The Identification of Nuclear Receptors Associated with Hepatic Steatosis to Develop and Extend Adverse Outcome Pathways

C.L. Mellor, F.P. Steinmetz and M.T.D. Cronin*

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, England

*Corresponding author: Tel. +44 151 231 2402; e-mail address: M.T.Cronin@ljmu.ac.uk (Mark Cronin)

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Abstract

The development of Adverse Outcome Pathways (AOPs) is becoming a key component of 21st century toxicology. AOPs provide a conceptual framework that links the molecular initiating event to an adverse outcome through organised toxicological knowledge, bridging the gap from chemistry to toxicological effect. As nuclear receptors (NRs) play essential roles for many physiological processes within the body, they are used regularly as drug targets for therapies to treat many diseases including diabetes, cancer and neurodegenerative diseases. Due to the heightened development of NR ligands there is increased need for the identification of related AOPs to facilitate their risk assessment. Many NR ligands have been linked specifically to steatosis. This paper reviews and summarises the role of NR and their importance with links between NR examined to identify plausible putative AOPs. The following NRs are shown to induce hepatic steatosis upon ligand binding: aryl hydrocarbon receptor, constitutive androstane receptor, oestrogen receptor, glucocorticoid receptor, farnesoid X receptor, liver X receptor, peroxisome proliferator-activated receptor, pregnane X receptor, and the retinoic acid receptor. A preliminary, putative AOP was formed for NR binding linked to hepatic steatosis as the adverse outcome.

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Introduction

AOP Development

The impact of 21st century life has led to increased demand for safer and more sustainable chemical products, whilst at the same time reducing the amount of animal testing (Patlewicz et al., 2015). Animal testing has been integral to toxicology studies for over 50 years; however, the need for more modern alternative toxicological approaches is now a key focus for toxicologists and regulatory scientists alike (NRC, 2007). For instance, the results gained from animal testing require many presumptions and extrapolations in order to predict human health effects and this process is still controversial due to both scientific and ethical reasons (NRC, 2007). Current chemical safety assessments require faster testing with the use of fewer animals, at the same time the number of chemicals being tested are rapidly increasing worldwide, making the cost of chemical safety assessment substantial (Patlewicz et al., 2015). The constraints of the modern chemical safety assessments, along with the increased cost of testing, have induced a new mind-set among toxicologist aiming to replace the more traditional extensive phenotypic animal testing with a more mechanistic based approach relying on the use of toxicokinetics, computational models and *in vitro* testing (Patlewicz et al., 2015; Vinken, 2013). The momentum of this change has led to big strides in the development of *in vitro* assays for use in high throughput screening and high content screening (seen in projects such as the United States Environmental Protection Agency's (US EPA's) ToxCast programme) (Cohen et al., 2010; NRC, 2007) and the development of computational approaches. Computational models are integral to the development of integrated alternative methods to identify organ level toxicity and ultimately leading towards the replacement of animal testing (Cronin and Livingstone, 2004). These models traditionally include the use of (quantitative) structure-activity relationships ((Q)SARs). QSARs are mathematical models that predict biological activity of chemicals from structure or physicochemical properties, whereas a SAR is a

qualitative association between a specific molecular substructure and biological activity (Cronin and Livingstone, 2004).

(Q)SARs are currently used successfully for the prediction of single dose acute toxicity and specific endpoints such and mutagenicity, however, the use of QSARs for the prediction of repeat dose systemic toxicity, carcinogenicity and reproductive toxicity poses a real challenge for computational scientists (Alder et al., 2010; Gocht et al., 2015; Hartung et al., 2011). This has led to the development of integrated testing strategies which use a combination of computational models (such as structural alerts, read across, QSARs and modelling) along with *in vitro* testing in order to develop alternative methods. The integration of alternative methods with pathways of toxicity has, in part, also catalysed the formation of the Adverse Outcome Pathway (AOP) framework concept (Ankley et al., 2010; Groh et al., 2015; Vinken, 2013, 2015). An AOP describes the causal linkage between a molecular initiating event (MIE) and an adverse outcome at individual or population levels (Patlewicz et al., 2015). The data-richness of an AOP is critical for driving its practical application therefore the process of data mining to find mechanistic connections is receiving considerable interest (Patlewicz et al., 2015). Repeat dose systemic organ toxicity is currently an area of considerable interest for AOP development, with the liver being prominently researched due to its integral links to drug/ chemical metabolism and effects. The structure of an AOP consists of a molecular initiating event (MIE) which is then linked via key events and ends in a particular adverse effect (as summarised in Figure 1) (Ankley et al, 2010; AOP wiki, 2015). As seen in Figure 1, the key events recorded within an AOP usually consist of the transition from the initiating event, to cellular, tissue and organ-level responses and may also be extrapolated to organism (or even population / ecosystem for environmental effects) (Vinken, 2013, 2015).

