-treatment (4 mg/kg b.w.) significantly inhibited sinusoidal transporters Ntcp, Oatp1 and 2, canalicular transporter Bsep mRNA expression but induced canalicular transporter Mrp2 mRNA in normal liver.

Similar to these findings, Bramow *et al.* also found that rapamycin pre-treatment (0.4 mg/kg/ day for 2 week) reduced Oatp1 mRNA expression (Bramow *et al.*, 2001). In contrast, this group also found that rapamycin pre-treatment reduced Mrp2, increased Oatp2 with no effect on Ntcp mRNA expression (Bramow *et al.*, 2001). In this study, the authors used lower dose of rapamycin with longer period of administration (0.4 mg/kg/ day for 2 week) compared to my study (4 mg/kg b.w. for 24 hour). Therefore, time course of drug administration and concentration of dose could be an explanation of the difference in the results.

Likely mechanisms for expression of bile acid transporters by rapamycin (in absence of ischemia reperfusion

This proposed mechanism involves inhibition of mTORC1 by rapamycin, thereby disrupting S6 kinase 1 mediated feedback inhibition of PI3-K (Fig. 4.8). This leads to increased activity of Akt phosphorylation, leading to activation of Akt. Then activated Akt mediates dissociation Keap1 from the Keap1-Nrf2 complex, translocation of Nrf2 to the nucleus and leads to binding Nrf2 to ARE in the promoter of bile acid transporters (Bsep, Mrp2 and others) (Weerachayaphorn *et al.*, 2014; Shen and Kong, 2009) (Fig. 4.8). However, our results indicate that rapamycin pre-treatment also inhibits bile acid transporter expression but this mechanism is still unclear.

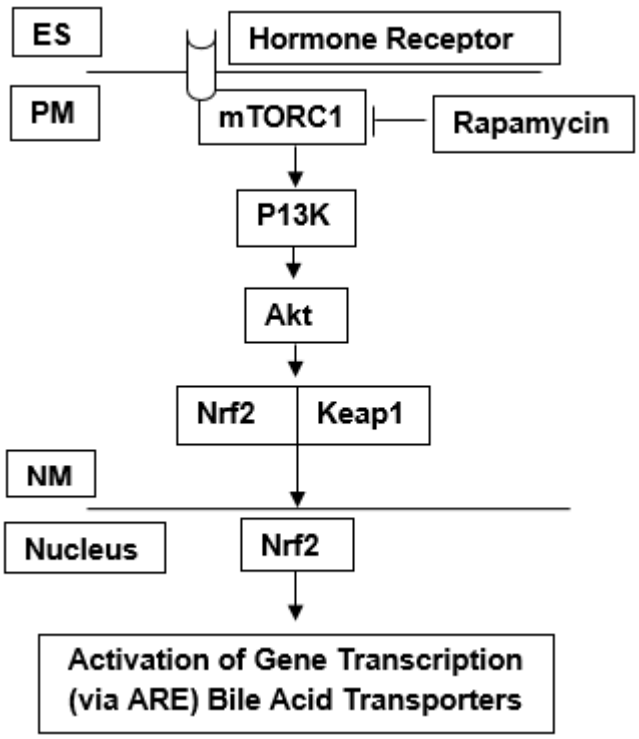

Figure 4.8: Proposed intracellular signaling pathways for regulation of bile acid transporter expressions caused by rapamycin. Rapamycin inhibits mTORC1 which leads to increased activity of Akt phosphorylation by disrupting feedback inhibition of PI3K which mediate Akt-PI3K-Nrf2 pathway. The symbols represent ES-Extracellular Space; PM-Plasma Membrane; NM-Nuclear Membrane; PI3K-Phosphatidylinositol 3 Kinase; Akt-Serine/Threonine Protein Kinase; mTORC1-Mammalian Target of Rapamycin Complex 1; S6K1-S6 Kinase-1; Nrf2-Nuclear Factor-Erythroid 2-Related Factor 2; Keap1- Ketch-like ECH Associated Protein 1; ARE-Antioxidant Response Element.

4.3.3 Effects of ischemia and reperfusion on expression of bile acid transporters following pre-treatment with rapamycin

Ischemia reperfusion increased sinusoidal transporter Ntcp and canalicular transporter Mrp2 mRNA expression. However, in the presence of rapamycin pre-treatment ischemia reperfusion increased only Mrp2 expression (see Tab. 4.1).

Likely mechanisms for expression of bile acid transporters by rapamycin-pretreatment in ischemia reperfusion liver

The Rapamycin/Akt/PI3K/Nrf2 pathway might be involved for induction of bile acid transporter expression by pre-treaatment with rapamycin in ischemia reperfusion liver which has been described in the General Introduction (see Fig 1.14.). However, it is still unclear which mechanism might be involved for inhibition of bile acid transporter expression. Activation of Nrf2 by rapamycin could structure a possible mechanism by which rapamycin modulates bile acid transporters expressions, and so, inhibits bile flow (Mann *et al.*, 2007; Zoncu *et al.*, 2011). Therefore, the correlation of the effects of rapamycin on increased antioxidant enzymes (HO-1 and Prx-1) expression and reduced bile flow and hence, liver function is not cleared.

4.3.4 Effects of rapamycin in cultured rat hepatocytes

Rapamycin caused a decrease in sinusoidal transporter Ntcp, Oatp1 and Oatp2 mRNA expression and increase in canalicular transporter Mrp2 and Bsep mRNA expression in isolated cultured rat hepatocytes (Tab. 4.1). In contrast, pre-treatment of rapamycin increased only Mrp2 mRNA expression in liver (absence of ischemia reperfusion) (Tab. 4.1). Similar to these findings, other laboratories have found an inhibition in Ntcp, Oatps mRNA expression in conventional culture of rat hepatocytes (Oswald *et al.*, 2010; Picard *et al.*, 2011; Liang *et al.*, 1993; Hoffmaster *et al.*, 2004). Mrp2 mRNA was increased in primary cultured rat hepatocytes (Rippin *et al.*, 2001; Hoffmaster *et al.*, 2004).

The actions of rapamycin on the expression of bile acid transporters are also very dependent on the exposure of time. This observation has been confirmed by others. Expression of Ntcp mRNA decreased within 72 hours in conventional culture of rat hepatocytes (Liang *et al.*, 1993). Mrp2 mRNA was increased to 154% at 24 and 48 hours, and decreased only afterwards in parallel with Mrp2 protein levels in primary

cultured rat hepatocytes (Rippin *et al.*, 2001). In contrast, Hoffmaster and colleagues who reported that expression of Mrp2 mRNA increased with time in culture (Hoffmaster *et al.*, 2004).

The results showed a biphasic dose response curve for the induction and inhibition by rapamycin on the expression of mRNA encoding Ntcp and Bsep and a monophasic dose response curve for the inhibition by rapamycin on the expression of mRNA encoding Oatp1 in isolated cultured rat hepatocytes (Tab. 4.1). These results suggest the concentration of rapamycin is one of the main reasons to get different results between *in vivo* and *in vitro* condition. The *in vivo* concentrations were high and on the high part of the cell dose response curves. One or more intracellular signaling pathways might be involved to induce or inhibit bile acid transporter expressions but we do not know exactly.

The proposed mechanism how rapamycin activates bile acid transporters expressions has been explained and drawn in the General Introduction (Fig. 1.14). However, the mechanism by which rapamycin inhibits bile acid transporter expression is still unknown.

4.4 CONCLUSION

Direct inhibition of bile acid transporters by rapamycin is unlikely to account for the observed *in vivo* inhibition of bile flow. Decreased expression of sinusoidal bile acid transporters is a likely explanation. Even though canalicular transporter expression is increased, the predominant effect is likely to be due to decreased sinusoidal transporter expression. This up-regulation and down-regulation depends on the time of exposure, dose, ischemia and reperfusion. The results described in Chapter 3 and 4 are for normal liver tissue. However, as discussed in the General Introduction, in HCC patients there is a significant amount of cancerous tissue. Moreover, rapamycin is being used to treat HCC patients; therefore, it is important to study actions of rapamycin on cancer tissue. This is the subject of the next two chapters.

CHAPTER V: EFFECTS OF RAPAMYCIN AND OLTIPRAZ ON THE EXPRESSION OF HEME OXYGENASE 1 AND PEROXIREDOXIN 1 IN TRANFORMED RAT LIVER CELLS

5.1 INTRODUCTION

As shown in Chapter 3, rapamycin induces HO-1 and Prx-1 mRNA expression in normal liver. A major factor in progression of cancer cell growth is inflammatory environment and generation of ROS by inflammation enhances cancer cell growth (Kim *et al.*, 2007; Hu *et al.*, 2013; Lee *et al.*, 2008). Therefore, induction of antioxidant enzymes (e.g. HO-1, Prx-1) would be a beneficial strategy to reduce ROS, resulting in inhibition of cancer cell growth (Lai *et al.*, 2008; Kim *et al.*, 2007).

As discussed in the General Introduction, rapamycin is in fact used clinically to treat HCC patients now (van Veelen *et al.*, 2011; Monaco, 2009; Geissier & Schlitt, 2009; Vivarelli *et al.*, 2010; Schnitzbauer *et al.*, 2010) and in the future oltipraz might be used for this purpose (Weerachayaphorn *et al.*, 2014; Cho *et al.*, 2009; Ko *et al.*, 2006). In untreated HCC patients there is a significant proportion of liver as tumor and properties of the tumor cells are very different from normal cells (Tomuleasa *et al.*, 2010; Thorgeirsson and Grisham, 2002; Farazi and DePinho, 2006). Therefore, it is important to know whether the actions of rapamycin on HO-1 and Prx-1 induction are the same or different in liver tumor tissue. This is particularly important for patients receiving rapamycin treatment over the long term.

Therefore, the aims of the experiments described in this chapter are:

1. To determine the effects of rapamycin and oltipraz on the expression of hemeoxygenase 1 and peroxiredoxin 1 in transformed rat liver cells.
2. To determine the doses of rapamycin needed to achieve this goal.

To achieve these goals H4IIE cells were used as a model of transformed (HCC) rat liver cells as discussed in the General Introduction. The effects of rapamycin on antioxidant

and bile acid transporter gene expression in rat liver and in isolated cultured rat hepatocytes were investiagted in the previous chapters. Therefore, in this chapter H4IIE rat liver cells were used as a model of HCC cells for comparison of results with normal rat liver or cells because H4IIE cells were originated from the Reuber hepatoma H-35 (Reuber, 1961) and treated as a good model of HCC cell. For these studies H4IIE cells without treatment were used as control. The relative gene expression of HO-1 and Prx-1 was measured by using RT-qPCR. The data of HO-1 expression will be presented first, and then followed by Prx-1 expression.

5.2 RESULTS

5.2.1 Relative expression of HO-1 and Prx-1 mRNA in H4IIE rat liver cells compared to that of isolated cultured rat hepatocytes

It is important to compare the expression level of HO-1 and Prx-1 mRNA in transformed H4IIE cells with primary rat hepatocytes before treatment for accurate translation of the drug effects. Therefore, the basal expression of HO-1 and Prx-1 in H4IIE cells with that in primary rat hepatocytes after 36-hour incubation was compared first. Differentiated H4IIE cells were used for the treatment of rapamycin and oltipraz to know any differences due to the de-differentiated state of the H4IIE cells. If the transformed (HCC) cells alter their characteristics it is important to understand what will be the effect of drugs. Thereby, the basal expression of HO-1 in differentiated H4IIE cells with undifferentiated H4IIE cells and primary rat hepatocytes was compared. Results are shown in Fig. 5.1. In this study, HO-1 (A) mRNA expression is nearly 3-fold lower in the H4IIE cells compared to that of primary cultured rat hepatocytes and differentiated H4IIE cells and Prx-1 (B) mRNA expression is nearly 25-fold lower in the H4IIE cells compared to that of primary cultured rat hepatocytes. These results indicate that after 10 days incubation in the presence of insulin (0.1 μM) and dexamethasone (0.1 μM) to induce differentiation, the basal HO-1 mRNA expression was increased to a level similar to that of primary rat hepatocytes.

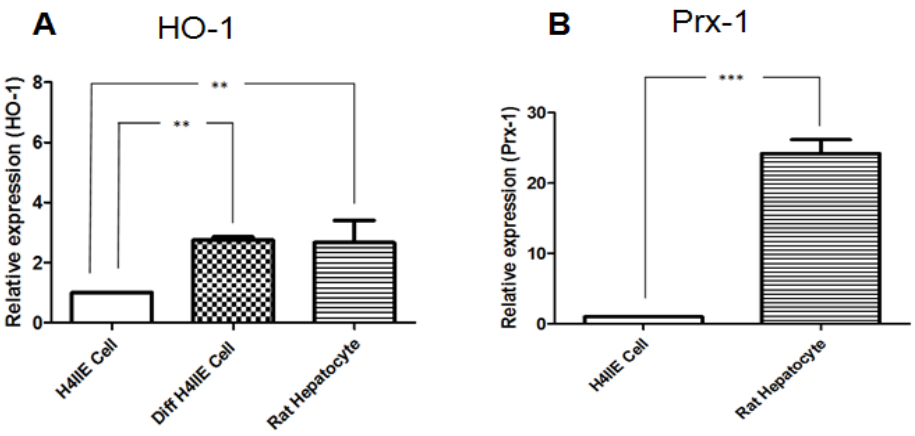

Figure 5.1: The relative expression of HO-1 (A) and Prx-1 (B) is lower in H4IIE cells than that of isolated rat hepatocytes in culture. Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in differentiated H4IIE cells and rat hepatocytes compared with H4IIE cells. The results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of H4IIE cells with isolated cultured hepatocytes are ** P<0.01 and *** P<0.001.

