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EXECUTIVE SUMMARY

SEURAT-1 is a research initiative to address the long term strategic target of "Safety Evaluation Ultimately Replacing Animal Testing" (SEURAT). SEURAT-1 is composed of six research projects, which will run for 5 years, combining the research efforts of over 70 European universities, public research institutes and companies addressing repeated dose toxicity in hepatic, cardiac, renal, neuronal, muscle and skin tissues. The SEURAT-1 strategy is to adopt a toxicological mode-of-action (MoA) framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental in vitro models that predict quantitative points of departure needed for safety assessment.

A mode-of-action could be described as a sequence of events (measurable parameters), starting with the interaction of an agent with a target biomolecule, through functional and anatomical changes resulting in adverse health effects. This report presents the definition and detailed documentation of chosen toxicological MoAs associated with repeated dose target organ toxicity as a first step in building a "prototype" safety assessment framework. In addition to providing a detailed description of the two chosen MoAs related to chronic liver toxicity, namely "MoA from Protein Alkylation to Liver Fibrosis" and "MoA from Liver X Receptor Activation to Liver Steatosis", the report also describes the working process leading to this result including the problems that have been encountered, such as scarcity of quantitative data and the difficulty in capturing and describing complex non-linear processes in a narrative manner.

For the elaboration of the pathways a multistep methodology was applied. After the selection of adverse outcome, molecular initiating event and the study of relevant physiology, the intermediate events were determined based on literature searches. The intention was to understand the normal biological/physiological processes and how these normal physiological processes can be dysregulated by reference chemicals known to induce either steatosis or fibrosis starting from the chosen molecular initiating events. The chemico-biological interaction of the chemical with the system (i.e. the molecular initiating event) and how such a stimulus could promote a series of events leading to the respective adverse outcomes (i.e. describe a toxicological MoA for these chemicals) was described in a qualitative way, graphically displayed and finally evaluated. The exercise followed as far as possible relevant WHO-IPCS and OECD guidance and the results have been introduced into the Wiki-based forum that has been developed by the US EPA and the JRC. A graphical representation of the described pathways using the Effectopedia tool developed within the context of the OECD adverse-outcome-pathway initiative is planned.

ABBREVIATIONS

Liver fibrosis

Ang II	Angiotensin II
CTGF	Connective Tissue Growth Factor
ECM	Extracellular Matrix
FasL	Fas Ligand
HSCs	Hepatic Stellate Cells
HGF	Hepatocyte Growth Factor
IL-1	Interleukin-1
KCs	Kupffer Cells
LPS	Lipopolysaccharide
MCP-1	Monocyte-Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MMP	Metalloproteinase
NFκB	Nuclear Factor kappa B
NK	Natural Killer cells
NOX	reduced NADPH Oxidase
PDGF	Platelet-Derived Growth Factor
ROS/NOS	Reactive Oxygen and Nitrogen Species
TIMPs	Tissue Inhibitors of Metalloproteinases
TGF-β1,	Transforming Growth Factor-beta 1
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-alpha
TRAIL	TNF-related Apoptosis-Inducing Ligand
VEGF	Vascular Endothelial Growth Factor

Liver steatosis

ABC	ATP Binding Cassette transporter
ACC	Acetyl-CoA carboxylase
AhR	Aryl hydrocarbon receptor
AOP	Adverse Outcome Pathway
ApoE	Apolipoprotein E
CETP	Cholesterylester Transfer Protein
ChREBP	Carbohydrate Response Element Binding Protein
CYP7A1	Cytochrome P450 isoform 7A1 - cholesterol 7a-hydroxylase
EAT	Estrogen, Androgen, Thyroid
ER	Estrogen Receptor
FA	Fatty Acid
FAS	Fatty Acid Synthase
FFA	Free Fatty Acid
FAT/CD36	Free Fatty Acid uptake transporter
HCV G1	Hepatitis C Virus Genotype 1
IE	Intermediate Effect

LDL	Low density lipoprotein
LF/HC diet	Low Fat / High Carbhydrate diet
L-PK	Liver pyruvate kinase
LPL	Lipoprotein Lipase
LXR	Liver X Receptor
LXRE	Liver X Receptor Element
NAFLD	Non Alcoholic Fatty Liver Disease
NASH	Non Alcoholic Steatohepatitis
NR	Nuclear Receptor
PPAR	Peroxisome Proliferator-activated Receptor
PPRE	Peroxisome Proliferator-activated Receptor Element
PXR	Pregnane X Receptor
RAR	Retinoid Acid Receptor
RXR	Retinoid X Receptor
SCD-1	Stearoyl-CoA desaturase 1
SREBP-1c	Sterol Response Element Binding Protein 1c
VDR	Vitamin D Receptor
VLDL	Very low density lipoprotein

CONTENTS

1. Preamble

The SEURAT-1 strategy is to adopt a toxicological mode-of-action (MoA) framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (in vitro) models that predict quantitative points of departure needed for safety assessment.

The purpose of the contract of work between Cosmetics Europe and the European Commission's Joint Research Centre (Contract No. 32485-COLIPA-2011-T1CD ISP) is to carry out a suite of inter-related research tasks overarching the six individual research projects of the SEURAT-1 cluster, intended to underpin the implementation of the SEURAT-1 strategy. The work programme covers three tasks, each with a number of defined sub-tasks, and involves the production of fourteen individual reports corresponding to the agreed deliverables and time-schedule, as laid down in Table I of Annex I of the contract's Technical Annex. The aim of the first task is to demonstrate "prototype" safety assessment frameworks based on a MoA approach, exploiting knowledge and tools derived from the SEURAT-1 cluster. The task is divided into 3 sub-tasks. The approach builds from 1) defining and describing a MoA to a sufficient extent to facilitate conducting a feasibility study to 2) predict selected types of repeated dose target organ toxicity based on an assembly of complimentary tools and test systems developed within the SEURAT cluster, the outcome of which will be used to 3) formulate a number of safety assessment scenarios.