FIGURE 1 HERE

A number of free-to-use tools are available for the scientific community to contribute towards the development of AOPs. These have recently been formalised into the Adverse Outcome Pathway Knowledge Base (AOP-KB) which was released in September 2014 (AOP-KB, 2015). The AOP-KB is an online space dedicated to gathering all the work undertaken worldwide on the development of AOPs into one easily accessible web-site. The AOP-KB is an Organisation for Economic Co-operation and Development (OECD) initiative involving collaborations with the European Commission's Joint Research Centre (JRC), US EPA and the US Army Engineer Research and Development Center (ERDC). It gathers four individually developed platforms in one place; the AOP wiki, AOP xplorer, intermediate effects database and effectopedia, as well as allowing for third party applications and plug-ins. The AOP-KB allows a contributor to build an AOP within the AOP wiki by entering information about MIE and key events associated with a particular AOP. As scientists understand that mechanistic pathways are diverse and not homologous, the AOP-KB allows the links between different MIEs, key events and AOPs forming a branching connection which can be visualised via the AOP xplorer platform. The AOP-KB allows users to gain insight into currently known AOPs, add information, comment on particular AOPs and review work that has already been carried out by other stakeholders/ users. The AOP wiki has been successful at engaging scientists to add details for mechanistic pathways they have data/ knowledge on allowing the progression of AOP development.

Due to their involvement in many essential processes within the body, the search for novel ligands for Nuclear Receptors (NR) has recently been intensified in order to elucidate possible preventative / therapeutic treatments for a wide range of diseases including diabetes, cancer, cardiovascular diseases, atherosclerosis, neurodegenerative diseases and obesity (Love, 2006). However, the induction of some NR ligands has been linked to the development of drug

induced liver injury (DILI) such as liver steatosis, due to the bio-activation of drugs (or metabolites) and / or the induction of hepatotoxic pathways (Love, 2006). The mechanisms behind these hepatotoxic pathways and the chemical structures of the ligands that induce them must first be understood before the definition of the characteristics for binding (which would be essential for the development of structural alerts) can take place. NRs are linked to onset of hepatic toxicity, especially hepatic steatosis.

Hepatic Steatosis

Hepatic steatosis is induced via the excessive accumulation of fats (triglycerides) within the hepatic parenchymal cells (hepatocytes) of the liver (Reddy and Roa, 2006; Zafrani, 2004; Nguyen et al., 2008). Hepatic steatosis usually occurs as the first stage of fatty liver disease, if the cause persists steatosis typically progresses to steatohepatitis (inflammation of the liver cells), cirrhosis (scarring of the liver) and liver cancer (Reddy and Roa, 2006). The onset of hepatic steatosis is associated with many different causes including alcoholism, diabetes, obesity and mitochondrial dysfunction (Reddy and Roa, 2006). Morphologically, hepatic steatosis presents as the accumulation of both large (macrovesicular) and small (microvesciular) intra-cytoplasmic fat droplets within hepatocytes which cause cytoplasm displacement. The macrovesicular fat droplets are found in cases of alcoholic, diabetic or obese patients and also present in cases of malnutrition such as immune deficiency syndrome. Macrovesicular steatosis gives rise to a large single vacuole of fat which fills the cytoplasm of hepatocytes and leads to the displacement of the nucleus (Zafrani, 2004). Microvesicular steatosis is present mostly in steatosis induced via β-oxidation of fatty acids (either mitochondrial or peroxisomal) and presents as the formation of many smaller fat droplets leaving the nucleus at the centre of the hepatocytes (Zafrani, 2004). The diagnosis of steatosis is made when lipid content in the liver exceeds 5–10% of the liver's total weight (Zafrani, 2004).

This critical review assesses NR induced hepatic steatosis considering the availability of information from extant AOPs. The purpose is to demonstrate, through relevant AOPs, the linkage of the receptor mediated molecular initiating event to adverse outcome. There are currently two AOPs listed on the AOP wiki that have hepatic steatosis as their adverse outcome (AOP wiki, April 2015). The first is aryl hydrocarbon receptor (AHR) leading to hepatic steatosis, for which there is strong evidence but it is currently under construction. The second is liver X receptor (LXR) activation to liver steatosis, which is under construction and the weight of evidence is unspecified (AOP wiki, April 2015).

NRs linked previously to the onset of liver injury are summarised in Table 1.The aim of this study was to utilise the information captured in Table 1, gathering the current knowledge of the mechanistic pathways of NRs, in order to investigate which are associated with the adverse outcome of hepatic steatosis.

TABLE 1 HERE

Nuclear Receptors

Those NRs associated with liver injury (Table 1) were studied with the objective of compiling knowledge to support the development and extension of an AOP with NR activation being the MIE and hepatic steatosis being the adverse outcome and ultimately allowing for the creation of (chemistry-based) structural alerts (although this is not the purpose of this investigation). The following summarises the pertinent information found within the literature for each NR:

Aryl Hydrocarbon Receptor (AHR)