5.2.2 Effects of Rapamycin on HO-1 and Prx-1 mRNA expression in H4IIE cells

Rapamycin in undifferentiated H4IIE cells on HO-1 expression

To observe the effect of rapamycin on HO-1 mRNA expression in undifferentiated H4IIE cells, cells were incubated with rapamycin (0.1µM and 0.5µM) for 36 hours which was similar treatment in previous chapters (Chapter 3 and 4). Here, CoPP was used as HO-1 inducer because CoPP is known to increase HO-1 expression (Amersi *et al.*, 1999; Wang *et al.*, 2009; Glanemann *et al.*, 2005; Wasserberg *et al.*, 2007). The results are shown in Fig. 5.2 (A). Compared to vehicle, rapamycin caused a 3-fold decrease at 0.1 µM and a 2-fold decrease at 0.5 µM in HO-1 mRNA expression. CoPP caused an increase HO-1 mRNA expression by 1.5 fold compared to that of vehicle, although this increase was not statistically significant.

Rapamycin in differentiated H4IIE cells on HO-1 expression

The inhibition by rapamycin of HO-1 expression in H4IIE cells was unexpected as the results of Chapter 3 showed that rapamycin induces HO-1 in primary rat hepatocytes. It was possible that this difference in rapamycin action was due to the de-differentiated state of the rat hepatocytes that exhibited losing properties they originally had, such as protein expression, or change shape (Schnabel *et al.*, 2002). Therefore, differentiated H4IIE cells were used to asses HO-1 expression by rapamycin.

To determine the effect of rapamycin on HO-1 mRNA expression in differentiated H4IIE cells cells were incubated with rapamycin (0.1µM and 0.5µM) for 36 hours like undifferentiated H4IIE cells. Differentiated cells by 10 days were used as control. Here, CoPP was also used as HO-1 inducer. The results are shown in Fig. 5.2 (B). Compared to vehicle, rapamycin caused a 1.5-fold decrease in HO-1 mRNA expression at 0.1 µM and a 2-fold reduction in HO-1 mRNA expression at 0.5 µM. CoPP increased HO-1 mRNA expression by 1.5 fold compared to that of vehicle. Thus, differentiation of the cells by incubation with insulin and dexamethasone did not alter their response to the actions of rapamycin on HO-1 expression. In undifferentiated cells, 0.5µM rapamycin did not give as much decrease as 0.1 µM rapamycin whereas in differentiated cells 0.5 µM rapamycin gave a further decrease.

Rapamycin in undifferentiated H4IIE cells on Prx-1 expression

To observe the effect of rapamycin on Prx-1 expression, undifferentiated H4IIE cells were incubated with rapamycin (0.1µM and 0.5µM) for 36 hours. The results are shown in Fig. 5.2 (C). Compared to vehicle, rapamycin caused a 1.5-fold decrease in Prx-1 mRNA expression at 0.5 µM.

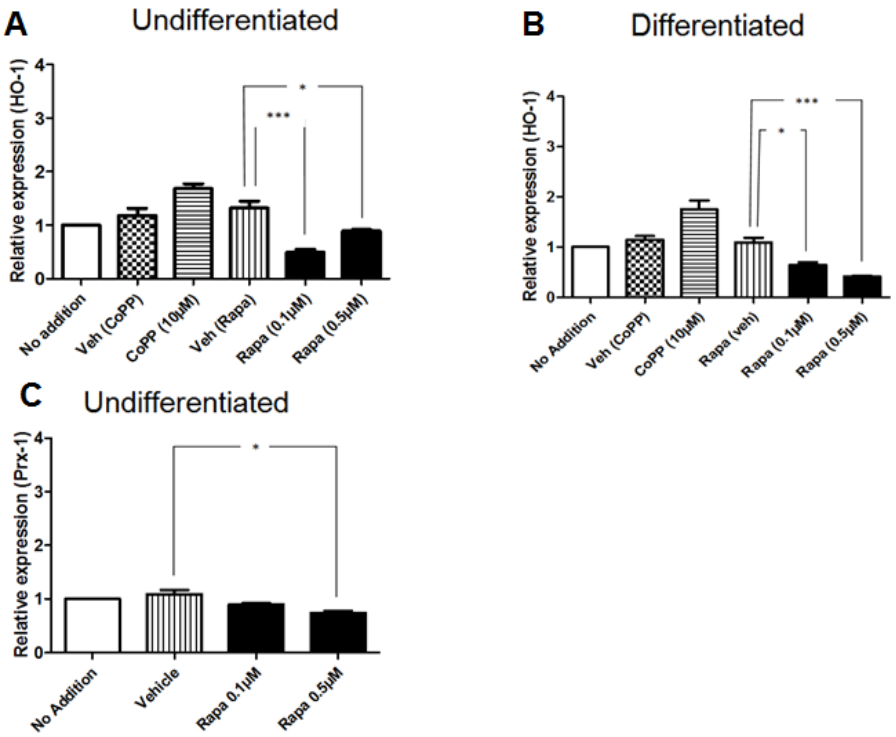

Figure 5.2: Rapamycin reduces HO-1 (A, B) and Prx-1 (C) mRNA expression in H4IIE cells. Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in H4IIE cells treated with rapamycin compared with vehicle. The results are expressed as the mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are *P<0.05 and *** P<0.001.

5.2.3 Effects of oltipraz on HO-1 and Prx-1 mRNA expression in H4IIE cells

To determine the effect of oltipraz on HO-1 mRNA expression in H4IIE cells, H4IIE cells were incubated with 25μM and 50μM oltipraz for 36 hours These two concentrations were selected on the basis of the results in published studies on the expression of antioxidant enzyme, glutathione transferase, treated by oltipraz in liver (Kim *et al.*, 2010). The results are shown in Fig. 5.3. Oltipraz caused an increase in HO-1 (A) mRNA expression at both concentrations (4-fold and 6-fold respectively). Oltipraz also caused an increase in Prx-1 (B) mRNA expression at both concentrations (1.5-fold and 2.5-fold respectively). Oltipraz induced increase in Prx-1 is much less than that in HO-1.

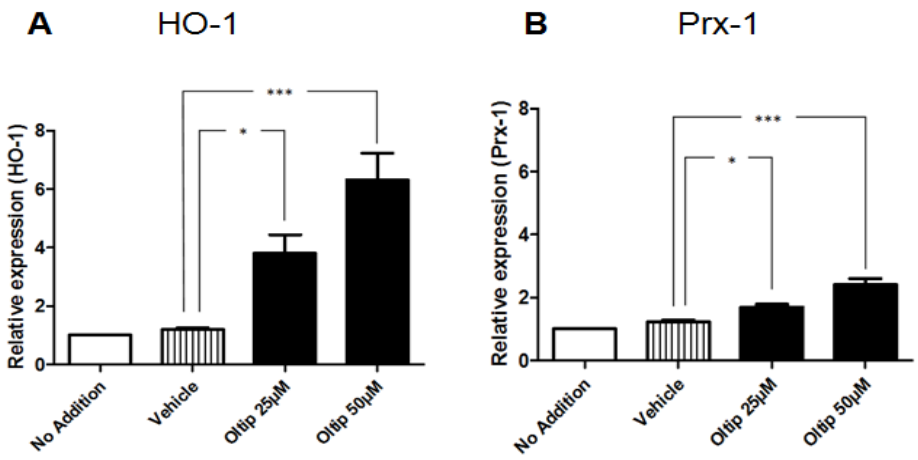

Figure 5.3: Oltipraz induces HO-1 (A) and Prx-1 (B) mRNA expression in H4IIE cells. Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in H4IIE cells treated with oltipraz compared with vehicle. The results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of oltipraz with vehicle are * $P<0.05$ and *** $P<0.001$.

5.2.4 Dose-response curve for the action of oltipraz on the expression of mRNA encoding HO-1 and Prx-1

To determine the concentration of oltipraz that gives half maximal value six concentrations of oltipraz (12.5, 25, 50, 75, 100 and 150μM) were used. The results are shown in Fig. 5.4. The results showed monophasic dose response curve of HO-1 (A) and Prx-1 (B) expression. The oltipraz concentration which gave half maximal (S0.5 value) induction was 50μM.

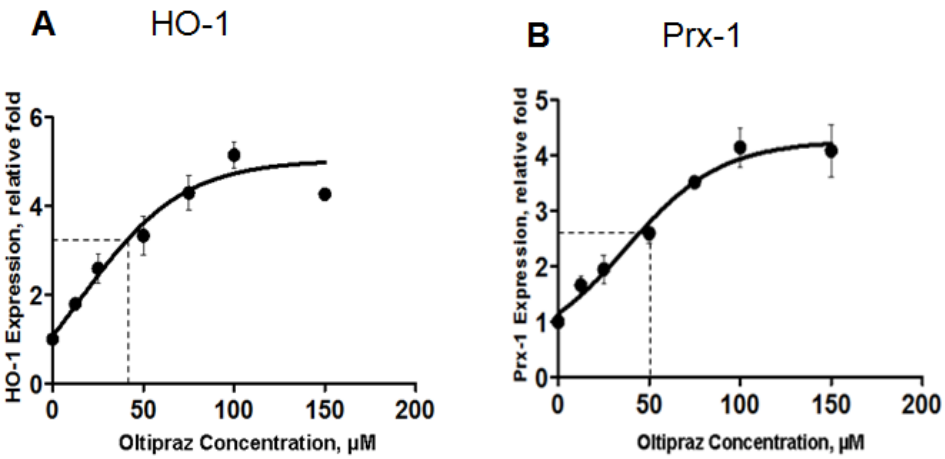

Figure 5.4: Dose-response curve by oltipraz for the induction of the expression of mRNA encoding HO-1 (A) and Prx-1 (B) in H4IIE cells. This line was achieved by fitting with linear regression equation (variable slope). Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in H4IIE cells treated with oltipraz compared with control. The results are expressed as mean ± SEM (n = 9, three individual experiments).

5.2.5 Effects of rapamycin on HO-1 and Prx-1 mRNA expression in presence of oltipraz

Since oltipraz gave a large induction of HO-1 and Prx-1 mRNA expression and rapamycin inhibited HO-1 and Prx-1 mRNA expression, it was important to understandwhether rapamycin counteracted this increase. The results for H4IIE cells with rapamycin on HO-1 and Prx-1 were unexpected. Two concentrations of rapamycin (0.1 and 0.5 µM) were used in the presence of one fixed oltipraz concentration (50 µM). 50 µM oltipraz to elevate HO-1 and Prx-1 expression was selected because this concentration gave half maximal induction on the basis of above dose response curve. The results are shown in Fig. 5.5. Oltipraz (50 µM) alone 6-fold and 3-fold increased in HO-1 and Prx-1 mRNA expression respectively. In the presence of 50 µM oltipraz, rapamycin caused a 3-fold and 2-fold decrease oltipraz activity in HO-1 mRNA expression at 0.1 and 0.5 µM respectively. In the presence of 50 µM oltipraz, rapamycin caused a 2-fold decrease oltipraz activity in Prx-1 mRNA expression at both concentrations.

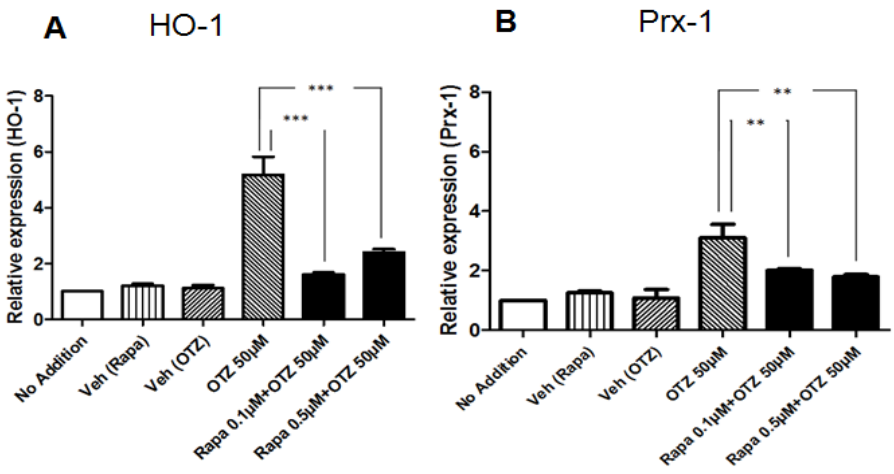

Figure 5.5: Rapamycin causes a large inhibition of HO-1 (A) and Prx-1 (B) mRNA expression when cells are co-incubated with oltipraz and rapamycin. Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in H4IIE cells treated with rapamycin and oltipraz compared with 50 μM oltipraz. The results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of treated cells with vehicle are *** P<0.001.

5.2.6 Dose response curve for rapamycin in presence of oltipraz

In H4IIE cells it was observed unexpectedly inhibition rather than increase in HO-1 and Prx-1 expression with rapamycin. The difference between H4IIE cells and primary rat hepatocytes might be due to the dose employed. Therefore, a dose response study with rapamycin was conducted. But, as the rapamycin effects on H4IIE cells were small the experiment was conducted in the presence of oltipraz to increase HO-1 and Prx-1 expression and then looked at the effects of rapamycin.

To do this six rapamycin concentrations (0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μM) and one fixed oltipraz concentration (50 μM) were used. Oltipraz 50 μM and no rapamycin was used as control in this case. The results are shown in Fig. 5.6. Rapamycin drastically inhibited the positive effects of oltipraz on HO-1 (A) and Prx-1 (B) mRNA expression, even at the lowest concentration of 0.01 uM rapamycin. It is noteworthy that the general shape of these curves was the same by rapamycin in the presence of oltipraz. In both curves, there seems to be some complexity; inhibition at the 0.01 μM concentration, then some recovery from inhibition.