This report represents the first contract deliverable (D1.1) providing detailed descriptions of chosen prototype MoAs related to repeated dose systemic toxicity in accordance with task 1.1 The Technical Annex to the contract describes this task as providing "scientific expert input to the definition and detailed documentation of chosen toxicological MoAs associated with repeated dose target organ toxicity, most likely the liver. The exercise will follow relevant WHO-IPCS and OECD guidance and templates specifically developed for formally describing a MoA. Interaction between SEURAT-1 specialists will be initially facilitated using the Wiki-based forum being developed by the US EPA and the JRC. The more elaborated and robust MoA descriptions will also be represented graphically within the Effectopedia tool developed within the context of the OECD adverse-outcome-pathway initiative."

2. Introduction

A mode-of-action has been described as the sequence of key events (measurable parameters), starting with the interaction of an agent with a target cell, through functional and anatomical changes resulting in cancer or other adverse health effects (Boobis et al, 2008).The main purpose of this task was to demonstrate how to appropriately describe and characterise a toxicological MoA for a specific adverse outcome which would inform the design of an integrated system for testing chemicals to further refine a MoA and/or associate a chemical with a specific adverse outcome. Consistent with the given task therefore this study focuses on MoA describing the pathway from the molecular initiating event to the adverse outcome in tissue/organ level.

The SEURAT-1 cluster is addressing repeated dose toxicity in hepatic, cardiac, renal, neuronal, muscle and skin tissues. Hepatotoxicity is of particular interest (Hengstler et al. 2012, Vinken et al. 2011). Moreover the JRC has a liver cell model in-house and is already exploring the development of suitable high content assays for evaluating responses to toxicants. Consequently it was decided to focus on toxicological MoAs leading to liver toxicity. Liver fibrosis and steatosis were chosen as adverse outcomes since they are typical of chronic or repeated-dose liver toxicity.

Recognising the importance of early engagement of many different scientific and regulatory communities that will be the users of the outcome of the SEURAT-1 projects it seemed logical to interface and contribute to the current international initiatives related to following MoA based approaches to safety assessment, particularly within the WHO/IPCS activities on a MoA framework and OECD's chemical management programme. Capturing and presenting the relevant data in appropriate formats for sharing across these communities is an important part of the goal in relation to stakeholder engagement and communication.

The OECD has recently adopted the term "Adverse Outcome Pathways" as an approach to capturing mechanistic/MoA data as a basis for understanding adverse effects and recognising the need to move towards more predictive toxicology, in particular with a view to using the information in the formation of categories of chemicals with shared MoA. Although the concept of AOPs is still developing and there are many definitions, at its simplest, OECD describe AOPs as a conceptual construct that portrays existing knowledge concerning the pathway linkage between a direct molecular initiating event and an apical adverse outcome at a biological level of organisation that is relevant to a regulatory decision. AOP thus incorporates MoA and will be the framework for all MoA based activities within OECD in the foreseeable future following endorsement by the policy making body of the OECD chemicals management programme in June 2011. A draft guidance document for developing and assessing the completeness of adverse outcome pathways was recently released (OECD, 2012) along with a suggested template for presenting the information. In addition, US EPA and JRC, on behalf the WHO/IPCS Mode of Action Steering Group, have been exploring the idea of a MoA-Wiki to share current MoA based knowledge on chemicals and engage the scientific community in hypothesising and substantiating new MoAs. Consequently it was agreed to explore the utility of both the OECD template and the MoA-Wiki by using the template to present the evidence for the chosen MoAs and to transfer the information to the MoA-Wiki, testing the compatibility between the two formats.

The AOP, as defined by the OECD in the guidance, has been extended to cover also exposure considerations and impacts up to the population level. The current analysis however is

focused on MoA from molecular initiating event up to tissue level effects, and explores the relevance of the template within these limits.

3. Methodology

In order to build the presented MoAs a **multistep methodology** was applied:

Step 1 Selection of the Adverse Outcome (AO)

Liver fibrosis and liver steatosis typically results from chronic injury and SEURAT-1 research activities are aimed at understanding the mechanisms behind repeated dose toxicity.

Step 2 Selection of the Molecular Initiating Event (MIE)

For fibrosis protein alkylation was chosen as the MIE because it is common to the two chemicals that have been chosen by the SEURAT Gold Compound Working Group as reference chemicals for liver fibrosis.

In relation to steatosis, nuclear receptor (NR) binding was chosen due to the globally increased concern in relation to chemicals that act on the endocrine system through such molecular initiating events which could be possibly considered as endocrine disrupting chemical (EDCs). From the six NRs that were initially considered as being involved in steatosis formation, liver X receptor (LXR) was chosen since the SEURAT Gold Compound Working Group proposed the LXR agonist T0901317 as reference chemical for liver steatosis.

Step 3 Study of the relevant physiology

The step of understanding normal physiological pathways is based on the study of existing relevant literature on biology and physiology.

Step 4 Determination of the Intermediate Effects (IEs) through literature search

A systematic literature search was performed for articles with significant data regarding intermediate events with emphasis on key studies and review papers. This search was performed in several literature databases such as PubMed and Scopus by using search terms like 'Fibrosis', 'Protein alkylation', 'Steatosis', 'Fatty liver', 'LXR', and many more as well as their combinations. The information was then analysed according to different levels of biological organization and MoAs were constructed based on the OECD draft guidance document and template (OECD 2012).