The AHR is a ligand-activated transcription factor that is involved in the regulation of the biological response to aromatic hydrocarbons (Nebert et al., 2004). Research has shown the AHR regulates xenobiotic metabolising enzymes, for example cytochrome P450. AHR is located within the cytosol when in its inactive form bound to its co-chaperones. Upon ligand

activation, the co-chaperones dissociate and AHR translocates to the nucleus. Once at the nucleus AHR undergoes dimerisation to AHR nuclear translocator (ARNT) which elicits a change to gene transcription (Elferink et al., 1990). Activation of the AHR is known to induce many toxic responses such as teratogenicity, immunotoxicity, tumour promotion and lethality (Pelclova et al., 2006). Due to its abundance within the liver, activation of the AHR has been shown to cause the onset of hepatic steatosis (Boverhof et al., 2006; Li et al., 1994; Niittynen et al., 2007). The AHR has been linked to hepatic steatosis though its ability to upregulate CD36 and to activate the PPARα receptor (see PPARα section). CD36 maintains uptake and intracellular trafficking of fatty acids and is also essential for the esterification of fatty acids into triglycerides. Up-regulation of CD36 via the AHR receptor causes increased fatty acid influx from the peripheral tissues (Figure 2) leading to the accumulation of triglycerides within the liver causing the formation of micro and macrovesciular fat droplets (He et al., 2011; Kawano et al, 2010). The accumulation of micro and macrovesciular fat droplets, as previously discussed, induces nucleus distortion, mitochondrial disruption and endoplasmic reticulum stress causing fatty liver which leads to hepatic steatosis when the lipid concentration reaches 5-10% of total liver weight (Dentin et al 2006; Miquilena-Colina et al.,2011).

Constitutive Androstane Receptor (CAR)

The CAR, also called the nuclear receptor subfamily 1, group I, member 3(NR1I3) (Base et al., 1994), is an orphan nuclear receptor that is an essential regulator of drug metabolising enzymes (Yamamoto et al., 2004). Recent studies have highlighted its importance for the control of enzymes such as CYP450s (Honkakoski et al., 1998; Sueyoshi et al., 1999), multidrug-resistant proteins (MRPs) (Cherrington et al., 2002; Kast et al., 2002) and UDP-glucuronosyltransferase (UGT) (Sugatani et al., 2001; Xie et al., 2003). Investigations have determined that CAR is

involved in the regulation of both bile acid (Saini et al., 2004) and bilirubin (Huang et al., 2004; Moreau et al., 2008; Xie et al., 2003) induced liver injury.

CAR forms a heterodimer with RXR upon ligand binding; this mediates transcriptional up-regulation of target genes (Suino et al., 2004). Analogous to PXR, CAR has a highly conserved DNA Binding Domain (DBD) and a moderately conserved Ligand Binding Domain (LBD) (Suino et al., 2004). Similar to the PXR NR, CAR activation can be induced via a wide range of chemicals such as the antiemetic chlorpromazine, the anti-inflammatory drug acetaminophen and the barbiturate phenobarbital (Moreau et al., 2008). Studies have shown that PXR and CAR share some chemical ligands with results showing them to have different ligand binding and activational properties (Maglich et al., 2009; Osabe et al., 2008). Investigations revealed that most CAR agonists, instead of inducing activation by direct binding, induce CAR translocation from the cytoplasm in to the nucleus (Maglich et al., 2009; Osabe et al., 2008). The predominant difference between CAR and other ligand-dependant nuclear receptors is that CAR is constitutionally active (Suino et al., 2004).

CAR activation has been shown to affect lipid homeostasis (Moreau et al., 2008). Studies have demonstrated that upon agonist binding and activation, CAR facilitates fat accumulation and leads to enhanced hepatic steatosis *in vivo* (Xie et al., 2003). The activation of CAR leads to increased expression of PPARγ (see this section) and Sterol Regulatory Element Binding Protein-1c (SREBP-1c) (Moreau et al., 2008; Wada et al., 2009). SREBP-1c expression leads to up-regulation of lipogenic enzymes essential for *de novo* lipogenesis (lipid production within the cell). Its activation via the CAR receptor leads to increased *de novo* synthesis (Figure 2) and causes lipid accumulation within hepatocytes (Azzout-Marniche et al., 2000 Foretz et al., 1999; Nguyen et al., 2007). Lipid accumulation can lead to the production of micro and macrovesicular fat droplets within hepatocytes if the CAR agonist is not removed, resulting in

a fatty liver which leads to the onset of hepatic steatosis (Azzout-Marniche et al., 2000 Foretz et al., 1999; Nguyen et al., 2007).

oEstrogen Receptor (ER)

The ER, which is also known as the nuclear receptor subfamily 3, group A, member 1/2 (NR3A1/2), is essential for the normal development and maintenance of the sexual and reproductive functions in both males and females (Heldring et al., 2007). ER signalling is linked to a wide range of physiological effects in biological systems such as the immune, cardiovascular, musculoskeletal and the central nervous system in both men and women (Gustafsson, 2005). The ER has two receptor subtypes ERα (NR3A1) and ERβ (NR3A2), which share 53% homology of their LBD (Gougelet et al., 2005). Although they share this homology, the two receptor subtypes have noticeable differences in their binding specificities (Gougelet et al., 2005). However, both ER receptor subtypes have strong affinity for 17β-estradiol with the main difference in binding being the strong affinity ERβ has for phytoestrogens (Kuiper et al., 1997).