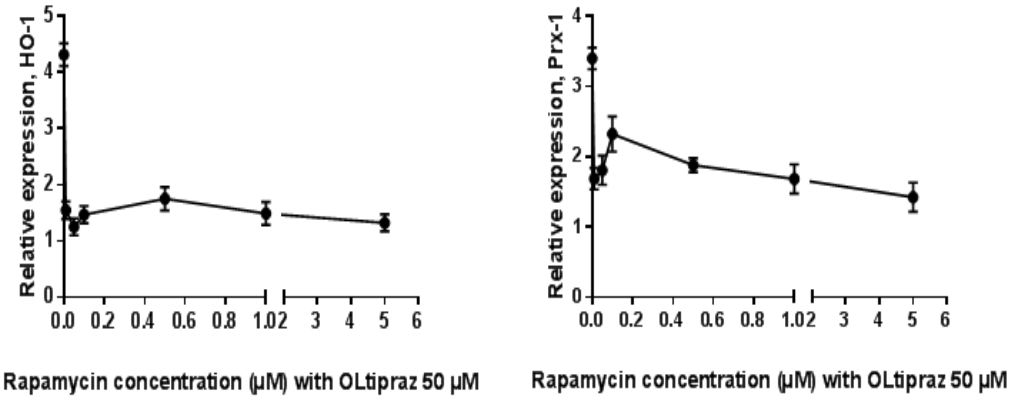

Figure 5.6: Dose response curve for the inhibition of HO-1 (A) and Prx-1 (B) expression by rapamycin in presence of the activator oltipraz. Relative fold changed in the mRNA expression of HO-1 and Prx-1 (normalized to β-actin expression) in H4IIE cells treated with rapamycin and oltipraz comparing with H4IIE cells treated with oltipraz 50μM. qPCR was normalized to β-actin and expressed as a ratio compared to no addition i.e. no rapamycin and no oltipraz. The results are expressed as mean ± SEM (n = 9, three individual experiments).

5.2.7 Effects of rapamycin and oltipraz on cell growth

In H4IIE cells, rapamycin did not increase HO-1 and Prx-1 expression but decreased it. So, it was important to look if this was due to rapamycin inducing cell damage or death. Therefore, in the next series of experiments, the effect of rapamycin was assessed on cell growth in H4IIE cells. To achieve this goal cells were incubated with rapamycin (0.1 and 0.5μM) for 36 hours and total cell number and cell viability (trypan blue exclusion) were measured. The results are shown in Fig. 5.7 (A). Rapamycin caused a decrease in total cell number by nearly 30% in both concentrations.

The effects of rapamycin were also assessed on cell growth in differentiated H4IIE cells. To do this, cells were differentiated first with 0.1μM insulin and 0.1μM dexamethasone for 10 days. These differentiated cells were then incubated with rapamycin (0.1 and 0.5μM) for 36 hours and total cell number and cell viability (trypan blue exclusion) were measured. The results are shown in Fig. 5.7 (B). Rapamycin caused an increase in total cell number at both concentrations. The total cell number was increased nearly 25% and 50% at 0.1μM and 0.5μM respectively in the differentiated H4IIE cells.

Next the effect of oltipraz was assessed on cell growth in H4IIE cells. H4IIE cells were incubated by oltipraz (25 and 50μM) for 36 hours and total cell number and cell

viability (trypan blue exclusion) were measured. The results are shown in Fig. 5.7 (C). Oltipraz caused a decrease in total cell number by nearly 25% at both concentrations.

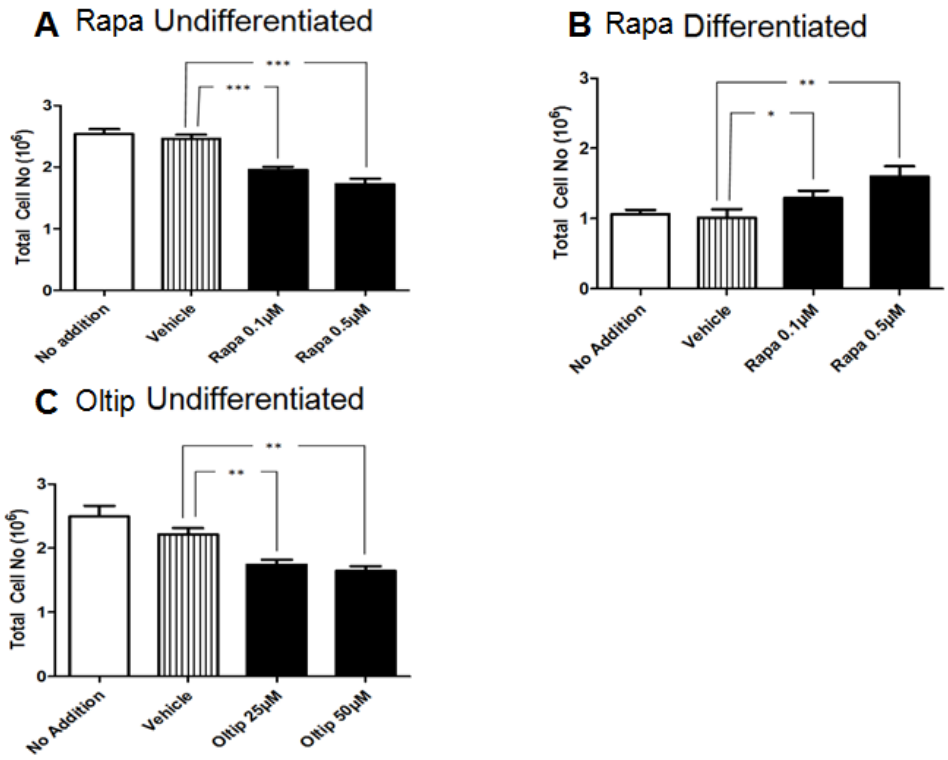

Figure 5.7: Rapamycin and oltipraz reduces total cell number in H4IIE cells after 36 hours incubation. Results were compared with vehicle. The results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are *** P<0.001.

The results of the effect of rapamycin on cell viability in H4IIE cells are shown in Fig. 5.8 (A). Rapamycin caused a decrease in cell viability by nearly 65% and 70% at 0.1µM and 0.5µM respectively. These results indicate that under the conditions tested rapamycin inhibited growth of undifferentiated cells and induced some cell damage.

Rapamycin also caused a decrease in the percentage of cell viability at 0.1 µM (Fig. 5.8 B). The percentage of viable cells was nearly 50% and 55% at 0.1µM and 0.5µM respectively in the differentiated H4IIE cells. These results indicate that under the conditions tested rapamycin increased growth of differentiated cells but did not alter the degree of cells damage.

Oltipraz caused an increase in cell viability by nearly 90 and 95 at 25µM and 50µM respectively (Fig. 5.8 C). These results indicate that under the conditions tested oltipraz inhibited growth of undifferentiated cells and protected the cells from damage or death.

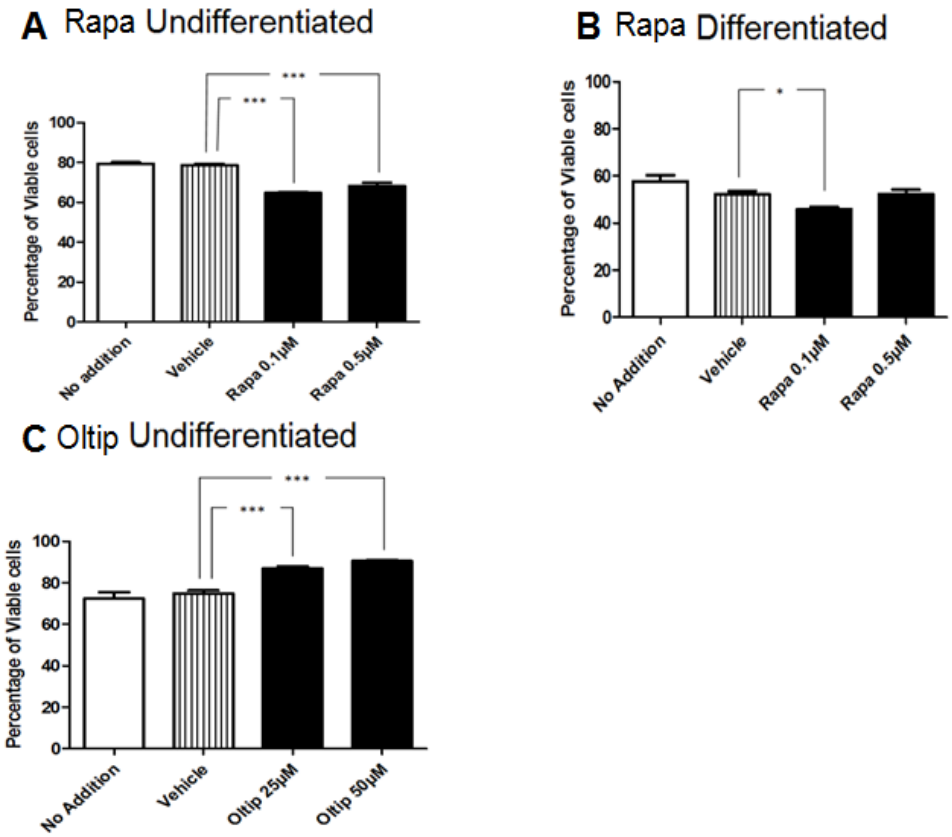

Figure 5.8: Rapamycin reduces the percentage of viable cells but oltipraz induces the percentage of viable cells after 36 hours incubation. Results are shown as percentage and compared with vehicle. The results are expressed as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are *P<0.05 and *** P<0.001.

5.3 DISCUSSION

The aim of this investigation was to determine the effects of rapamycin and oltipraz on the expression of heme oxygenase 1 and peroxiredoxin 1 in cultured rat hepatocytes and H4IIE rat liver cells. The main observation is that in H4IIE cells, basal expression of HO-1 and Prx-1 mRNA was lower than that in isolated cultured rat hepatocytes. In contrast to its effect on cultured rat hepatocytes, rapamycin blunted HO-1 and Prx-1 mRNA expression (Table 5.1). Oltipraz increased HO-1 and Prx-1 mRNA expression in H4IIE cells similar to that in rat hepatocytes. The S0.5 for rapamycin inhibition was 0.01µM and for oltipraz was 50µM. Differentiation restored basal HO-1 mRNA

expression in H4IIE cells but was not able to restore the rapamycin-mediated induction of HO-1 mRNA seen in hepatocytes. Rapamycin decreased total cell number and cell viability in H4IIE cells while oltipraz decreased total cell number but increased cell viability. In contrast, rapamycin increased total cell number in differentiated H4IIE cells.

Table 5.1: Effects of rapamycin and oltipraz on HO-1 and Prx-1 mRNA expression in H4IIE rat liver cells

Antioxidant enzymes	Actions in H4IIE cells	Compared to Rat Hepatocytes
Rapamycin		
HO-1	3-fold decreased	3-fold increased
Prx-1	1.5-fold decreased	3-fold increased
Oltipraz		
HO-1	6-fold increased (Monophasic dose response)	Not tested
Prx-1	2.5-fold increased (Monophasic dose response)	Not tested
Action of rapamycin following oltipraz (compared to oltipraz)		
HO-1	3-fold decreased	Not tested
Prx-1	2-fold decreased	Not tested

5.3.1 Effects of rapamycin on HO-1 and Prx-1 expression in H4IIE cells

The results showed that incubation with rapamycin decreased HO-1 and Prx-1 expression in transformed rat liver (H4IIE) cells which is in contrast to the observed induction of HO-1 and Prx-1 expression in normal rat liver cells. Similar to these findings, Hu *et al.* found an inhibition in HO-1 mRNA expression in human carcinoma (Eca109) cells (Hu *et al.*, 2013). The degree of mRNA expression in Prx1 was significantly reduced in MEF cell line (Kim *et al.*, 2007).

The results of differentiated H4IIE cells for increasing HO-1 and Prx-1 mRNA expression were unexpected. Differentiation by 0.1µM insulin and 0.1µM

dexamethsone by 10 days did not convert rapamycin activity on the inhibition of HO-1 and Prx-1 expression to activation. However, the basal expression of HO-1 was increased, similar to rat hepatocytes. These results indicate that rapamycin activity may be influenced by insulin and dexamethasone but the mechanism by which this occurs is unknown.

Likely explanation

In the previous chapter (chapter 3), it was observed that rapamycin induced HO-1 and Prx-1 mRNA expression in normal rat liver cells (Fig 3.9). However, in this chapter it was found that rapamycin reduced HO-1 and Prx-1 mRNA expression in H4IIE cells. The possible explanation would be the following:

1. H4IIE rat tumor liver cells derived from Reuber Hepatoma H-35 (Pitot *et al.*, 1964). As discussed in the General Introduction, there are mutations in numerous proteins including intracellular signaling pathways, and these may alter the pathways affected by rapamycin in unknown ways.
2. mTORC1 is the key regulator of rapamycin action and mTORC1 has dual function which can activate and inhibit Akt activity by phosphorylation and dephosphorylation (Sun *et al.*, 2005; Sarbassov *et al.*, 2006) that can be accountable for alteration of mTOR dependent signal transduction.
3. There is some evidence that the activation site of Nrf2 may be altered in mutated cells which can interrupt the Nrf2-Keap1 signal transduction pathway (Padmanabhan *et al.*, 2006; Singh *et al.*, 2008; Mitsuishi *et al.*, 2012).