Step 5 Graphic representation of the MoA

After the selection of the adverse outcome and the molecular initiating event, and the determination of intermediate effects, a simplified flow diagram for each of the two MoAs was constructed. This representation is considered helpful for an overview of the proposed MoAs.

Step 6 Evaluation

As a final step an evaluation of the proposed MoAs was made according to the OECD 2012 guidance, using criteria like the number of the available studies, the quality of the key studies, the consistency between the findings, the plausibility and the relevance of the models (in vitro, in silico, in vivo) used.

The two MoAs have been independently elaborated by two different researchers and although the same methodology was generally applied, the detailed approach was slightly different. This was due in part to the different professional background of the authors. The level of detail in data collection and presentation varies and while one presentation tries to follow the OECD draft template as close as possible, the other one applies only the parts of the template that appeared relevant for the respective context.

4. MoA from Protein Alkylation to Liver Fibrosis

4.1. Introduction

Liver fibrosis is a reversible wound healing response to a variety of chronic injuries including toxic injury from chemicals. It results from an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition.
Pathogenic fibrosis typically results from chronic injury with sustained production of growth factors and fibrogenic cytokines in which inflammation, tissue destruction, and repair processes occur simultaneously.

Hepatocytes are targets for hepatotoxic agents. After an acute liver injury hepatocytes are regenerated to substitute the apoptotic/necrotic cells. Responding to repeated injury regeneration fails and hepatocytes are substituted with ECM as a result of repeated cycles of hepatocytes injury and repair.

When the injury is limited in time fibrotic regression and restoration of normal tissue structure occurs. Multiple cycles of repair lead to net accumulation of ECM with damage of normal tissue structure and function. Fibrosis progresses from collagen bands to bridging fibrosis to cirrhosis. Fibrous bands may disrupt normal blood flow, leading to portal hypertension and extensive scarring is the setting for unregulated growth and neoplasia.
(Bataller and Brenner 2005, Lee et al 2011, Guo and Friedman 2007).

Liver fibrosis results from a complex interplay between various hepatic cell types, various receptors and signaling pathways, but is always strongly associated with hepatocyte death, TGF-β1 (transforming growth factor – beta 1) expression, oxidative stress and chronic inflammation (Brenner 2009).

SEURAT reference chemicals:
Allyl alcohol and Carbon tetrachloride (CCl_4) were chosen as reference standards for liver fibrosis. Both chemicals act via production of reactive aldehydes, either generated from interaction of CCl_4 with endogenous lipids or by generation of acrolein as a metabolite of allyl alcohol, though the location of observed toxicity is different (perivenous rather than periportal, respectively).

Allyl alcohol was selected as a Gold Compound because of a relatively well-defined alkylating MoA that causes cytotoxicity and fibrosis. Its toxicity appears to be exclusively mediated by acrolein formed from allyl alcohol as a result of oxidation by alcohol dehydrogenase. Acrolein is a highly reactive α,β- unsaturated aldehyde and readily alkylates model proteins in vitro. Acrolein is selective for sulfhydryl groups, including glutathione. Acrolein also alkylates nitrogen nucleophiles, primarily lysine and deoxyguanosine. Alkylation of proteins is assumed to be the event actually leading to cell injury.

Typical cytotoxic effects are observed in the periportal region of the liver (due to oxygen-dependent bioactivation); consecutively periportal fibrosis is observed after repeated administration. Allyl alcohol toxicity is accompanied by oxidative stress, collapse of mitochondrial membrane potential, and lipid peroxidation.

Carbon tetrachloride
Hepatic toxicity is due to biotransformation of CCl_4 to the trichloromethyl free radical (CCl_3) via the cytochrome P450 system. CCl_3 further leads to alkylation of proteins and DNA along with lipid peroxidation. Necrosis is located in the perivenous region, where cytochrome P450 activity is high. Chronic exposure may result in fibrosis and cirrhosis.
(SEURAT-1 Gold Compound Selection Tables)

4.2. Summary of the events

Events that correspond to one level of biological organisation are marked in red, while those events that relate to several levels and are interrelated to other intermediate events are marked in blue.

4.2.1 Molecular Initiating Event

Protein alkylation

4.2.2 Intermediate Events – cellular

4.2.2.i Hepatocyte injury and apoptosis / necrosis
Adducts, free radicals, oxidative damage ←→ oxidative stress *
Release of cytokines, chemokines → inflammatory signaling ←→ inflammation **
→ activation of Kupffer cells (KCs)
→ activation of stellate cells (HSCs)
→ activation of endothelial cells
→ activation of platelets

4.2.2.ii Activation of hepatic macrophages (KCs)
by engulfed apoptotic bodies, ROS / NOS (reactive oxygen, nitrogen species)
→ expression of chemokines and cytokines most importantly TGF-β1, PDGF
→ inflammatory signaling ←→ inflammation **
→ ROS ←→ oxidative stress *

4.2.2.iii TGF-β1 expression
by activated KCs (and sinusoidal epithelial cells, platelets, eventually also by activated HSCs) TGF-β1 is a key mediator and the most pro-fibrotic cytokine
→ directly activates HSCs (via Smad 2/3)
→ regulates the activation of CTGF

→ stimulates the synthesis of multiple ECM proteins (including collagen) and
→ inhibits ECM degradation (inhibits the synthesis of MMPs and
induces the production of TIMPs)

4.2.2.iv Stellate cell activation

by TGF-β1, engulfed apoptotic bodies and ROS
→ expression of new receptors: PDGF – and TGF-β1– receptor
→ expression of new proteins: α -smooth muscle protein
→ production and deposition of collagenous ECM (stimulated by TGF-β1)
→ cytokine production → TGF-β1 - perpetuates the activated state
→ MMPs - degradation of ECM proteins
→ TIMPs - inhibiton of ECM degradation
→ CTGF - endothelial cell growth and stimulation of extracellular matrix production
→ proliferation (stimulated by growth factors, mainly PDGF)
→ increased contractility (driven mainly by endothelin-1)
→ HSC chemotaxis (driven by chemoattractants)
→ inflammatory signaling ←→ inflammation **
→ production of ROS (by NADPH oxidase) ←→ oxidative stress *

4.2.3 Intermediate Events – tissue

Progressive **collagen accumulation** and changes in ECM composition
Changes in composition of ECM directly stimulate fibrogenesis;
ECM provides a reservoir for growth factors and MMPs.