ERα blockers/ antagonists are used therapeutically for the treatment of breast cancer. ER agonists have been linked to the development of hepatotoxicity such as hepatic steatosis due to their ability to inhibit respiration leading to increased accumulation of triglycerides within the liver (Bandypadhyay et al., 2006; Foster, 2012; Lelliot et al., 2005; Moya et al., 2010; Shimizu et al., 2007).

Studies have shown that both ER-α agonists and antagonists can lead to the onset of hepatic steatosis, although the mechanisms behind this are not yet fully understood (Lelliott et al., 2005; Moya et al., 2010). It was determined that ERα activation causes increased malonyl-CoA levels within the cell (Bandypadhyay et al., 2006; Lelliott et al., 2005). Malonyl-CoA is essential for energy homeostasis; its regulation determines the rate of cellular fatty acid β oxidation within

the mitochondria and fatty acid synthesis within the cytosol (Foster, 2012). At lower concentrations, malonyl-CoA acts as a substrate for the enzyme malonyl coenzyme A-acyl carrier protein transacylase during fatty acid synthesis, a rate limiting step for the elongation of fatty acids. When the concentration of malonyl-CoA within the cytosol is high, it acts to inhibit the carnite acyltransferase 1 transporter which prevents the transport of fatty acids into the mitochondrial matrix and therefore inhibits mitochondrial β oxidation (Foster, 2012; Lelliot et al., 2005). ERα activation causes increased concentrations of malonyl-CoA causing the inhibition of mitochondrial β oxidation and thus preventing the breakdown of fatty acids. This leade to the accumulation of triglycerides which, as previously discussed, can result in the onset of hepatic steatosis (Bandypadhyay et al., 2006; Foster, 2012; Lelliot et al., 2005; Moya et al., 2010)

Farnesoid X receptor (FXR)

The FXR, which is also known as the nuclear receptor subfamily 1, group H, member 4/5 (NR1H4/5) (Forman et al., 1995), is an essential modulator of lipid and glucose homeostasis (Claudel et al., 2005). The FXR has two receptor subtypes FXRα (NR1H4) and FXRβ (NR1H5). The endogenous ligand for the FXR receptor is bile acids (Laffitte et al., 2002). After FXR ligand binding has occurred, FXR binds to DNA segments termed FXR response elements (FXREs), which can elicit activation or repression of the FXREs. FXR can act as a monomer or can form a heterodimer with RXR (Claudel et al., 2003). Upon activation via bile acids, the FXR regulates bile acid synthesis, bile acid transport, bile acid conjugation and also impacts on lipid and glucose metabolism (Claudel et al., 2005). The FXR is highly expressed within the liver and intestine (Howard et al., 2000). Studies have demonstrated that the FXR can also be activated via triterpenoids (e.g. forskolin) (Zhao et al., 2004) and polyunsaturated fatty acids (e.g. arachidonic acid) (Iser et al., 1975). The weak FXR agonist ursodeoxycholic acid is the only bile acid used therapeutically. Ursodeoxycholic acid is used for the treatment of gallstone

disease and cholestatic liver diseases (Claudel et al., 2005). As the FXR plays a pivotal role in energy and bile acid metabolism, it has been identified as a promising target for the treatment of dyslipidaemia and liver disorders (Claudel et al., 2005). However, this would require the development of highly specific FXR modulators and will require extensive research before it becomes a reality (Claudel et al., 2005). Chenodeoxycholic acid, demonstrated to be the most naturally potent ligand of the FXR, was taken to clinical trials to be used as a potential therapy for the treatment of gallstone disease and/ or hypertriglyceridemia (Biddie et al., 2002). However, the adverse side effects such as diarrhoea and liver toxicity such as hepatic steatosis lead to the withdrawal of this drug as a therapy and has shown the need for further understanding of the effects FXR activation induces (Biddie et al., 2002). FXR activation is linked to increased expression of PPAR-α (see this section) causing increased accumulation of triglycerides within the liver culminating in hepatic steatosis (Figure 2) (Pineda Torra et al., 2003).

Glucocorticoid Receptor (GR)

GR, also known as the nuclear receptor subfamily 3, group C, member 1(NR3C1) is essential to the maintenance of various metabolic and homeostatic functions within the body (Anbalagan et al., 2012). Cortisol is the naturally occurring ligand for the GR within mammals, it is produced via the adrenal gland and is a cholesterol-derived steroid hormone (Lewis-Tuffin et al., 2007). Upon ligand binding the GR undergoes a conformational change that triggers its translocation to the nucleus, where it induces transcriptional activation of target genes (Buttgereit et al., 2012). Post-translational modifications can also occur (such as phosphorylation, acetylation and ubiquitination), these can alter the function of the GR target genes (Vilasco et al., 2011).

Therapeutically GR agonists were first used in 1940 for the treatment of rheumatoid arthritis (cortisone) (Anbalagan et al., 2012). Since then they have been developed and used for many

other chronic inflammatory conditions such as eye infections, asthma (budesonide), skin infections (dexamethasone) and for immunosuppression (prednisone) in transplant patients. GR agonists have also been developed for the treatment of certain cancers due to their antiproliferative and antiangiogenic properties (prednisone) (Chourbaji et al., 2008).