5.3.2 Effects of oltipraz on HO-1 and Prx-1 expression in H4IIE cells

From these investigations, it was found that oltipraz increased HO-1 and Prx-1 mRNA expression in H4IIE cells. In contrast, rapamycin inhibited HO-1 and Prx-1 mRNA expression in H4IIE cells and induced HO-1 and Prx-1 mRNA expression in normal rat hepatocytes. The effects of oltipraz on HO-1 and Prx-1 mRNA expression in normal rat hepatocytes were not investigted due to time limitations. Therefore, the exact effect of oltipraz on HO-1 and Prx-1 expression in liver is still unknown. Experiments with isolated cultured hepatocytes would be important for future experiments.

Likely mechanisms for expression of HO-1 and Prx-1 by oltipraz

The proposed intracellular signaling pathway by which oltipraz regulates antioxidant enzyme (HO-1 and Prx-1) expression in liver has been described in the General Introduction Chapter (see Fig 1.16). The likely explanation of increased HO-1 and Prx-1 mRNA expression by oltipraz compared to rapamycin action on decreased HO-1 and Prx-1 expression would be due to increase Nrf2 activation by oltipraz in H4IIE cells. Several studies provide evidence about the activation of Nrf2 by oltipraz (Weerachayaphorn *et al.*, 2009; Maher *et al.*, 2007).

5.3.3 Effects of rapamycin and oltipraz co-administration on HO-1 and Prx-1 expression

It was observed that rapamycin inhibited oltipraz activity on HO-1 and Prx-1 mRNA expression in H4IIE cells when rapamycin and oltiptaz were co-administered. This result is very unlikely. At this moment it is unclear the reason why and how rapamycin inhibited oltipraz activity on HO-1 and Prx-1 expression in H4IIE cells.

Likely mechanisms for expression of HO-1 and Prx-1 by rapamycin in presence of oltipraz

There is no direct evidence found how rapamycin reduces oltipraz activity on HO-1 and Prx-1 mRNA expression in H4IIE cells. However, to sum up the available data it is proposed that rapamycin inhibits oltipraz activity by disrupting Nrf2 translocation from the Nrf2 complex, and leads to binding Nrf2 to ARE in the promoter of the HO-1 and Prx-1 genes (Fig. 5.9).

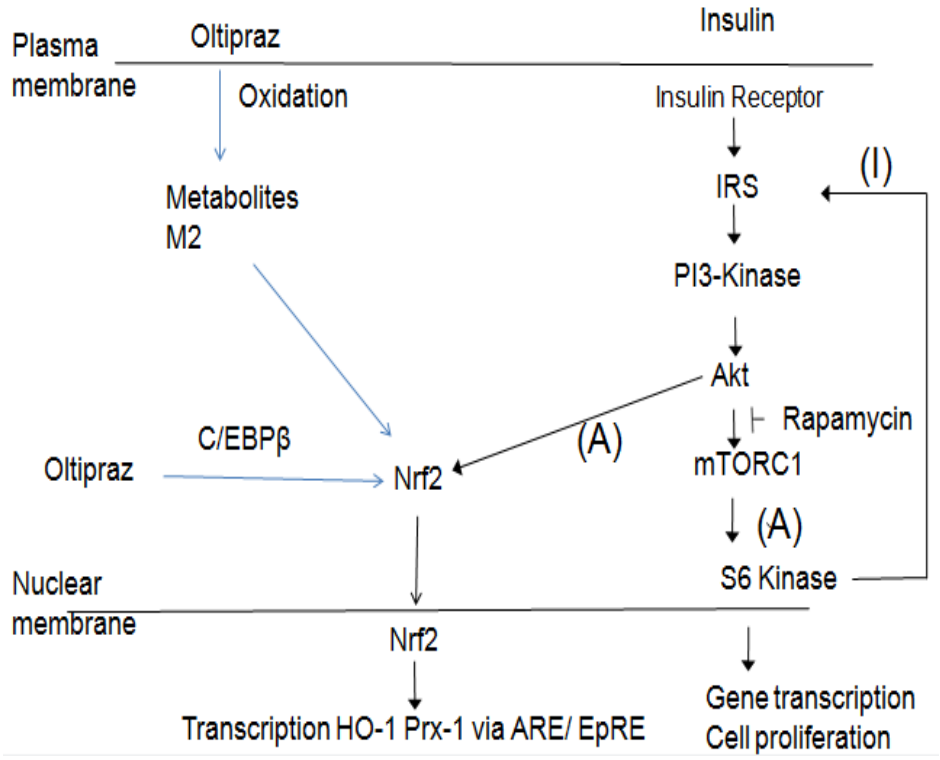

Figure 5.9: Proposed intracellular signaling pathways for regulation of HO-1 and Prx-1 expression in H4IIE cells caused by rapamycin and oltipraz co-administration. Rapamycin inhibits mTORC1 which leads to increased activity of Akt phosphorylation by disrupting feedback inhibition of PI3K which mediate Akt-PI3K-Nrf2 pathway. Oltipraz derivative M2 activates Nrf2 by dissociating Keap1 blocking PI3K-p70S6 kinase pathway (Cho *et al.*, 2009; Ko *et al.*, 2006). This dissociated Nrf2 translocates to the nucleus and promotes Nrf2 binding to the promoter region of target gene EpRE/ARE which leads to the expression of HO-1, Prx-1. The symbols represent C/EBPTβ- CCAAT/Enhancer Binding Protein β; PI3K- Phosphoinositadyl 3 kinase; mTORC1- Mammalian Target of Rapamycin Subunit C1; Nrf2- Nuclear Factor-Erythroid 2-Related Factor 2; Keap1- Ketch-like ECH Associated Protein 1; EpRE/ARE- Ferritin H Electrophile/Antioxidant Responsive Element; HO-1- Hemeoxygenase-1; Prx-1- Peroxiredoxin-1.

5.3.4 Effects of rapamycin on cell proliferation in H4IIE cell culture

In this study, rapamycin (0.1 and 0.5μM for 36-hour incubation) inhibited total cell number and also inhibited the percentage of viable cells in transformed H4IIE (HCC) cells. Similar to this observation, various laboratories also found that rapamycin inhibited different transformed or HCC (PLC5, HuH7 and BMOL-TAT) cell lines (Shirouzu *et al.*, 2010; Heuer *et al.*, 2009; Bridle *et al.*, 2009). In addition, it was reported by various groups of studies that rapamycin had been slowed down the proliferation of different types of cells *in vitro* (Zhu *et al.*, 1999; Gabele *et al.*, 2005; Schreml *et al.*, 2007; Jankiewicz *et al.*, 2006; Hafizi *et al.*, 2004; Bridle *et al.*, 2009).

This inhibition was also dependent on dose and time of incubation (Shirouzu *et al.*, 2010; Heuer *et al.*, 2009).

These results suggest that rapamycin inhibits cell growth or activates apoptosis or necrosis. The Trypan Blue results (percentage of viable cell) indicate that rapamycin induces some cell damages of some sort but it is unknown whether this is apoptosis and or necrosis. It is noteworthy that the percentage of viable cells in control cells was 80%, which was unexpected. Although it is unclear the reason behind this issue but it is important to consider the observed effect of rapamycin and oltipraz begins. Apoptosis or necrosis induced by extracellular signaling pathways in *in vitro* culture would be a possible explanation to reduce viability of cells in culture. It is still unestablished from these results if there is any correlation between inhibition of cell proliferation and HO-1 and Prx-1 expression.

Rapamycin (0.1 and 0.5µM for 36-hour incubation) also induced total cell number in differentiated H4IIE cells but inhibited percentage of viable cells. These results indicate that differentiation by insulin and dexemethasone may have a great impact on rapamycin activity but still it is unclear.

Likely Mechanisms for inhibition of cell growth by rapamycin

The likely mechanism how rapamycin inhibits cell proliferation and percentage of viable cells has been described in the General Introduction (see in Fig. 1.12). The likely explanation of inhibition of cell growth by rapamycin would be due to disruption of 70S ribosomal protein S6 kinase (S6K1) activity in H4IIE cells. Several studies provide evidence about the disruption of PI3K-Akt-S6K1 pathway and inhibition cell growth by rapamycin (Sehgal, 2003; Kist *et al*., 2012; Ashwarth and Wu, 2014).

5.3.5 Effects of oltipraz on cell proliferation in H4IIE cell culture

Oltipraz (25 and 50 µM for 36-hour incubation) decreased total cell number but increased the percentage of viable H4IIE cells, opposite to the effect of rapamycin. Similar to this observation, Cho *et al.* found that oltipraz enhanced liver regeneration in an animal model of bile duct ligation (Cho *et al.*, 2009).

These results indicate that oltipraz may inhibit cell proliferation or activate apoptosis or necrosis in transformed liver (HCC) cells. The Trypan Blue results indicate that oltipraz may protect liver cells from cell damage. This observation suggests that using oltipraz would be a beneficial drug for the HCC patients who are waiting for liver resection or transplantation.

Likely mechanism

The likely mechanism how oltipraz regulates cell growth in liver cells has been described in the General Introduction (see Fig 1.17). The likely explanation of inhibition of cell growth by oltipraz would be due to blocking PI3K-p70S6 kinase pathway (Cho *et al.*, 2009).

5.4 CONCLUSION

Taken together, the results of this investigation suggest that rapamycin is not a good inducer of HO-1 and Prx-1 expression in transformed (HCC) liver cells since it inhibited expression of these enzymes. However, it can inhibit transformed (HCC) cell proliferation which would be beneficial for the HCC patients to reduce tumor growth. However, oltipraz is a good inducer of HO-1 and Prx-1 expression in transformed (HCC) liver cells and inhibitor of tumor growth or HCC cell proliferation.

CHAPTER VI: EFFECTS OF RAPAMYCIN AND OLTIPRAZ ON THE EXPRESSION OF BILE ACID TRANSPORTERS IN TRANSFORMED RAT LIVER CELLS

6.1 INTRODUCTION

As described in the General Introduction, rapamycin is used to treat patients with advanced stage HCC in order to reduce growth of cancer cells (Ashworth and Wu, 2014; Toso *et al.*, 2010) and potentially oltipraz can also be used in this way (Ruggeri *et al.*, 2002; Helzlsouer and Kensler, 1993; Johnson *et al.*, 2002; Kensler *et al.*, 2003; Merrell *et al.*, 2008). As shown in Chapter 3, rapamycin induces antioxidant HO-1 and Prx-1 expression in normal liver and this can be of potential value in reducing damage due to ischemia reperfusion injury in liver surgery. However, this was also associated with inhibition of bile acid transporter expression, as shown in Chapter 4, and therefore may lead to poorer outcome.

In HCC patients treated with rapamycin, there is a large amount of tumor tissue as well as normal tissue in liver. In these patients, rapamycin is expected to act on not only on normal liver cells but also on HCC cells. Moreover, the results in Chapter 5 show inhibition of HO-1 and Prx-1 expression in liver tumor tissue, which is the opposite of what happens in normal liver. However, oltipraz shows an induction of HO-1 and Prx-1 expression in liver tumor tissue. Therefore, it was considered important to know the effect of rapamycin and oltipraz on bile acid transporters expression in liver tumor tissue. H4IIE cells were used here as a model of liver tumor tissue which was described before in the General Introduction. Several published papers report that some bile acid transporters are not expressed in liver tumor and or may be expressed at levels different from those in normal liver (Weerachayaphorn *et al.*, 2009; Ko *et al.*, 2006; Cho *et al.*, 2009). Therefore, before investigating the effect of rapamycin and oltipraz, the expression of bile acid transporters in H4IIE cells and primary hepatocytes was necessary.

Aim:

Therefore, the aims of these experiments described in this chapter are:

i. To detect which bile acid transporters are expressed in H4IIE cells,
ii. To determine the relative expression of bile acid transporters in H4IIE cells compared to hepatocytes, and
iii. To determine the effects of rapamycin and oltipraz in expressions of bile acid transporters in H4IIE cells.

To achieve these goals, the major bile acid transporters in H4IIE cells were investigated which have been described as in General Introduction and Chapter 4 (Fig. 4.1). Among bile acid transporters, sinusoidal transporter Ntcp, Oatp1, Oatp2, Oatp9 and canalicular transporter Mrp2, Bsep, Mdr1a, Mdr1b, Mdr2, Bcrp were selected for studies.
The data of sinusoidal transporter expression will be presented first, and then followed by canalicular transporter expression.

6.2 RESULTS

6.2.1 Detection and comparison of bile acid transporter expression in H4IIE cells with that in rat hepatocytes by conventional PCR

In this chapter, expression of bile acid transporters was compared in H4IIE cells and primary rat hepatocytes.

Sinusoidal transporters expressions Ntcp, Oatp1, Oatp2 Oatp9 were presented in Fig. 6.1. In contrast to primary hepatocytes, the sinusoidal transporter, Oatp9 was not detected in H4IIE cells (see Fig. 6.1; Tab. 6.1).