4.2.4 Intermediate Events – overlapping various levels

4.2.4.i.* Oxidative Stress

ROS generation from hepatocytes, KCs, HSCs, inflammatory cells
→ hepatocyte apoptosis
→ KC activation
→ HSC activation of KCs
→ macrophage activation ←→ inflammation **

4.2.4.ii ** Chronic Inflammation

Inflammatory signaling from injured hepatocytes, activated KCs and HSCs
→ hepatocyte injury
→ KC activation
→ HSC activation
→ ECM degradation, production and remodeling
←→ oxidative stress *

4.2.5 Adverse Effect - organ

Liver fibrosis

4.3. Analysis of the MoA

4.3.1 Molecular Initiating Event (MIE)

A molecular initiating event is the initial point of chemical-biological interaction within the organism that starts the adverse outcome pathway **(**OECD 2012**).** Bioactivation of a chemical may produce both protein-alkylating metabolites and reactive oxygen species with the various consequences of oxidative stress and the potential of hepatocyte injury, a necessary requirement for the initiation of the fibrotic process (Liebler 2008).

The MIE for the two reference chemicals is protein alkylation, leading to structural and functional cell injury. Protein alkylation disturbs the cellular redox balance, which leads to disruption of multiple biochemical pathways in exposed cells, which in turn can trigger the death of exposed cells via either apoptosis and/or necrosis (Kehrer and Biswal 2000).

4.3.2 Intermediate Events (IE)

These are events along the pathway between the molecular initiating event and the apical outcome that are toxicologically relevant to the apical outcome and experimentally quantifiable (OECD 2012).

4.3.2.i Hepatocyte Injury and Apoptosis - cellular

Chemicals or their metabolites undergo or promote a variety of chemical reactions with direct effects on cellular organelles or indirect influence on cellular structures through the activation and inhibition of signaling kinases, transcription factors, and gene-expression profiles, which may lead to cell death caused by either cell shrinkage and nuclear disassembly (apoptosis) or swelling and lysis (necrosis) (Tarantino et al. 2009). Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death (Kissileva and Brenner 2007, Orrenius et al. 2011). The pathogenic contribution of necrosis to hepatic fibrosis is unclear. Specific inflammatory pathways of necrosis have not been identified. Necrosis may simply represent a more severe cellular response to injurious stimuli, but the relative potencies of necrosis compared with apoptosis in stimulating fibrogenesis are unknown (Jaeschke et al. 2002, 2004, Friedman 2008).

Our reference chemicals damage hepatocytes via both covalent binding to liver proteins and lipid peroxidation accompanied by oxidative stress and collapse of mitochondrial membrane potential which triggers apoptotic cell death (Tanel et al. 2007, Boll et al. 2001, Manibusan et al. 2007).

Damaged hepatocytes release reactive oxygen species (ROS), cytokines (like TGF-β1, TNF-α - Tumor Necrosis Factor-alpha) and chemokines which lead to oxidative stress, inflammatory signaling and activation of KCs (resident macrophages), HSCs, endothelial cells and platelets (Friedman 2008, Bataller and Brenner 2005, Kisseleva and Brenner 2007, 2008). ROS generation in hepatocytes results from oxidative metabolism of the toxicant by NADH oxidation and Cytochrome P450 2E1 and through lipid peroxidation (Friedman 2008).

Apoptotic hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies. Upon engulfment of apoptotic bodies HSCs and KCs are activated and reduced NADPH oxidase (NOX) is induced in HSCs (Kisseleva and Brenner 2007, Friedman 2008). Apoptotic cells also release the nucleotides ATP and UTP, which can bind to purinergic

receptors (P2Y2) on macrophages and HSCs, enhancing collagen secretion by HSCs (Malhi et al. 2010).

Enhanced hepatocyte apoptosis is tightly connected with inflammation and fibrosis, but the relationship between apoptosis and fibrosis is also bidirectional, wherein fibrosis may in turn stimulate apoptosis by inducing pro-apoptotic gene expression in parenchymal cells. For example, fibrosis accompanying tissue injury may lead to up-regulation of Fas/ Fas L (Fas ligand) (Canbay et al. 2004).

4.3.2.ii Activation of Hepatic Macrophages (Kupffer cells) - cellular
Following engulfment of apoptotic bodies, KCs become activated, providing a major source of inflammatory mediators including cytokines, chemokines, lysosomal and proteolytic enzymes and a main source of TGF-β1 (the most potent profibrogenic cytokine). In addition latent TGF-β1 can be activated by KC-secreted MMP-9 (matrix metalloproteinase-9) (Friedmann 2002, Stalnikowitz 2003).