Upon agonistic binding to the GR anti-angiogenic, anti-inflammatory, proapoptotic and anti-proliferative effects are induced in the musculoskeletal (Bultink et al., 2013; Weinstein et al., 2010), nervous (Tronche et al., 1999), visual (Edelman, 2010; Kiernan et al., 2009), cardiovascular (Fardet et al., 2012), immune (Silverman et al., 2012; Zen et al., 2011), reproductive (Harris et al., 2011), integumentary (Coenraads et al., 2012; Sevilla et al., 2012), respiratory systems (Hakim et al., 2012), and on glucose/ liver metabolism (Rose et al., 2010) within the body.

Studies have shown that when under stress the GR signalling induces glycogenolysis and gluconeogenesis within the liver to replenish glucose levels (Biddie et al., 2012; Jia et al., 2009). This is an adverse effect of GR agonists and has been linked to the onset of Cushing's disease, Addison's disease and to the development of hepatic steatosis (Jia et al., 2009; Letteron et al., 1997). GR agonist have been shown to induce hepatic steatosis due to their ability to both inhibit mitochondrial fatty acid β oxidation and to upregluate CAR expression (see CAR section), both actions lead to increased accumulation of triglycerides within the liver which can lead to hepatic steatosis (Figure 2) (Jia et al., 2009; Letteron et al., 1997)

Liver X Receptor (LXR)

The LXR, also known as the nuclear receptor subfamily 1, group H, member 2/3 (NR1H2/3), is an adopted orphan NR that upon activation forms a heterodimer with RXR to induce transcriptional activation of its target genes (Janowski et al., 1996). The LXR became an adopted NR when it was discovered that endogenous oxysterols serve as LXR ligands (Schultz

et al., 2000). LXR has two isoforms LXRα (NR1H3) and LXRβ (NR1H2), which are both essential for the regulation of cholesterol and lipid metabolism (Rippa et al., 2000). Studies have determined that LXRα is expressed mainly in the liver and small intestine whereas LXRβ is expressed ubiquitously, with only low expression within the liver (Rippa et al., 2000). Investigations have shown that upon LXR activation, intestinal cholesterol absorption (Rippa et al., 2002) and hepatic cholesterol synthesis (Rippa et al., 2000) are reduced, whereas the expression of genes involved in cholesterol mobilisation (Peet et al., 1998), bile acid synthesis (Zaghini et al., 2002), reverse cholesterol transport (Peet et al., 1998) and cholesterol excretion into the bile (Grefhorst et al., 2002) are increased. Treatments with LXR agonists have shown to be preventative against atherosclerosis during *in vivo* studies. This has led to the creation of a potential therapeutic drug target against atherosclerosis (Rippa et al., 2000). However, LXR activation has been linked to the development of hepatic steatosis (Janowski et al., 1996). LXR activation is shown to cause up-regulation of: carbohydrate responsive element binding protein (ChREBP), SREBP-1c, fatty acid synthase (FAS) and SCD1. ChREBP is crucial for mediating the body's response to glucose on glycolytic and lipogenic genes, it is required for the induction of FAS (responsible for transporting triglycerides into cells) and acetyl-CoA carboxylase (ACC) (essential enzyme involved in *de novo* synthesis). Therefore, the induction of ChREBP via LXR agonists leads to increased accumulation of lipids inside hepatocytes as more lipids are transport into the cell via FAS induction and more lipids are produced via ACC induction (Dentin et al., 2006). Similarly, increased SREBP-1c mRNA expression also leads to up-regulation of lipogenic enzymes ACC and FAS (Nguyen et al., 2007). Up-regulation of the SCD1 enzyme leads to increased fatty acid production as it is the rate limiting step in the production of unsaturated fatty acids (Zhang et al., 1999). In summary, LXR activation induces all the above mentioned pathways, these collectively cause the accumulation of *de novo* fatty acids within the liver resulting in the production of mico and macrovesicular fat droplets within

hepatocytes, thus creating a fatty liver and ultimately leading to hepatic steatosis (Kawano et al., 2010; Rosen et al., 2008; Zafrani, 2004).

Peroxisome Proliferator-Activated Receptor (PPAR)

The PPARs, also known as Nuclear receptor subfamily 1, group C, member 1-3 (NR1C1-3), are orphan receptors that form heterodimers with RXR upon ligand-induced activation, leading to transcriptional regulation of target genes (Issemann et al., 1990). There are three PPAR isoforms, namely PPARα (NR1C1), PPARβ (NR1C2), and PPARγ (NR1C3) (Kliewer et al., 1994). PPARα was the first to be discovered 20 years ago (Lee et al., 1995). PPARα mediates increased hepatic peroxisome expression causing increased concentration and density of peroxisomes within the liver (Peters et al., 2005). PPARβ and PPARγ share a sequence homology to PPARα however they are not involved in peroxisome proliferation (Issemann et al., 1990). Studies have shown that PPARβ and PPARγ can interfere with NF-Kβ and API signalling through protein-protein interactions (Peters et al., 1997; Satoh et al., 2013). All PPAR isoforms are encoded for by separate genes, have different expression levels within various tissues and are expressed by all mammalian species (Dreyer et al., 1992). PPARα is essential for the down regulation of apolipoprotein CII mRNA expression However; the mechanism behind this pathway is not understood (Misrahi et al., 1987).