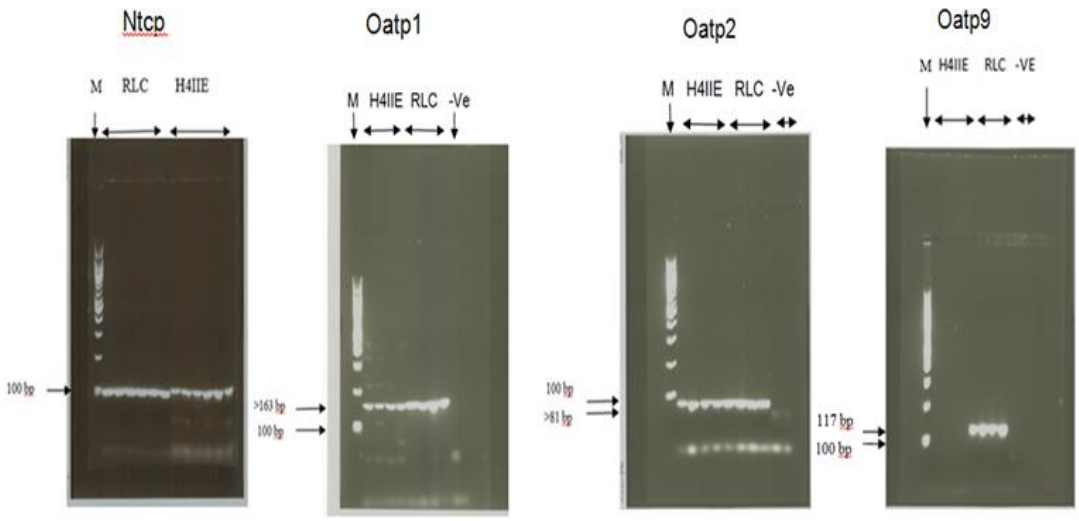

Figure 6.1: Detection of sinusoidal bile acid transporters expression in H4IIE and rat liver cells by PCR. PCR was conducted three times for each gene and these are representative images of each gene. Nuclease free water (NFW) was used as negative control. The sequence of the primers used for each bile acid transporter and PCR product size has been described as in Materials and Methods Chapter (see Tab.-2.1). The symbol represents RLC-Rat Liver Cells (primary hepatocytes).

Canalicular transporters expressions Mrp2, Bsep, Mdr1a, Mdr1b, Mdr2, Bcrp were presented in Fig 6.2. Each transporter was detected with the PCR primers used in hepatocytes except Mdr1a which was detected in H4IIE cells (see Fig 6.2; Tab. 6.1). In contrast to primary hepatocytes, the canalicular transporters, Bsep and Bcrp, were not detected in H4IIE cells (see Fig. 6.2; Tab. 6.1).

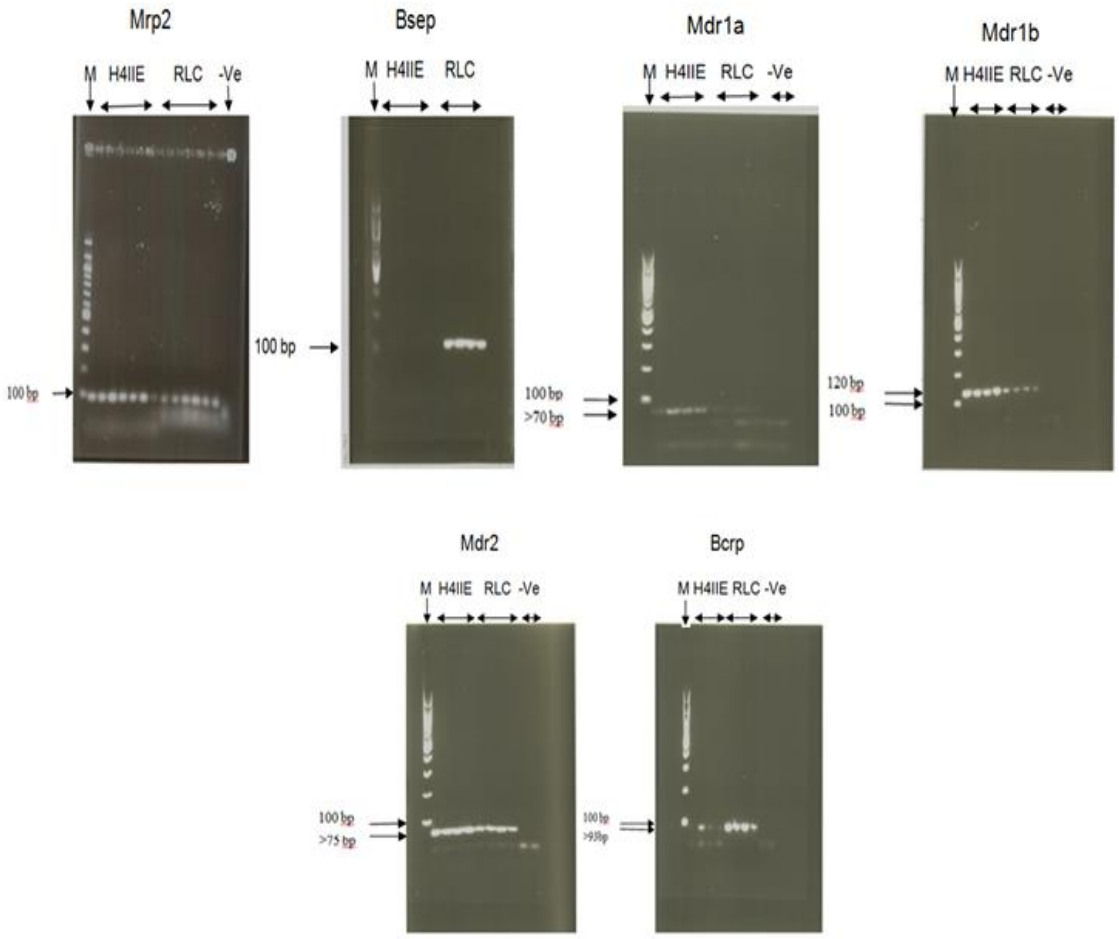

Figure 6.2: Detection of canalicular bile acid transporter expression in H4IIE and rat liver cells by PCR. PCR was conducted three times for each gene and these are representative images of each gene. Nuclease free water (NFW) was used as negative control. The sequence of the primers used for each bile acid transporter and PCR product size has been described as in Materials and Methods Chapter (see Tab.-2.1). The symbol represents RLC-Rat Liver Cells (primary hepatocytes).

Table 6.1: Results summary of the bile acid transporter mRNA expression detected in H4IIE rat liver cells and primary rat hepatocytes.

Bile Acid Transporter	Expression in rat hepatocytes	Expression in H4IIE cells
Sinusoidal Transporter		
Ntcp	Expressed	Expressed
Oatp1	Expressed	Expressed
Oatp2	Expressed	Expressed
Oatp9	Expressed	Not Expressed
Canalicular Transporter		
Mrp2	Expressed	Expressed
Bsep	Expressed	Not Expressed
Mdr1a	Not Expressed	Expressed
Mdr1b	Expressed	Expressed
Mdr2	Expressed	Expressed
Bcrp	Expressed	Not Expressed

6.2.2 Relative expression of mRNA encoding bile acid transporter in H4IIE rat liver cells compared to that in isolated cultured rat hepatocytes

Relative expression of sinusoidal transporters Ntcp, Oatp1, Oatp2 and canalicular transporter Mrp2 mRNA were measured in H4IIE (undifferentiated) cells. The expressions of some bile acid transporters were investigated in differentiated H4IIE cells. All cells (H4IIE cells, differentiated H4IIE cells and isolated primary rat hepatocytes) were incubated for 36 hours without any treatment. RT-qPCR was conducted to assess the expression of bile acid transporters. The results are shown in Fig. 6.3 and Tab. 6.2.

The relative basal expression of Ntcp (A) mRNA in H4IIE cells and differentiated H4IIE cells was nearly 30-fold lower than that of primary rat hepatocytes. Differentiation did not increase Ntcp expression in H4IIE cells.

The relative expressions of Oatp1 (B) and Oatp2 (C) mRNA in H4IIE cells were 25 and 14- fold lower, respectively than that in primary rat hepatocytes.

The relative expression of Mrp2 (D) in H4IIE and differentiated H4IIE cells was similar and was nearly 3- fold higher than that in primary rat hepatocytes.

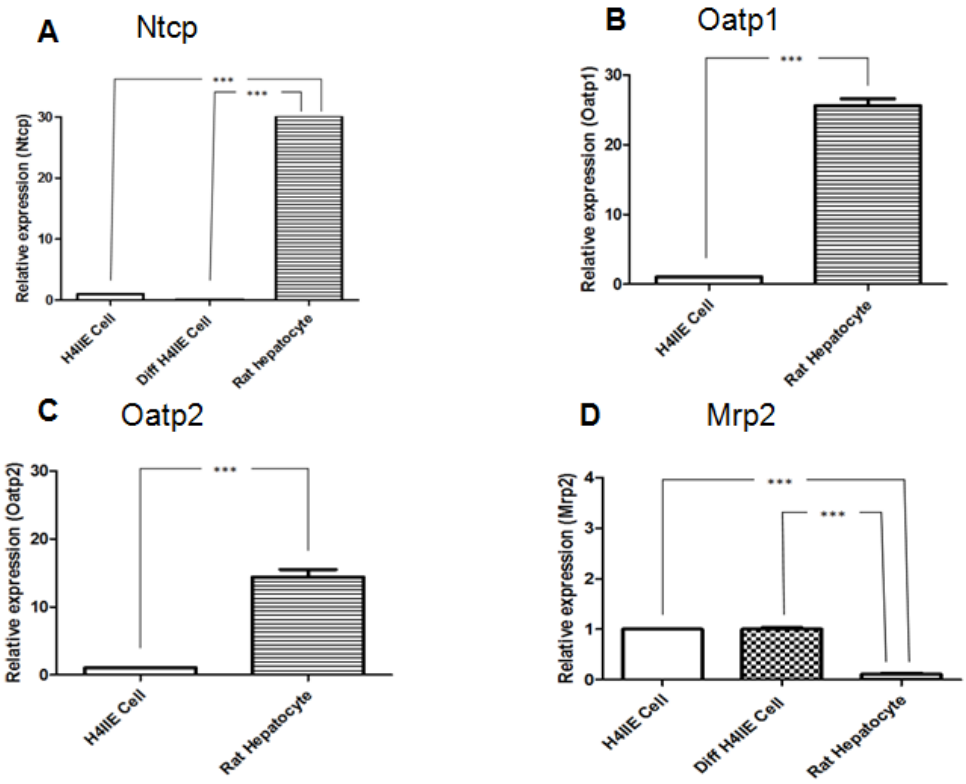

Figure 6.3. Relative expression of Ntcp (A), Oatp1 (B), Oatp2 (C) are lower but Mrp2 (D) mRNA in H4IIE cells is substantially higher than that in primary rat hepatocytes. Relative fold changed in the mRNA expression of Ntcp, Oatp1, Oatp2 and Mrp2 (normalized to β-actin expression) in differentiated H4IIE cells and primary rat hepatocytes compared with H4IIE cells. The results are presented as mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of differentiated H4IIE cells and primary rat hepatocytes with H4IIE cells are *** P<0.001.

Table 6.2: Summary of the relative mRNA expression of bile acid transporters in H4IIE rat liver cells compared to that in freshly isolated rat hepatocytes.

Transporter	Expression in Undifferentiated H4IIE cells relative to that in rat hepatocytes	Effect of differentiation
Sinusoidal		
Ntcp	30-fold lower	No change
Oatp1	25-fold lower	Not tested
Oatp2	14-fold lower	Not tested
Canalicular		
Mrp2	3- fold Higher	No change

6.2.3 Effects of rapamycin and oltpraz on sinusoidal bile acid transporter encoding Ntcp, Oatp1 and Oatp2 mRNA expression in H4IIE rat liver cells

Effects of rapamycin

To investigate the effect of rapamycin in H4IIE cells on sinusoidal transporters mRNA expression, H4IIE cells were incubated with rapamycin (0.1 and 0.5 µM) for 36 hours which was similar to previous chapter's treatment. Differentiated H4IIE cells were used to determine the Ntcp mRNA expression by rapamycin. Results are shown in Fig. 6.4.

Ntcp mRNA expression

Rapamycin caused a 2.5-fold increase in expression of Ntcp mRNA (Fig 6.4 A). Similar results were obtained with differentiated H4IIE cells (Fig 6.4 B).

Oatp1 expression

Rapamycin caused a 2-fold and 4-fold increase in Oatp1 mRNA expression at 0.1 and 0.5µM, respectively compared to vehicle (Fig. 6.4 C).

Oatp2 expression

Rapamycin caused a decrease in Oatp2 mRNA expression by 2-fold and 2.5-fold at 0.1 and 0.5µM, respectively compared to vehicle (Fig. 6.4 D).

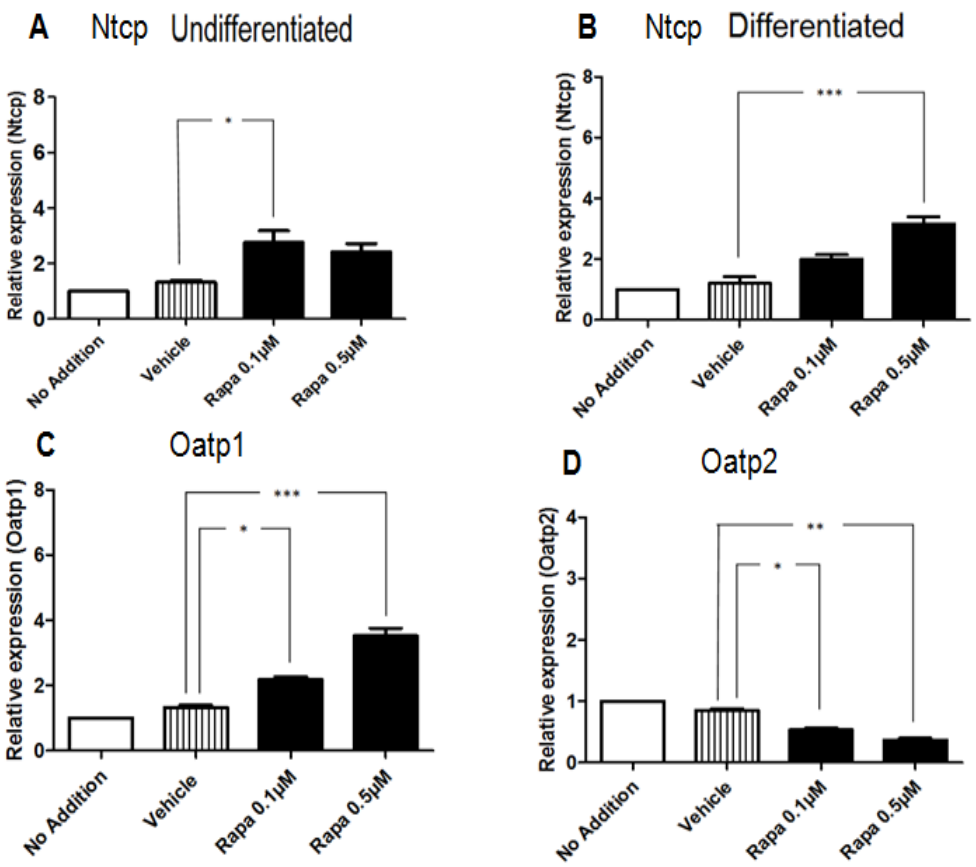

Figure 6.4: Rapamycin induces Ntcp (A, B) and Oatp1 (C) mRNA expression but reduces Oatp2 (D) mRNA expression in H4IIE cells. Relative fold changed in the mRNA expression of Ntcp, Oatp1 and Oatp2 (normalized to β-actin expression) in H4IIE cells treated with rapamycin compared with vehicle. The results are expressed as the mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are * P<0.05, **P<0.01 and *** P<0.001.