Furthermore, activated KCs are an important source of ROS like superoxide (generated by NOX), but they also produce nitric oxide (NO) the counterbalance for the stimulatory effects of ROS. KCs express TNF-α, IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for HSCs and induce the expression of platelet-derived growth factor (PDGF) receptors on HSCs cells which further enhances HSC-proliferation. Expressed TNF-α, TRAIL (TNF-related apoptosis-inducing ligand), and FasL are pro-inflammatory agents and capable of inducing receptor-mediated apoptosis in hepatocytes. Secreted gelatinase degrades collagen type IV and might also trigger the phenotypic change of HSCs (Guo and Friedman 2007, Kolios et al. 2006).

4.3.2.iii TGF-β1 Expression - cellular
TGF-β1 is the most potent profibrogenic cytokine and plays a central role in fibrogenesis, mediating a cross-talk between parenchymal, inflammatory and collagen expressing cells and thereby further amplifying the response.

TGF-β1 is released by activated KCs, sinusoidal endothelial cells, and platelets; in the further course of events also activated HSCs express TGF-β1. Hepatocytes do not produce TGF-β1 but are implicated in intracellular activation of latent TGF-β1 (Kisseleva and Brenner 2007, 2008, Poli 2000, Liu et al. 2006). Platelets are the first cells recruited to sites of injury, they initiate coagulation and release the growth factors TGF-β1 and PDGF (Henderson and Iredale 2007). TGF-β1 induces its own mRNA to sustain high levels in local sites of liver injury.
The effects of TGF-β1 are classically mediated by intracellular signaling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory (Parsons 2007, Friedman 2008). Smad1/5/8, MAP kinase and PI3 kinase are further signaling pathways in different cell types for TGF-β1 effects. TGF-β1 activates HSCs, stimulatig ECM synthesis and suppresses ECM degradation. It stimulates collagen transcription in stellate cells and connective tissue growth factor (CTGF) in hepatocytes and induces the expression of TIMP-1, an inhibitor of the collagen cleaving enzymes MMP-8 and MMP-13. TGF-β1 further recruits inflammatory cells, portal fibroblasts and circulating myofibroblasts to injured liver and triggers apoptosis of hepatocytes (Gressner et al. 2002, Stalnikowitz 2003).

4.3.2.iv Stellate Cell Activation - cellular
Stellate cell activation means a transdifferentiation from a quiescent vitamin A–storing cell to

a proliferative and contractile myofibroblast. The HSC is the central effector in hepatic fibrosis and undergoes activation through a two-phase process.

The initiation phase after liver injury with generation of hepatocyte apoptotic bodies (engulfed by HSCs), ROS, and paracrine stimulation from neighbouring cell types (KCs, sinusoidal endothelium cells, and platelets) makes the cell sensitized to additional activation by up-regulating various receptors. Subsequently, HSCs are able to secrete autocrine and paracrine growth factors (such as TGF-β1), chemokines, and ECM proteins (type I collagen) (Friedman 2008, 2000).

Maintenance of HSC activation is termed the perpetuation phase, and involves changes in HSC behaviour. In response to TGF-β1 activated HSCs up-regulate collagen synthesis (mainly type I collagen). Together with decreased matrix degradation (expression of degrading MMPs is down-regulated while their inhibitors TIMPs are up-regulated) ECM composition changes and further stimulates HSC activation and production of TGF-β1. Also increased mechanical stiffness of the ECM activates HSCs through integrin signaling (Lotersztajn et al. 2005, Bataller and Brenner 2005).

In response to growth factors (including PDGF, VEGF and thrombin) HSCs proliferate.
Increased contractility (endothelin-1 and nitric oxide are the key opposing counter-regulators that control HSC contractility, in addition to angiotensinogen II, and others) – leading to increased portal vascular resistance - and chemotaxis (driven by chemoattractants including PDGF - enhancing the accumulation in areas of injury) are features of the activated HSC phenotype (Lee et al. 2011, Friedman 2010). HSCs amplify inflammation through the release of chemoattractants for neutrophils and monocytes (MCP-1 and colony-stimulating factor). Synthesis of TGF-β1 promotes activation of neighbouring quiescent HSCs, whereas the release of HGF (hepatocyte growth factor) stimulates regeneration of adjacent hepatocytes (Poli 2000)

4.3.2.v. Oxidative Stress - overlapping
Oxidative stress plays a crucial role in liver fibrogenesis by inducing hepatocyte apoptosis, activation of KCs and HSCs (Poli 2000, Parola and Robino 2001).

Development of oxidative stress is associated with an increase in ROS, including superoxide, hydrogen peroxide, hydroxyl radicals and aldehydic end products that both initiate and then perpetuate fibrosis. ROS may be derived from hepatocytes, KCs, HSCs, and inflammatory cells and are generated through lipid peroxidation, from hepatocyte Cytochrome P450 2E1 and NOX in activated KCs and HSCs. ROS stimulate HSC in a paracrine manner through activation of redox-sensitive intracellular signaling which results in increased collagen production (Friedman 2008, Lee et al. 2011, Kisseleva and Brenner 2007, Parsons 2007).

Oxidative stress products have shown to be able to induce the synthesis of fibrillar ECM even in the absence of significant hepatocyte damage and inflammation (increased procollagen I gene expression in activated human HSC) (Pinzani and Rombouts 2004).

Under conditions of oxidative stress macrophages are activated which can lead to a more enhanced inflammatory response (Kirkham 2007). Oxidative stress can activate a variety of transcription factors like NF-κB, PPAR-γ whose activation can lead to the expression of genes for the production of growth factors, inflammatory cytokines and chemokines (Reuter et al. 2010).

4.3.2.vi. Chronic Inflammation – overlapping

The inflammatory response plays an important role in driving fibrogenesis, since persistent inflammation precedes fibrosis. Inflammatory and fibrogenic cells stimulate each other in amplifying fibrosis. Chemokines and their receptors provoke further fibrogenesis, as well as interacting with inflammatory cells to modify the immune response during injury.