PPARγ agonists are used to regulate glycaemia in type II diabetics. PPARα agonists are used to treat atherosclerosis. PPARγ and PPARα dual agonists are used to improve glycaemia and the condition of the cardiovascular system in patients with type II diabetes. PPARα and PPARγ ligands have been shown to cause hepatic steatosis due to lipid accumulation (Honkakoski et al., 1998; Misrahi et al., 1987). The mechanisms behind PPARα and PPARγ induced hepatic steatosis differ:

PPARα

The production of energy (in the form of ATP) in the liver is controlled by both mitochondrial and peroxisomal β-oxidation (Nguyen et al., 2007; Reddy and Roa, 2005). Peroxisomal β-oxidation involves the oxidation of long or very long chain fatty acids and results in less ATP production than mitochondrial β-oxidation. One of its key enzymes is the fatty acyl-CoA oxidase (AOX) which catabolises the dehydrogenation of fatty acids into acetyl –CoA (Hashimoto et al., 1999).

The PPAR-α receptor acts as a sensor for fatty acids and is responsible for the transcriptional activation of the AOX enzyme in the liver. Upon antagonistic binding to the PPARα, down regulation of the AOX enzyme follows leading to the inhibition of microsomal β-oxidation (Figure 2). This causes an accumulation of fatty acids within the liver, if PPARα antagonism continues, the build-up of triglycerides will also continue which will eventually lead to the production of micro and macrovesciular intra-cytoplasmic fat droplets within hepatocytes. As discussed previously, micro and macrovesciular fat droplets induce nucleus distortion, mitochondrial disruption and endoplasmic reticulum stress (Figure 2) (Nguyen et al., 2007; Reddy and Roa, 2005; Zafrani, 2004). These effects result in a fatty liver which becomes diagnosable as hepatic steatosis when lipid weight is between 5-10% of the total liver weight (Kawano et al., 2010; Rosen et al., 2008; Zafrani, 2004).

PPARγ

PPARγ activation leads to increased expression of SREBP-1c, increased expression of fatty acid translocase CD36 (CD36), and cellular differentiation (Al Sharif et al., 2014; Miquilena-Colina et al., 2011; Rippa et al., 2000) (Figure 2). Increased SREBP-1c mRNA expression leads to up-regulation of lipogenic enzymes such as ACC (catalyses elongation of the fatty acid chain in *de novo* lipogenesis) and FAS (multifunctional enzyme responsible for catalysing many steps in *de novo* lipogenesis) (Azzout-Marniche et al., 2000 Foretz et al., 1999; Nguyen

et al., 2007). CD36 maintains uptake and intracellular trafficking of fatty acids and is also essential for the esterification of fatty acids into triglycerides. The increased expression of SERBP-1c and CD36 are known to induce accumulation of *de novo* fatty acids. *De novo* lipogenesis is the process of lipid production inside the cytosol of a cell and is necessary for energy homeostasis within mammals (Nguyen et al., 2007). Increased production of *de novo* fatty acids has been demonstrated to cause accumulation of triglycerides within the liver which ultimately results in the onset of hepatic steatosis (Figure 2) (Postic and Girard, 2008; Rippa et al., 2000; Peraza et al., 2006).

Pregnane X Receptor (PXR)

The PXR is also known as the steroid Nuclear receptor subfamily 1, group I, member 2(NR1I2) (Venkatesh et al., 2011). PXR is an adopted orphan nuclear receptor that upon ligand binding forms a heterodimer with RXR, this mediates transcriptional up-regulation of target genes (Gonzalez et al., 1991).

PXR can be activated by both small (e.g. estradiol 268Da) and large molecules (e.g. rifampicin 823 Da). The naturally occurring ligands for PXR are steroids pregnenolone and progesterone. The synthetic ligands that have been shown to bind to PXR are glucocorticoid agonists and antagonists (Bertilsson et al., 1998). The PXR is known to bind a large range of structurally unrelated chemicals. X-ray crystal structures of the PXR-LBD have shown it to have a larger LBD compared to other NRs. This may account for its promiscuous binding (Watkins et al., 2001). Therefore, PXR has low substrate specificity and can be activated by many different chemicals.