Effects of oltipraz

To determine the effect of oltipraz on sinusoidal bile acid transporter mRNA expression in H4IIE cells two doses of oltipraz (25μM and 50μM) were used for 36 hours which was similar to Chapter 5 on HO-1 and Prx-1 expression. Results are shown in the Fig. 6.5.

Ntcp expression

Oltipraz caused a 2-fold and 4-fold decrease in Ntcp (A) mRNA expression at 25 μM and 50 μM respectively. At the higher concentration of oltipraz, a greater decrease was observed.

Oatp1 expression

Oltipraz caused a decrease in Oatp1 (B) mRNA expression by 2- and 3-fold at 25 µM and 50 µM, respectively compared to vehicle.

Oatp2 expression

Oltipraz caused an increase in Oatp2 (C) mRNA expression by 1.5-fold at 25 µM and decrease by 2-fold at 50 µM compared to vehicle.

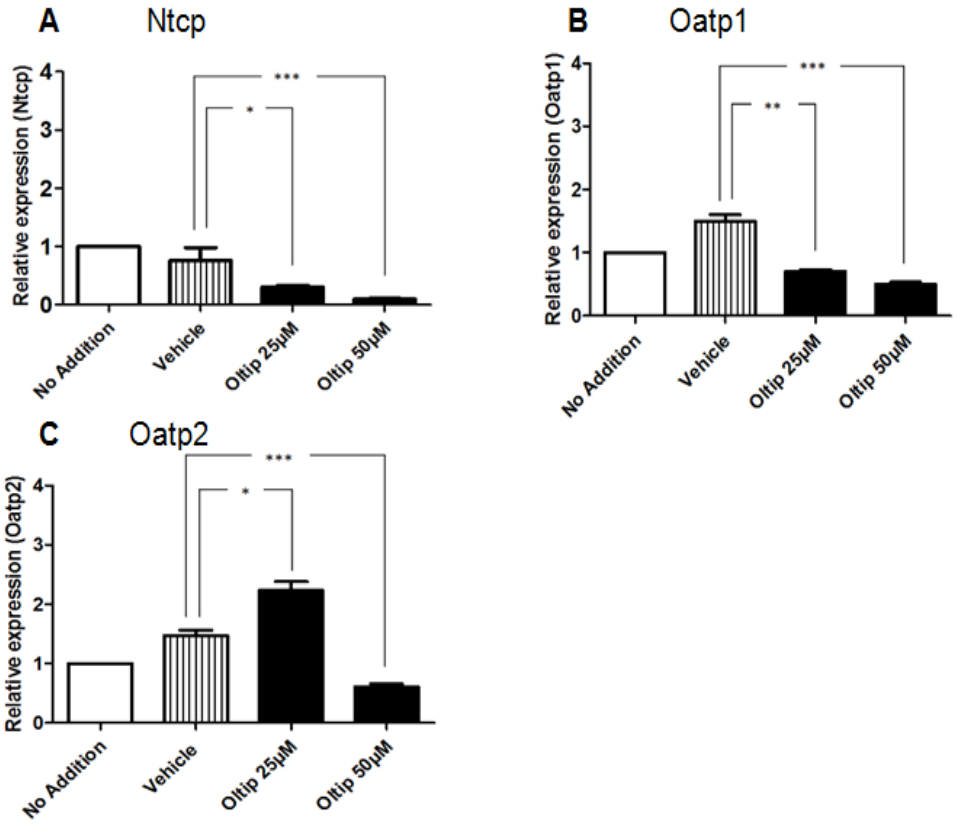

Figure 6.5: Oltipraz reduces Ntcp (A), Oatp1 (B) and Oatp2 (C) mRNA in H4IIE cells. Relative fold changed in the mRNA expression of Ntcp, Oatp1 and Oatp2 (normalized to β-actin expression) in H4IIE cells treated with oltipraz compared with vehicle. The results are expressed as the mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of oltipraz with vehicle are * P<0.05, ** P<0.01 and *** P<0.001.

6.2.4 Effects of rapamycin and oltipraz on canalicular transporter encoding Mrp2 mRNA expression in H4IIE rat liver cells

Effects of rapamycin

To observe the effect of rapamycin on Mrp2 mRNA in H4IIE cells and differentiated H4IIE cells, cells were treated with 0.1 and 0.5µM rapamycin for 36 hours similar to previous experiments. Results are shown in Fig. 6.6. Rapamycin caused an increase in Mrp2 mRNA expression by 3-fold (Fig 6.6 A) and 2- fold (Fig 6.6 B) at 0.5µM in undifferentiated and differentiated H4IIE cells, respectively.

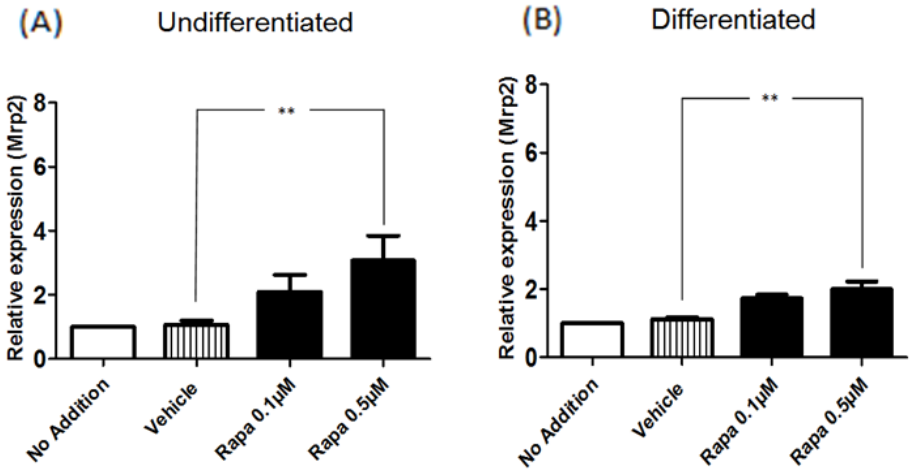

Figure 6.6: Rapamycin induces Mrp2 mRNA expression in H4IIE (undifferentiated and differentiated) cells. Relative fold changed in the mRNA expression of Mrp2 (normalized to β-actin expression) in H4IIE cells treated with rapamycin compared with vehicle. The results are expressed as the mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of rapamycin with vehicle are ** P<0.01.

Effects of oltipraz

To observe the effect of oltipraz on Mrp2 mRNA expression in H4IIE cells, 25µM and 50µM oltipraz were used for 36 hours similar to the above experiments. Results are shown in Fig. 6.7. Oltipraz caused an increase in Mrp2 mRNA expression by 2-and 3-fold at 25µM and 50µM, respectively.

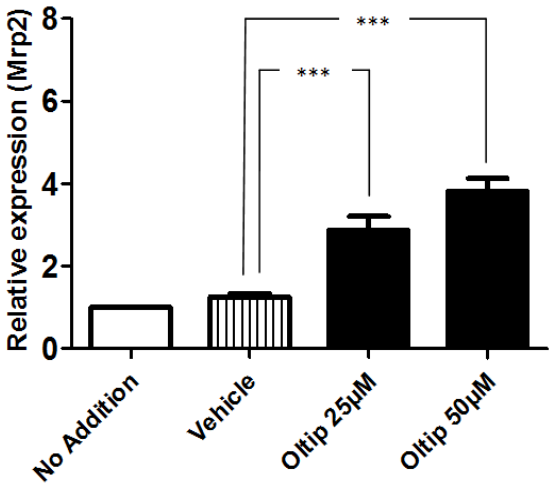

Figure 6.7: Oltipraz induces Mrp2 mRNA expression in H4IIE cells. Relative fold changed in the mRNA expression of Mrp2 (normalized to β-actin expression) in H4IIE cells treated with oltipraz compared with vehicle. The results are expressed as the mean ± SEM (n = 9, three individual experiments). Degrees of significance determined using ANOVA with Post Hoc Tukey's test for comparison of oltipraz with vehicle are *** P<0.001.

6.2.5 Dose-response curve by oltipraz on the expression of mRNA encoding Ntcp, and Mrp2 in H4IIE cells

To determine dose response curve on Ntcp and Mrp2 expression that gives half maximal value different concentrations of oltipraz were used. The results are shown in Fig. 6.8. The results show a monophasic dose response curve but Ntcp shows decreased expression with higher expression (A) and Mrp2 shows increased expression with higher concentration (B). The oltipraz concentration which gave half maximal (S0.5 value) induction and inhibition was 50 µM.

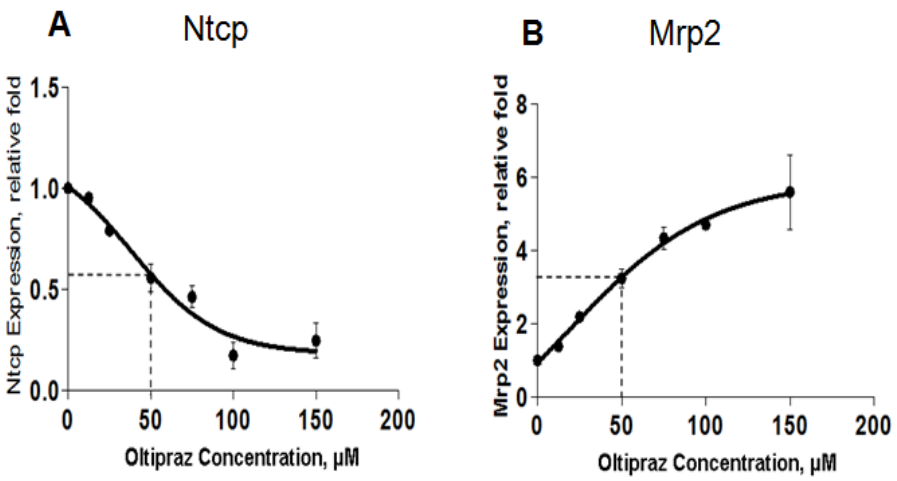

Figure 6.8: Dose-response curve for the reduction by oltipraz of the expression of mRNA encoding Ntcp in H4IIE cells. This line was achieved by fitting with linear regression equation (variable slope). Relative fold changed in the mRNA expression of Ntcp (normalised to β-actin expression) in H4IIE cells treated with oltipraz compared with H4IIE vehicle. The results are expressed as the means ± SEM (n = 9, three individual experiments).

6.3 DISCUSSION

The aims of these investigations were to detect the expression of bile acid transporters, to determine the relative expression and the effects of rapamycin and oltipraz on the expression of bile acid transporters in transformed liver cells. The main observations are that (i) expression of some bile acid transporters (Oatp9, Bsep and Bcrp) were not detected (see Table 6.1) and (ii) basal expression of bile acid transporters were significantly different in transformed liver cells compared to primary rat hepatocytes (see Table 6.2). Treatment with rapamycin caused an induction in the mRNA expression of bile acid transporters (with the exception of Oatp2) in H4IIE cells. However, oltipraz caused an inhibition in the mRNA expression of bile acid transporters (with the exception of Mrp2) in H4IIE cells.

Table 6.3: Summary of the effects of rapamycin and oltipraz on bile acid transporters mRNA expressions in H4IIE rat liver cells

Transporters	Action of rapamycin (compared to vehicle)	Effect of rapamycin in isolated cultured hepatocytes for 36 hours	Action of oltipraz (compared to vehicle)
Sinusoidal			
Ntcp	2.5-fold increased	3-fold decreased (Biphasic curve)	3-fold decreased
Oatp1	4-fold increased	5-fold decreased (Monophasic curve)	3-fold decreased
Oatp2	2-fold decreased	3-fold decreased	2-fold increased
Canalicular			
Mrp2	3-fold increased	4-fold increased	3-fold increased

6.3.1 Detection and comparison of bile acid transporters in H4IIE cells with that in isolated cultured rat hepatocytes

It was observed that some of the bile acid transporters were not expressed (Oatp9, Bsep, Bcrp) in H4IIE cells compared to normal primary hepatocytes. In contrast to these findings, Schäfer *et al.* found Bsep mRNA expression in H4IIE cells under osmotic conditions (Schäfer *et al.*, 2007). The reason that expression of some bile acid transporters were not observed in H4IIE cells could be due to: (i) lack of Bsep, Bcrp and Oatp9 mRNA expression in H4IIE cells, (ii) the mRNA in H4IIE cells may contain splice variants or mutations, altering the primer binding site, (iii) the promoter site of the gene in H4IIE cells may be rapidly cleaved or degraded at the site of primer binding, (iv) transcription factor or binding site may be altered or mutated in H4IIE cells, (v) the mRNA expression may be below the level of detection, (vi) a repressor may be bound within the promoter binding site and/or (vii) feedback inhibition may be occurring (Crocenzi *et al.*, 2008; Kubitz *et al.*, 2004).