In addition to already existing leucocytes, liver injury results in a massive accumulation of recruited inflammatory cells, with contemporaneous activation of the resident inflammatory cell pool (Henderson and Iredale 2007, Bataller and Brenner 2005).

HSCs are central modulators of hepatic inflammation and immunity. Activated HSCs secrete inflammatory chemokines that amplify infiltration by inflammatory cells, they interact directly with immune cells through expression of adhesion molecules (mediated by TNF-α and facilitating the recruitment of inflammatory cells), and they modulate the immune system through antigen presentation. Responding to stimulation by pro-inflammatory cytokines including TNF-α and MCP-1 (induced by KC-initiated increased NFκB activity) HSCs secrete various cytokines (like macrophage colony-stimulating factor (M-CSF), MCP-1 and IL-6) leading to an amplified acute phase response with further activation of macrophages. Signaling of HSCs in response to either LPS or endogenous TLR4 ligands down-regulates the protein activin membrane-bound inhibitor (BAMBI), a transmembrane suppressor of TGF-β1 (Friedman 2008, Lee et al. 2011).

Other inflammatory cells regulating progression and resolution of fibrosis include T-cells, dendritic cells, endothelial cells and natural killer cells (NK) which exert an anti-fibrotic activity by inhibiting and/or killing activated HSCs by inducing apoptosis through production of interferon γ. NFκB signals interactions between HSCs and myofibroblasts, and immune cell subsets (Novitskiy et al. 2005).

Oxidant stress and apoptotic parenchymal cells are strong inducers of the immune system. Apoptotic hepatocyte DNA can interact with TLR9 expressed on HSCs, which then can repress HSC migration and increase collagen production (Friedman 2008, Lee et al. 2011). In chronic inflammation activated neutrophils, macrophages and Kupffer cells are a major source of ROS. Inducible NO synthase (iNOS) is upregulated in almost all liver cell populations, including HSC during chronic inflammation and, consequently, NO generation is increased that might interact with O_2 to generate reactive nitrogen species, thus increasing oxidative stress (Parola and Robino 2001).

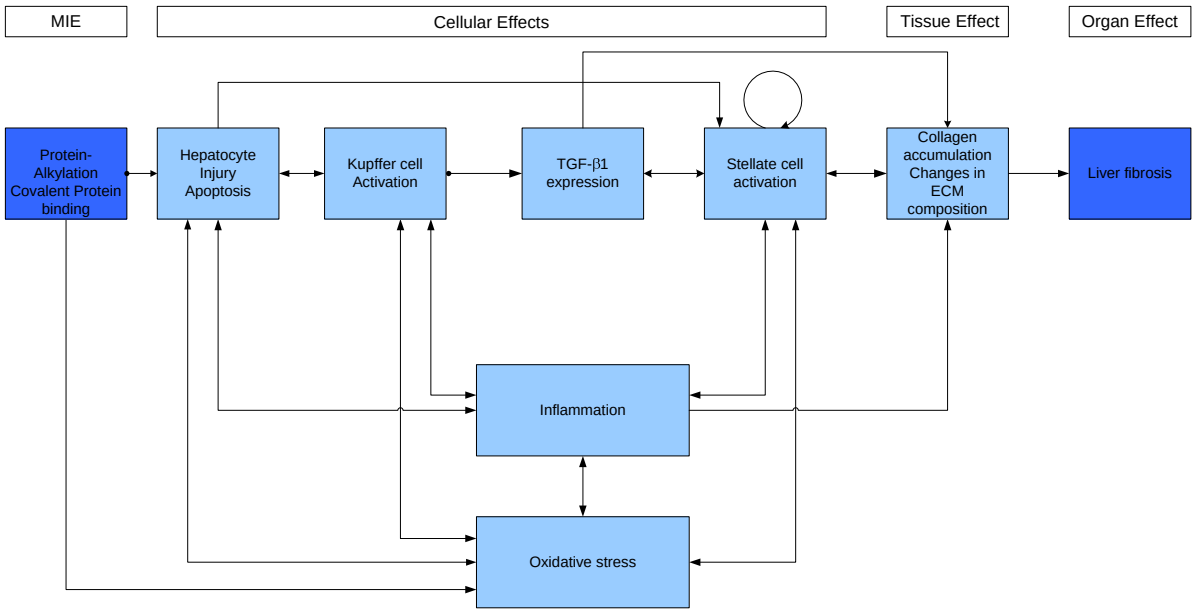
4.3.2.vii. Progressive Collagen Accumulation and changes in ECM composition - tissue

HSCs generate fibrosis not only by increasing cell number, but also by increasing matrix production per cell. The basement membrane-like matrix is normally comprised of collagens IV and VI, which is progressively replaced by collagens I and III and cellular fibronectin during fibrogenesis. These changes in ECM composition initiate several positive feedback pathways that further amplify fibrosis such as increasing matrix stiffness which is a stimulus for HSC activation. In addition, matrix-provoked signals link to other growth factor receptors through integrin-linked kinase and transduce signals via membrane-bound guanosine triphosphate binding proteins, in particular Rho67 and Rac, to the actin cytoskeleton that promote migration and contraction of HSCs.

Besides the transition of quiescent HSCs into activated HSCs and then further into contractile myofibroblasts (which are the primary collage producing cells), other cells may also transdifferentiate into fibrogenic myofibroblasts in liver injury.