Research has demonstrated that the PXR is essential for the modulation of hepatic drug metabolism (Li et al., 2012). Metabolic clearance especially via the phase I cytochrome p450 enzymes (CYP450), is vital for endogenous (bile acids) and exogenous (xenobiotics)

detoxification and essential to the survival of an organism (Waxman et al., 1999). Both the CYP3A and CYP2B isoenzymes mediate the metabolism of many clinical drugs and can be induced by substrate binding, therefore they are up-regulated to meet hepatic requirements (Smith et al., 1993). The PXR and CYP3As are both highly expressed within the liver (Bertilsson et al., 1998). Studies have determined that PXR up-regulates hepatic CYP3A expression along with other CYP450 enzymes. This was demonstrated via the use of PXR Knock Out (KO) mice. The mice were both viable and fertile, however upon dosing with xenobiotics the PXR KO mice developed severe hepatotoxicity as CYP3A could not be up-regulated. These studies established that PXR is the central mediator of CYP3A induction (Hoekstra et al., 2009; Smith et al., 1993; Xie et al., 2000). Studies showed that upon ligand binding PXR forms a heterodimer with RXR and binds to the Direct Repeat 3 (DR3) site on the CYP3A promoter this mediates transcriptional activation (Bertilsson et al., 1998). The mechanisms underlying PXR activation are still unknown.

It has been shown that PXR agonists induce a decrease in plasma LDL cholesterol levels (Bitter et al., 2014) and the onset of hepatic steatosis (Bitter et al., 2014; Kaisimanickam et al., 2013) *in vivo*. Research has shown that PXR agonists increase the expression of PPARα (see PPAR section) which leads to increased triglyceride accumulation within the liver which can lead to hepatic steatosis (Figure 2) (Moreau et al., 2007).

Retinoic Acid Receptor (RAR)

The RAR, also called nuclear receptor subfamily 1, group b, member 1-3 (NR1B1-3), is divided into three subtypes: RARα (NR1B1), RARβ (NR1B2) and RARγ (NR1B3). Bound together with the retinoid X receptor (RXR) as a heterodimer, RAR regulates genetic expression as transcriptional repressors in absence of ligands. RAR is important for regulating of cellular proliferation and differentiation (Liu et al., 2014). All three subtypes of the RAR are

activated by *all-trans* retinoic acid and *9-cis* retinoic acid, which are derivatives of vitamin A (Liu et al., 2014). Ligands are used in pharmacological treatment of dermal diseases, such as *Acne vulgaris*, *Psoriasis vulgaris*, *Keratosis pilaris* and specific types of cancer, e.g. acute promyelocytic leukemia (Alizadeh et al., 2014; Leyden et al., 2005). Effects of RAR agonists include changes in lipid metabolism, which may cause hepatic steatosis and eventually liver inflammation, fibrosis and finally liver failure. Teratogenic effects and neural disorders, such as nausea and headache, have been also reported from retinoids (Biesalski et al., 1989; Moya et al., 2010; Shalita et al., 1988). Moya et al (2010) demonstrated that the retinol forms of RAR agonists are able to induce steatosis in hepatocytes, however, the retinoic acid forms were not able to induce this same effect. The difference in these toxic responses are not yet understood but the mechanistic route for the onset of steatosis is suggested to be via the up regulation of lipid synthesis leading to increased accumulation of triglycerides within the liver (Figure 2) (Moya et al., 2010).

AOP Formulation

All the findings from the NR literature are summarised in Table 2 below. As can be seen the AHR, AR, CAR, ER, FXR, GR, LXR, PPAR, PXR, RAR, RXR and THR are all associated with hepatic injury, however, only AHR, CAR, ER, FXR, GR, LXR, PPAR, PXR, RAR and RXR (Table 2 - highlighted with *) have been shown to induce hepatic steatosis upon binding. Upon studying the mechanistic information gathered it was noted that activation of those NR associated with the onset of hepatic steatosis followed a similar chain of events. Activation of these NRs leads to triglyceride accumulation within the hepatocytes which then ultimately results in hepatic steatosis. Using the evidence found within the literature and the AOPs found on the AOP wiki, an AOP pathway for NR induced hepatic steatosis is proposed (Figure 2).

TABLE 2 HERE

FIGURE 2 HERE

The AOP pathway reported in Figure 2 has been adapted from the diagram available on the AOP wiki website (AOP, May 2015). The AOP pathways 1-7 (blue arrows) are extracted from the original figure and AOPs 7-10 (green and orange arrows) have been added. The AOPs 1-6 that have been highlighted green and the arrows that are green show the NR pathways that have been studied within this critical review and further strengthen the evidence for these AOP pathways. For all AOPs within Figure 2, the chemical is the NR agonist and the MIE is the binding of these agonists within the NR binding pocket to initiate activation of the NR. The key event is triglyceride accumulation and the adverse outcome is hepatic steatosis. The key events of this AOP (Figure 2) are affirmed via the literature search carried out within this review; however, the molecular processes between the MIE and the key events should be explored further.

Conclusion

The links between the chemical structure of NR ligands and the biological responses of the NR they bind to have been elucidated, this has provided knowledge of the toxicological pathways linked to these NR, specifically to the adverse outcome of hepatic steatosis. However, more work will need to be carried out to form this AOP, with the next essential step being the formation of structural alerts. This critical review has given an example of how current AOPs can be extended and developed via the use of literature review in order to bring together different (*in vitro*, *in vivo* and *in silico*) existing mechanistic / toxicological knowledge for a particular AOP. To conclude, this study has highlighted the importance of the promotion of the AOP concept to the scientific community to gain wider participation and has shown the way in which AOP formation can be developed and extended using current knowledge.