In these studies, Bcrp was not detected in rat hepatocytes. Borght *et al.* also observed similar results (Borght *et al.*, 2006). In contrast, Tanaka *et al.* found low mRNA

expression of Bcrp in rat hepatocytes (Tanaka *et al.*, 2004). Bcrp is expressed at higher levels in the endothelium of small intestine, colon and brain rather than hepatocytes in rat (Tanaka *et al.*, 2004). However, Bcrp mRNA expression was detected in H4IIE cells while Bsep mRNA was not. This maybe due to the fact that H4IIE cells are derived from rat liver tumor tissue (Reuber, 1961).

6.3.2 Expression of bile acid transporters in H4IIE cells relative to that in rat hepatocytes

This study suggested that the relative expression of bile acid transporters (mRNA) was substantially different between the H4IIE cells and primary hepatocytes (See Table 6.2). Major sinusoidal transporters Ntcp, Oatp1 and Oatp2 expression were significantly lower in H4IIE cells compared to hepatocytes, which may suggest lower capacity to transport bile fluid. Differentiation of H4IIE cells treated by 0.1µM insulin and 0.1µM dexamethasone for 10 days did alter bile acid transporter expression to some extent but this expression was not the same as rat hepatocytes.

6.3.3 Effects of rapamycin on bile acid transporter expression in H4IIE cells

It was observed that rapamycin (0.1 and 0.5µM for 36-hour incubation) induced bile acid transporters expression except Oatp2 expression in H4IIE cells. Very little information has been reported about the effects of rapamycin on bile acid transporters in transformed liver cells. Picard *et al.* observed inhibition of Oatps expression in human HepaRG cell by rapamycin (10µM for 2 min) (Picard *et al.*, 2011). A similar observation was found by Oswald and his colleagues (Oswald *et al.*, 2010).

These results provide information that rapamycin may increase bile flow in transformed liver (H4IIE) cells i.e. in HCC cells, thus, rapamycin may improve liver function in HCC cells by increasing BA transporter expression.

<u>Likely mechanisms for expression of bile acid transporters by rapamycin in H4IIE cells</u>

The proposed or suggested mechanism how rapamycin may regulate bile acid transporter expression in rat liver cells has been described in the General Discussion and Chapter 4 (Fig 1.14). Similar effect of rapamycin on Mrp2 and Oatp2 mRNA expression was observed in isolated rat hepatocytes and H4IIE cells (see Tab. 6.3). Compared to isolated rat hepatocytes, rapamycin increased Ntcp and Oatp1 expression

in H4IIE cells whereas rapamycin decreased Ntcp and Oatp1 expression in isolated rat hepatocytes (see Tab. 6.3). The mechanism by which rapamycin changes expression in Ntcp and Oatp1 mRNA in H4IIE cells is unclear. However, the likely explanation could be in the following way:

1. Bile acid transporter expression is regulated by different regulatory mechanisms which depends on various transcription factors (such as RARα, RXRα, HNF4α, HNF1α for Ntcp expression; HNF4α, HNF1α for Oatp1 expression; PXR, CAR, RXRα, HNF1α for Oatp2 expression; PXR, CAR, FXR, RARα, RXRα, for Mrp2 expression) (Taruner *et al*., 2003); FXR, NR1H4, RXR for Bsep expression (Ananthanarayanan *et al*., 2001). mRNA, protein levels and transport activity are developmentally regulated and altered during enterohepatic circulation of bile acids (Ananthanarayanan *et al*., 2001). Rapamycin may alter these regulatory mechanisms in HCC cells (Chen *et al*., 2013; Crocenzi *et al*., 2008).

2. mTORC1 is the key regulator of rapamycin action and mTORC1 has dual function which can activate and inhibit Akt activity by phosphorylation and dephosphorylation (Sun *et al*., 2005; Sarbassov *et al*., 2006) that can be accountable for alteration of mTOR dependent signal transduction.

3. There is evidence that the activation site of Nrf2 may be altered in mutated cells which can interrupt Nrf2-Keap1 signal transduction pathway (Padmanabhan *et al*., 2006; Singh *et al*., 2006; Mitsuishi *et al*., 2012).

4. The dynamics of H4IIE cells are not represented in the *in vivo* environment accurately (Kubitz *et al*., 2004).

6.3.4 Effects of oltipraz on bile acid transporter expression in H4IIE cells

The results showed that oltipraz inhibited Ntcp, Oatp1, Oatp2 mRNA expression but induced Mrp2 mRNA expression. There is no literature found that oltipraz has changed the expression of these bile acid transporters in H4IIE cells. This investigation suggests that oltipraz may reduce bile flow in HCC patients due to decreased expression of some important bile acid transporters, resulting cholestatic effect may appear in HCC patient.

Likely mechanisms for expression of bile acid transporters by oltipraz in H4IIE cells

The intracellular mechanism how oltipraz may alter bile acid transporter expression is described in the General Introduction (Fig. 1.16.). The likely explanation of increased bile acid transporter expression by oltipraz would be due to increase Nrf2 activation by oltipraz in H4IIE cells. Several studies provide evidence about the activation of Nrf2 by oltipraz (Weerachayaphorn *et al.*, 2009; Maher *et al.*, 2007).

6.4 CONCLUSION

On the basis of mRNA expression, transformed liver cells are predicted to exhibit a lower capacity to transport bile acids than normal hepatocytes. The actions of rapamycin on expression of some bile acid transporters in transformed liver cells differ from those in rat hepatocytes. However, on the basis of mRNA expression, our results suggest that oltipraz inhibits bile acid transport in transformed liver (HCC) cells.

CHAPTER VII: GENERAL DISCUSSION

7.1 RESULTS SUMMARY

Results of this study have shown that rapamycin increased HO-1 and Prx-1 mRNA expression in rat liver *in vivo* and in cultured rat hepatocytes. In contrast, rapamycin inhibited HO-1 and Prx-1 mRNA expression in transformed rat liver (H4IIE) cells. However, oltipraz induced HO-1 and Prx-1 mRNA expression in H4IIE cells similar to rat hepatocytes. Some bile acid transporters were not detected in H4IIE cells. Rapamycin inhibited bile acid transporters expression except Mrp2 in rat liver *in vivo* but induced Mrp2 and Bsep expression in primary rat hepatocytes. In contrast, rapamycin induced bile acid transporters expression except Oatp2 in H4IIE cells. Oltipraz inhibited bile acid transporters expression except Mrp2 similar to primary rat hepatocytes treated by rapamycin.

These results suggest that rapamycin and oltipraz have the same effect on canalicular transporter expression in normal and transformed rat liver cells. However, rapamycin and oltipraz differ in their effect on expression of sinusoidal transporters. It seems that inhibition of sinusoidal transporters *in vivo* may impede entry of bile acids and other ions to the hepatocytes during the enterohepatic circulation, thereby accounting for the inhibition of bile flow recovery after ischemia reperfusion injury (Kist *et al.*, 2012; Tanaka *et al.*, 2006; Fouassier *et al.*, 2007). H4IIE (transformed) cells exhibit a lower capacity to transport bile acids and are less responsive to the actions of rapamycin in inducing expression of antioxidant enzymes. Evidence in the literature suggests that clinical pre-treatment with rapamycin or oltipraz is a useful strategy to protect the liver from ischemia reperfusion injury following liver surgery (Asworth and Wu, 2014; Toso *et al.*, 2010; Kim *et al.*, 2010; Bodeman *et al.*, 2013;). However, the results reported in this thesis suggest that pre-treatment with rapamycin or oltipraz is associated with the inhibition of bile acid transporter expression.

Table 7.1: Comparison of rapamycin concentrations for actions in liver cells in culture and *in vivo*

Parameter or condition	Effect of rapamyin	Conc. of rapamycin which gives half maximal response
Rat hepatocytes		
HO-1 mRNA	Induction	0.05 µM (biphasic)
	Inhibition	0.5 µM
Prx-1 mRNA	Induction	0.05 µM (biphasic)
	Inhibition	0.5 µM
Ntcp mRNA	Induction	1.0 µM (biphasic)
Oatp1 mRNA	Inhibition	0.05 µM (monophasic)
Bsep mRNA	Induction	0.05 µM (biphasic)
	Inhibition	1.0 µM
H4IIE cells		
HO-1 mRNA	Induction	0.5 µM (monophasic)
Prx-1 mRNA	Induction	0.1 µM (monophasic)
Liver *in vivo*		
Treatment of liver surgical patient (Shinke *et al.*, 2013)	Blood concentration after 24 h	0.01-0.027 µM

Table 7.2: Comparison of oltipraz concentrations for actions in culture and *in vivo*

Parameter or condition	Effect of oltipraz	Conc. of oltipraz which gives half maximal response
H4IIE cells		
HO-1 mRNA	Induction	50 µM
Prx-1 mRNA	Induction	50 µM
Ntcp mRNA	Inhibition	50 µM
Mrp2 mRNA	Induction	50 µM
Treatment of liver surgical patients (Kim *et al.*, 2010)	Blood concentration range	35 µM

7.2 Evaluation of pre-treatment of liver with rapamycin as a strategy for reducing ischemia reperfusion injury following liver surgery

As discussed in the General Introduction, ischemia and reperfusion injury generates a large amount of ROS resulting in oxidative stress and tissue damage after liver surgery. It is very important to restore redox balance between radical-generating and radical-scavenging capacity to protect liver from additional oxidative injury (Weerachayaphorn *et al.*, 2014; Copple *et al.*, 2010). Theoretically, the induction of HO-1 and Prx-1 is predicted to reduce ROS and hence, be useful. Specific pathways can be activated to reduce ROS by inducing stress response pathway involves enhancing the antioxidant gene expressions including HO-1 and Prx-1 (Klaassen and Reisman, 2010; Yerushalmi *et al.*, 2001; Kim *et al.*, 2007). Besides this, induction of HO-1 and Prx-1 expression by pre-treatment with pharmacological agent can be a viable strategy to reduce ROS and protect liver from damage.

In the present study, it was observed that pre-treatment with rapamycin can induce HO-1 and Prx-1 expression in normal liver after liver surgery. It is thought that this induced HO-1 and Prx-1 can reduce ROS production through inactivation of Kupffer cells and neutrophils. This results in reduction of ROS-induced apoptosis and necrosis and thereby, protects liver from excessive damage. Thus, rapamycin pre-treatment can protect liver from damage by removing ROS during ischemia reperfusion injury. Therefore, pre-treatment by rapamycin induces anti-oxidant enzymes which could be beneficial for patients who will undergo liver surgery.

However, pre-treatment with rapamycin also inhibits bile flow due to decreased expression of sinusoidal transporters which means bile acid cannot pass into hepatocytes to the bile duct. This creates an accumulation of bile acid in the liver that will be toxic (Weerachayaphorn *et al.*, 2014). Exposure of hepatocytes to these increasing levels of toxic bile acids can result in production of ROS, leading to oxidative stress and progressive liver damage (Copple *et al.*, 2010). Decreased flow of bile acid causes synthesis of excessive extracellular matrix proteins that result in liver fibrosis (Weerachayaphorn *et al.*, 2014; Hemmann *et al.*, 2007).

However, there is some limitation of this mechanism (Copple *et al.*, 2010). Results of this study have been shown that rapamycin can decrease Oatps expression in normal

liver that will not be detrimental for the post-surgery setting. Because rapamycin has a very low affinity for Oatps and the role of Oatps is insignificant as compared with passive diffusion at low concentration of rapamycin (Oswald *et al.*, 2010; Picard *et al.*, 2011). Therefore, rapamycin pre-treatment might be effective after liver surgery when rapamycin blood concentration will be range within 0.015 µM (Shinke *et al.*, 2013).

7.3 Evaluation of treatment with rapamycin as a strategy for the HCC patients who are ineligible for surgery or wait for surgery

Not all the HCC patients are eligible for liver transplantation or resection due to metastatic, extensive tumor involvement, invasion of the hepatic or portal vein or advanced hepatocelluar disease (Zollner *et al*., 2005). Inflammation and further tumor or HCC development cause adverse condition for the HCC patients. In HCC patients, upto 70% tissue can be tumor or cancer tissue (Colli *et al.*, 2006). It is important to reduce inflammation and tumor or HCC growth for the patients who are required to go for surgery or who are ineligible for surgery. Therefore, using immunosuppressant and antitumor agent is a viable strategy for the HCC patients to reduce inflammation and further tumor or HCC development.

Data of this study indicate that the basal expression of antioxidant enzymes (HO-1 and Prx-1) and bile acid transporters in transformed liver cells (HCC cells) is lower than that of primary hepatocytes. This result suggests that HCC cell has lower capacity to induce antioxidant enzymes and to transport bile acid and drug from blood into bile duct. However, our data indicate that in cancer tissue, rapamycin does not induce (HO-1 and Prx-1) expression but induce bile acid transporter expression. Inhibition of transformed liver cell (HCC cell) proliferation treated by rapamycin was observed in this study. These results are consistent with others reports who showed that rapamycin has ability to inhibit tumor growth in HCC patient (Liao *et al.*, 2015; Ashworth and Wu, 2014; Menon *et al.*, 2013; Heuer *et al.*, 2009). But, it is still unclear whether antioxidant enzyme is correlated with tumor re-growth or not. This result suggests that rapamycin treatment may not be a beneficial therapeutic agent for late stage or advanced stage HCC patients who will undergo liver resection or transplantation but indicate improved liver function. However, using rapamycin may be beneficial after post-transplantation settings when HCC cells will be removed by inducing antioxidant enzymes. Therefore, rapamycin should not be considered to be a unique

chemotherapeutic agent, but considered as an aid to prevent tumor or HCC development at early stage of HCC.