Additional sources of ECM include bone marrow (which probably gives rise to circulating fibrocytes), portal fibroblasts, EMT (epithelial–mesenchymal cell transition) from hepatocytes and cholangiocytes and MET (mesenchymal-to-epithelial transition), in which mesenchymal cells give rise to epithelium (Friedman 2010, 2008, Lee et al. 2011).

4.4. Flow diagram of the intermediate events associated with the MoA



4.5. Subjective evaluation of the strength of scientific evidence

Event	Scientific Support	Strength of Evidence
MIE Protein Alkylation	Liebler DC "Protein Damage by Reactive Electrophiles: Targets and Consequences" Chem. Res. Toxicol. 2008; 2221(1): 117-128 Kehrer JP, Biswal S. "The Molecular Effects of Acrolein" Toxicol. Sciences 57, 6-15 (2000)	well established A well-accepted toxic mechanism for causing cell injury.
IE Hepatocyte Injury and Apoptosis	Malhi H. et al. "Hepatocyte Death: A Clear and Present Danger" Physiol. Rev. 90:1165-1194. 2010 Canbay A. et al. "Apoptosis: The Nexus of Liver Injury and Fibrosis" Hepatology, Vol. 39, No. 2, 2004 Orrenius S.et al. "Cell Death Mechanisms and Their Implications in Toxicology" Toxicol. Sciences 9(1);3-19(2011) Jaeschke H. "Inflammation in Response to Hepatocellular Apoptosis" Hepatology 2002;35:964–966	very strong Emerging concepts implicate apoptosis as a keystone in the genesis of hepatic inflammation and fibrogenesis.
IE Activation of Hepatic Macrophages (Kupffer cells)	Kolios G. et al. "Role of Kupffer Cells in the Pathogenesis of Liver Disease" World J Gastroenterol 2006 December 14; 12(46): 7413-7420 Kisseleva T, Brenner D. "Mechanisms of Fibrogenesis" Minireview Experimental Biology and Medicine 2008,233:109-122	very strong Kupffer cells are the main source of TGF-β1 and a major source of ROS and inflammatory mediators. Their activation is directly related to hepatocyte injury and apoptosis.
IE TGF-β1 Expression	Liu X. et al. "Therapeutic Strategies Against TGF-β Signaling Pathway in Hepatic Fibrosis" Review Liv Int 2006:26: 8-22 Gressner et al. "Roles of TGF-β in Hepatic Fibrosis" Front Biosci. 2002 Apr 1;7:d793-807	very strong TGF-β1 is considered the most potent profibrogenic cytokine and several reviews assign this cytokine a central role in fibrogenesis, especially in stellate cell activation.
IE Stellate Cell Activation	Kisseleva T, Brenner D." Role of Hepatic Stellate Cells in Fibrogenesis and the Reversal of Fibrosis" Journal of Gastroenterology and Hepatology 22 (2007) Suppl. 1; S73–S78 Friedman SL "Hepatic Fibrosis -- Role of Hepatic Stellate Cell Activation" MedGenMed. 2002 Jul 15; 4(3):27. Friedman SL "Mechanism of Hepatic Fibrosis" Review Nat Clin Pract Gastroenterol Hepatol. 2004 Dec;1(2):98-105.	very strong Activated HSCs (myofibroblasts) are the primary collagen producing cell and the key cellular mediators of fibrosis (a nexus for converging inflammatory pathways leading to fibrosis).

Event	Scientific Support	Strength of Evidence
IE Oxidative Stress	Parsons Ch. et al. "Molecular Mechanisms of Hepatic Fibrogenesis - Oxidative Stress and Cytokine Response in Hepatic Fibrogenesis" Journal of Gastroenterology and Hepatology 22 (2007) Suppl. 1; S79–S84 Poli G. "Pathogenesis of Liver Fibrosis: Role of Oxidative Stress" Molecular Aspects of Medicine 21 (2000) 49 – 98 Parola M, Robino G. "Oxidative Stress-Related Molecules and Liver Fibrosis. A Review" J Hepatol 2001; 35:297–306.	strong Oxidative stress plays a crucial role in liver fibrogenesis by inducing hepatocyte apoptosis, activation of KCs and HSCs. Oxidative stress-related molecules act as mediators to modulate tissue and cellular events responsible for the progression of liver fibrosis.
IE Chronic Inflammation	Stalnikowitz DK "Liver Fibrosis and Inflammation. A Review" Annals of Hepatology 2003; 2(4) 159-163 Henderson NC, Iredale JP "Liver Fibrosis: Cellular Mechanisms of Progression and Resolution" Clinical Science (2007) 112, 265-280	very strong The whole fibrinogenic cascade is initiated and maintained by inflammatory mediators. Damaged hepatocytes release inflammatory cytokines that activate Kupffer cells and stimulate the recruitment of inflammatory cells which produce profibrotic cytokines and chemokines that further activate fibroblastic cells.
IE Progressive Collagen Accumulation	Lee U., Friedman SL "Mechanisms of Hepatic Fibrogenesis" Best Practice & Research Clinical Gastroenterology 25 (2011) 195–206	very strong Liver fibrosis results from an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition; the latter initiates several positive feedback pathways that further amplify fibrosis.

Event	Scientific Support	Strength of Evidence
Adverse Event Human Liver Fibrosis	Lee WM , "Drug-Induced Hepatotoxicity" N Engl J Med (2003);349:474-85. Russmann S. et al., " Current Concepts of Mechanisms in Drug-Induced Hepatotoxicity" Current Medicinal Chemistry (2009), 16, 3041-3053 3041 Jaeschke H., "Mechanisms of Hepatotoxicity" Toxicological Sciences (2002) 65, 166–176 Mehta N. et al. " Drug-Induced Hepatotoxicity" Medscape E-Medicine Updated: Jun 27, 2012 http://emedicine.medscape.com/article/169814-overview Malhi H. et al. "Hepatocyte Death: A Clear and Present Danger" Physiol Rev (2010) 90: 1165–1194 Ramachandran R. et al."Histological patterns in drug-induced liver disease" J Clin Pathol (2009) 62:481–492.	well established: It is generally accepted that any chronic form of liver damage, including any drug causing sub-massive hepatocellular injury, can result in myofibroblast activation, leading to hepatic fibrosis and cirrhosis in humans.