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Declaration of Interest

The authors declare that there are no conflicts of interest

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Table 1: **Nuclear Receptors associated with liver injury and their abbreviations**

Nuclear receptor name	Abbreviation
Arhyl Hydrocarbon Receptor	AHR
Constitutive Androstane Receptor	CAR
oEstrogen Receptor	ER
Farnesoid X Receptor	FXR
Glucocorticoid Receptor	GR
Liver X Receptor	LXR
Peroxisome Proliferator-Activated Receptor	PPAR
Pregnane X Receptor	PXR
Retinoic Acid Receptor	RAR

Table 2: Summary of the effects on the liver following activation of Nuclear Receptors

Nuclear receptor	Agonist effect on liver	Antagonist effect on liver
AHR	Induces hepatic steatosis*	-
CAR	Induces hepatic steatosis*	-
ER	Induces hepatic steatosis*	Induces hepatotoxicity
FXR	Induces hepatic steatosis*	-
GR	Induces hepatic steatosis*	
LXR	Induces hepatic steatosis*	-
PPAR	PPAR γ Induces hepatic steatosis*	PPARα
PXR	Induces hepatic steatosis*	-
RAR	Induces hepatic steatosis*	-
RXR	Induces hepatic steatosis*	-

Figure Titles

Figure 1. Structure of an AOP adapted from Ankley et al (2010)

Figure 2. Updated AOP for steatosis, developing that presented by Vinken (2013)

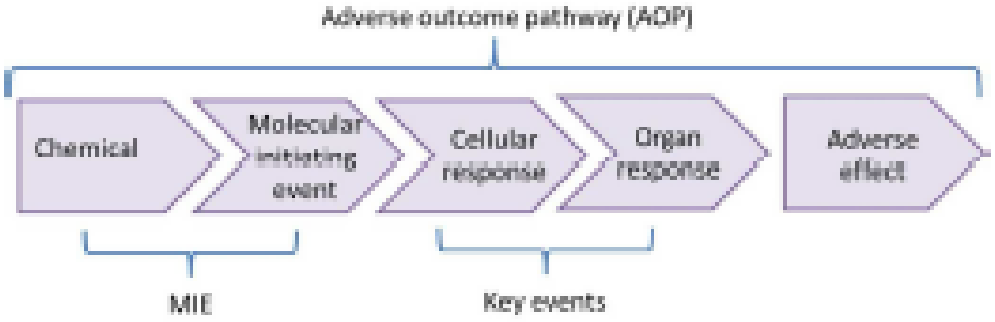

Figure 1

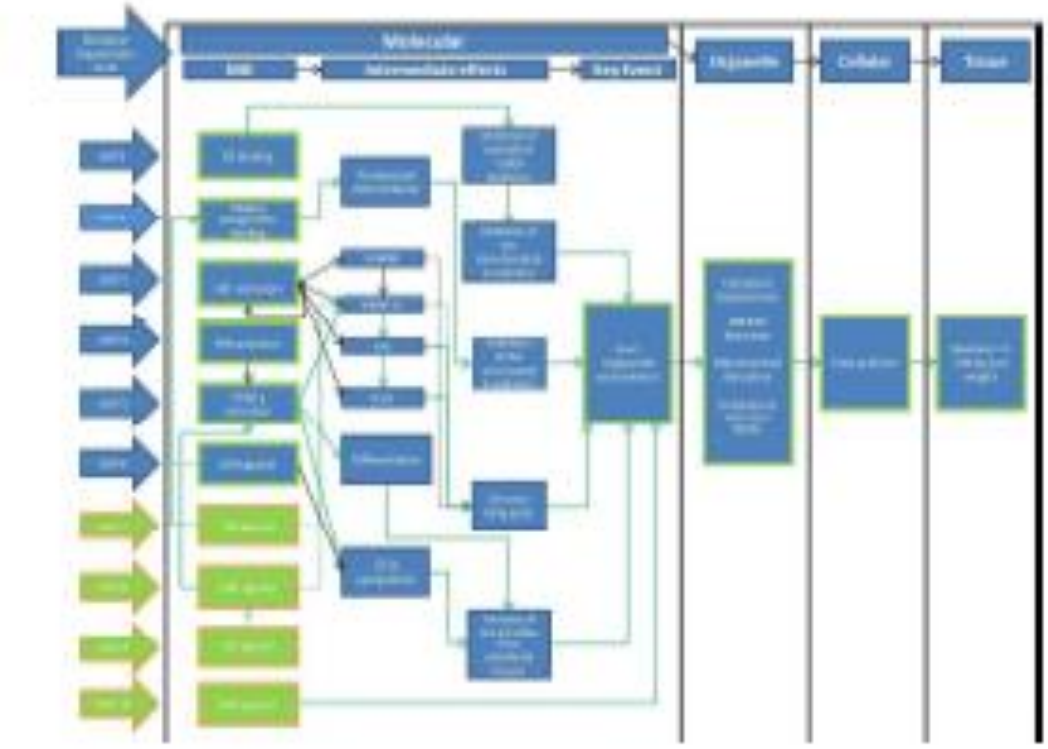

Figure 2