On the other hand, rapamycin increased bile acid transporter expression in H4IIE cells or HCC cells. While present in excess, bile acid can create a loss of gap junction proteins thereby reducing permeability and a fail of the bile osmotic gradient (Trauner *et al.,* 1998). Thereby, reduction in movement across membranes can cause accumulation and cellular swelling (Trauner *et al.,* 1998). This result suggests that using rapamycin for a long time can cause accumulation of bile acids in the liver which will be toxic in HCC patients that can turn into liver damage. Therefore, using rapamycin for an extended time may be detrimental in HCC patients who will not go surgery but it is warranted to investigate the clinical effect of rapamycin on HCC patient in context to impact of bile acid accumulation. However, treatment by rapamycin may improve liver function by increasing bile acid transporter expression in HCC patient. This increased bile acid transporter expression may contribute against chemoresistance in HCC patients. Thus, it is still unclear using rapamyin could be beneficial without any detrimental effects.

7.4 Evaluation of treatment with rapamycin as a strategy for the post surgery HCC patients

As discussed in the General Introduction, liver transplantation or resection is the best option for the treatment of advanced HCC or metastatic cancer. Graft survival and reducing posttransplantation complication is the principal concern of this option. HCC or tumor recurrence is the major posttransplantation problem which limits the success of liver transplantation or resection. There is no standard therapy to resolve all the issues. However, calcineurin inhibitors are considered the central immunosuppressive therapy for liver transplantation or resection. However, HCC or tumor recurrence is the main reason to limit the usage of these drugs. mTOR inhibitors are considered candidates as effective immunosuppressants and agents for reducing tumor recurrence.

These studies indicate that treatment by rapamycin can induce antioxidant enzyme HO-1 and Prx-1 expression that will be beneficial to reduce inflammation after post transplantation or resection of liver and increase graft survival from inflammation. It has been also reported by these studies that rapamycin can reduce HCC cell

proliferation suggested reducing tumor recurrence after posttransplantaion settings. However, rapamycin can reduce cell viability suggesting that it may not be an ideal therapy for liver regeneration.

On the other hand, decreased bile acid transporter expression is associated with the induction of antioxidant enzyme (HO-1 and prx-1) expression by rapamycin. These results suggest that treatment by rapamycin for long time may appear cholestatic effect after posttransplantation or resection. Moreover, it is presumed that decreased bile acid transporter expression in hepatocytes can cause drug resistance or chemoresistance. Thus, treatment with rapamycin can be beneficial but optimum dose should be determined to minimise detrimental effects associated with decreased bile acid transporter expression.

7.5 Clinical effect of rapamycin concentration and time exposure on antioxidant enzymes and bile acid transporters

The degree of antioxidant enzyme and bile acid transporter expression is dependent on the concentration and time of exposure (Shinke *et al.*, 2013; Kist *et al.*, 2012). The concentrations of rapamycin by incubating for 36 hours which gave half maximal expression of antioxidants and bile acid transporters are in Table-7.1. Monophasic and biphasic dose response curve were observed in this investigation. The clinical rapamycin blood concentration seems to be about 0.01 to 0.027 µM after 24 hours (Picard *et al.*, 2011; Campone *et al.*, 2009; Yao *et al.*, 2010; Shinke *et al.*, 2013). The observed concentrations (0.01-5.0µM for 36 hour) of this study are above in the range of rapamycin blood levels usually observed in post-transplantation settings. This would be a likely explanation for unexpected results in these studies.

The observed biphasic curves indicate that different concentrations can have noticeably different effects in expression of antioxidant enzymes and bile acid transporters. This result suggests that increased blood concentration of raapamycin may not be effective, however, turn into detrimental outcome. Therefore, it is highly significant to maintain rapamycin blood concentration to get beneficial or optimum therapeutic result.

7.6 Evaluation of treatment with oltipraz as a strategy for the HCC patients who are ineligible for surgery or wait for surgery

As discussed above and in the General Introduction, all HCC patients are not eligible for liver transplantation or resection by surgery due to advance staged HCC. However, at an early stage of HCC patients who will undergo liver surgery it is essential to minimize immunosuppressive regimens and HCC or tumor development for improved prognosis. Calcinurin inhibitors or mTOR inhibitors are currently used as immunosuppressive agents for reducing inflammation or tumor growth but both have some limitations. As discussed above, rapamycin treatment cannot induce HO-1 and Prx-1 expression in HCC cells.

These studies have shown that oltipraz induces HO-1 and Prx-1 mRNA expression in transformed rat liver cells (HCC cells) which is very important to reduce inflammation by reducing ROS production in HCC patient. Thus, oltipraz can protect from progressive liver damage by balancing redox generation. This result suggests that oltipraz may reduce cancer cell growth by balancing inflammatory environment through reducing redox generation. Moreover, oltipraz can decrease proliferation and cell death in HCC cells. Thus, treatment by oltipraz can be a beneficial strategy to protect liver by reducing inflammation or HCC growth in the HCC patients.

In this study, oltipraz can reduce H4IIE cell proliferation but increase viable H4IIE cells. This result suggests that oltipraz may not be a beneficial agent for liver regeneration after liver surgery for HCC patient. But, it is yet unknown about tumor or HCC recurrence after oltipraz treatment of HCC patient who will go liver surgery or transplantation.

In contrary, oltipraz inhibits sinusoidal bile acid transporters expression in HCC cells which are responsible in obstructing entry of bile acids in the hepatocytes. This result suggests that inhibition of increasing bile acids in hepatocytes can protect from hepatic accumulation of toxic bile acids, hepatocellular injury and fibrosis (Weerachayaphorn *et al.*, 2012). Therefore, pre-treatment of oltipraz can be a beneficial strategy for the late staged or advanced staged HCC patients. However, there is possibility to induce cholestasis by decreasing bile acid transportation.

Bile flow and bile acid transporter expression *in vivo* were not measured in this study. Therefore, it is still unclear whether oltipraz can inhibit bile flow due to inhibition of sinusoidal bile acid transporter expression. However, Weerachayaphorn *et al.* showed that oltipraz can increase bile flow rates due to increased canalicular bile acid transporter expression (Weerachayaphorn *et al.*, 2014).

7.7 Clinical effect of oltipraz concentration and time exposure on antioxidant enzymes and bile acid transporters

In this investigation, higher doses of oltipraz (upto 150 μM for 36 hours) were used and a monophasic dose response curve of antioxidant enzyme and bile acid transporter expression was observed. The concentration of oltipraz which gave half maximal expression of antioxidant and bile acid transporter expression are shown in Table. 7.2. The concentration of oltipraz which gave half maximal induction of antioxidant enzyme and bile acid transporter expression was 50 μM in H4IIE cells. This concentration is in the range employed clinically (30~50 μM, Kim *et al.*, 2010). This result suggests that implementation of higher doses will not be detrimental but it is important to use optimum doses. Higher doses and a long-dosing interval of oltipraz dosage regimens have been found to be more efficacious in preventing cancer in human studies (Wang *et al.*, 1999; Ko *et al.*, 2006; Jackson and Groopman, 1999; Jacobson *et al.*, 1997).

7.8 Some limitations of this research project addressed

7.8.1 Using qPCR data

The transcription data is useful for identifying potential candidates for follow-up work at the protein level. However, changes in gene expression level are frequently not reflected at the protein level. Therefore, there is a poor correlation between mRNA and its relevant protein level. It is reasonable to see high protein level with high mRNA level but, the protein concentration is affected by many steps in transcription and translation as well as degradation. The half-life of different proteins can vary from minutes to days - whereas the degradation rate of mRNA would fall within a much tighter range (2-7 hours for mammalian mRNAs vs 48 hours for protein) (Vogel and Marcotte, 2012). Thus, current results are warranted to complete the protein expression level of antioxidant enzymes and some important bile acid transporters for confirmation of rapamycin and oltipraz effects on both rat liver and transformed liver cells.

7.8.2 Lack of evidence for increased levels of Akt-pT308/pS473 or Nrf2 expression

As discussed in the General Introduction, Nrf2 is the key regulator of the Akt-Nrf2 pathway which is controlled by the activation or inhibition of antioxidant enzymes and bie acid transporter expression in normal and transformed liver cells (Fig. 1.14 and 1.16). Current studies showed that Nrf2 can cross-talk with other pathways that are important for cell survival (Wakabayashi *et al.*, 2010). However, in this research project, Nrf2 was not measured. It is very important to determine whether rapamycin and oltipraz can activate antioxidant enzyme and bile acid transporter expression through the Akt-Nrf2 pathway or not. Thus, measurement of Akt and Nrf2 expression level through immunoblotting or immunofluorescence microscopy needs to be completed.

7.9 PROPOSED FUTURE EXPERIMENTS TO INVESTIGATE THE ACTIONS OF RAPAMYCIN AND OLTIPRAZ ON THE LIVER

This thesis provides novel information regarding the effects of rapamycin and oltipraz on antioxidant enzyme and bile acid transporter mRNA expression in normal and transformed liver cells. Several important questions remain unclear that can be addressed for future work.

7.9.1 Future experiments to investigate whether pre-treatment with oltipraz as a potential strategy to reduce ischemia reperfusion injury

For achieving this goal, the list of investigations should be the following-

- Rat hepatocytes in normal culture
- Test the expression of antioxidant enzymes and bile acid transporters
- Cultured rat hepatocytes under hypoxic conditions
- Test the ability of oltipraz to protect cells from hypoxic damage

  rat liver *in vivo*
- Test the ability of oltipraz to protect liver from ischemia reperfusion injury

7.9.2 Future experiments to investigate whether pre-treatment with rapamycin as a potential strategy to reduce ischemia reperfusion injury

For achieving this goal, the list of investigations should be the following-

- Pre-treatment with rapamycin in rat liver *in vivo* at different time points

- Test the ability of rapamycin at different concentrations to protect liver from ischemia reperfusion damage *in vivo*

7.9.3 Future experiments to investigate intracellular mechanisms of action of rapamycin and oltipraz on antioxidant enzyme and bile acid transporter expression

For achieving this goal, the list of investigations should be the following-

- Confirm the results obtained from mRNA by immunoblotting assays for protein expression and enzymatic activity assays for enzyme activities
- Test the effect of rapamycin and oltipraz on Akt and transcription factor Nrf2 by immunoblotting assays or immunofluorescence microscopy
- Test the effect of rapamycin on PI3 kinase

7.10 GENERAL CONCLUSION

To sum up all the results, it can be concluded that pharmacological pre-treatment with rapamycin may not be so effective in reducing ischemia reperfusion injury since the induction by rapamycin of antioxidant enzyme expression in normal liver cells is modest; while in transformed cells expression of antioxidant enzymes is inhibited. The inhibition of bile flow associated with pre-treatment with rapamycin is likely due to inhibition of the expression of sinusoidal bile acid transporters in normal liver cells. In the clinical treatment of HCC patients with rapamycin, attention needs to be paid to the blood concentration of the drug as the actions of rapamycin on expression of both antioxidant enzymes and bile acid transporters are very dependent on the concentration of rapamycin (and presumably also time of exposure). The ability of transformed liver cells to transport bile acids is likely impaired compared to that of normal liver cells, and the actions of both rapamycin and oltipraz on the expression of bile acid transporters are mixed. Further studies are warranted, to determine conditions under which pre-treatment with oltipraz can be effective to reduce ischemia reperfusion injury with minimal effects on bile flow.

Pre-treatment with rapamycin (to reduce ROS) is theoretically a useful strategy to protect the liver from ischemia reperfusion injury following liver surgery. Pre-treatment with rapamycin is associated with the inhibition of bile acid transporter expression. This is potentially detrimental to the outcome of patients following liver surgery.

Transformed liver cells (hepatocarcinoma cells) exhibit a lower capacity to transport bile acids and are less responsive to the action of rapamycin in inducing expression of antioxidant enzymes than normal hepatocytes. Oltipraz is a promising chemotherapeutic agent for the treatment of HCC patients by inducing antioxidant gene expression, although bile acid transporter expression is also blunted.

APPENDICES

I. Presentation in scientific congresses as a result of Ph.D. studies

Afroz F, Padbury R, Nieuwenhuijs V, Barritt J: Regulation by rapamycin of heme oxygenase-1 expression in liver cells. ASMR Medical Research Week, SA Scientific Meeting 2012, held at The Adelaide Convention Centre, South Australia, Australia, 6th June, 2012. Abstract no P4, Page no. 119 (poster presentation).

Afroz F, Padbury R, Nieuwenhuijs V, Barritt J: Regulation by rapamycin and Nrf2 of the expression of anti-oxidant enzymes and bile acid transporters in liver cells. ASMR Medical Research Week, SA Scientific Meeting 2013, held at The Adelaide Convention Centre, South Australia, Australia, 5th June, 2013. Abstract no OP18, Page no. 65 (oral presentation).

Afroz F, Padbury R, Nieuwenhuijs V, Barritt J: Regulation by rapamycin of the expression of bile acid transporters in liver cells. ASCEPT 2013, Annual Scientific Meeting, held at RACV City Club, Melbourne, Australia, 1-4 December 2013. Abstract no P552, Page no. 140 (poster presentation).

II. Awards during Ph.D.

International Postgraduate Research Scholarship (IPRS) (2011). Australian Government scholarship to support international PhD student at Flinders University, South Australia.

Australian Postgraduate Award (APA) (2012). Australian Government scholarship for postgraduate studies.

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