4.6. Other fibrogenic signaling pathways influencing hepatic stellate cell activation

Adipokine pathways:
Adipokines are secreted mainly by adipose tissue, but also by resident and infiltrating macrophages and are increasingly recognised as mediators of fibrogenesis.

Leptin promotes HSC fibrogenesis and enhances TIMP-1 expression and further acts as a pro-fibrotic through suppression of peroxisome proliferator-activated receptor-γ (PPARγ), an anti-fibrogenic nuclear receptor that can reverse HSC activation. The expression of leptin receptor is up-regulated during HSC activation and leptin activity is therefore increased through enhanced signaling. Downstream effects include increased release of TGF-β1 from KCs. The counter-regulatory hormone adiponectin is reduced in hepatic fibrosis (Lee et al. 2011, Friedman 2010).

Neuroendocrine pathways
The fibrogenic function of HSCs is also influenced by neurochemical and neurotrophic factors. Upon chronic liver injury, the local neuroendocrine system is up-regulated, and activated HSCs express specific receptors, most prominently those regulating cannabinoid signaling. Activated stellate cells are additionally a key source of the endogenous cannabinoid, 2-AG, which drives increased CB1 receptor signaling. Stimulation of the CB1 receptor is profibrogenic, whereas the CB2 receptor is anti-fibrotic and hepatoprotective.
Opioid signaling increases proliferation and collagen production in HSCs. Serotonin has a pro-fibrotic effect that synergizes with PDGF signaling. Also thyroid hormones enhance

activation of HSC (through increased p75NTR and activation of Rho), thereby accelerating the development of liver fibrosis (Friedman 2008, 2010, Lee et al. 2011).

Renin–angiotensin pathway in HSCs induces ROS and hepatic fibrosis. Angiotensin II (Ang II) promotes inflammation and activation of HSC, upon which they themselves are capable of Ang II synthesis. NOX is a key downstream signaling mediator of Ang II in activated HSC. Activated NOX directly up-regulates collagen expression, produces ROS in response to cytokine induced redox-sensitive stimulation and also mediates inflammatory responses in Kupffer cells (Kisseleva and Brenner 2007, Friedman 2010, Lee et al. 2011).

4.7. Similarities of fibrogenesis in different organs

The complex mechanism of fibrogenesis does not only affect a single organ, but causes a systemic response which may equally damage other organs and tissues. The described findings in liver fibrosis parallel those in studies of fibrogenesis in lung and kidney and other organs with the same cells and soluble factors involved (Friedman 2002, 2010). For example our reference compound CCl_4 equally affects lymphoid organs, lungs and kidneys (Kisseleva and Brenner 2008). Pathogenic fibrosis in any organ typically results from chronic injury in which inflammation, tissue destruction, and repair processes occur simultaneously. The main pathway from injury leads via inflammatory response to the production and secretion of profibrotic cytokines (TGF-β1 being by far the most important for human fibrotic diseases (Poli 2000) and chemokines by inflammatory cells and further to the activation of fibroblastic cells which turn into collagen-producing myofibroblasts.

Myofibroblasts origin from resident mesenchymal cells (e.g. hepatic stellate cells), epithelial cells (Epithelial - mesenchymal transition - EMT), Endothelial cells (Endothelial – mesenchymal transition –EndMT), and from circulating fibrocytes (from bone marrow stem cells) (Kisseleva and Brenner 2008).

Fibrosis may affect lung, kidney, heart and blood vessels, eye, skin, pancreas, intestine, brain and bone marrow. Multi-organ fibrosis might occur due to mechanical injury (scar tissue), or may be drug- or radiation-induced (Wynn 2007, 2008, Sivakumar and Das 2008, Chatziantoniou and Dussaule 2005, Liu 2011).

Findings also suggest **common conserved pathways across different species** which initiate and significantly modulate the progression of liver fibrosis (Weber et al 2010, Iredale 2007 Constandinou 2005, Tsukamoto et al. 1990).

5. MoA from Liver X Receptor Activation to Liver Steatosis

5.1. Introduction

Liver steatosis (fatty liver) is characterized by the accumulation of lipid droplets (mainly triglycerides) in the hepatocytes which can be identified histologically as either microvesicular or macrovesicular accumulation (Amacher 2011). Steatosis is the output of the disturbance on the homeostasis of hepatic lipids which depends on the dynamic balance of several pathways including fatty acid (FA) uptake, de novo FA synthesis, β-oxidation, and

very low-density lipoprotein (VLDL) secretion (Zhu et al. 2011). The diagnosis of steatosis is made when fat in the liver exceeds 5–10% by weight (Reddy & Rao 2006). Despite the fact that steatosis is not adverse per se and it is usually reversible once the cause of the problem is diagnosed and corrected it is considered as one of the first manifestations of possible hepatotoxicity. The importance of steatosis is highlighted from the fact that it is a prerequisite for the development of non-alcoholic fatty liver disease (NAFLD) as according to the 2 hits theory different pathogenic factors lead firstly to steatosis and secondly to hepatic damage (‘‘the second hit’’) (Day & James 1998). NAFLD is the most common cause of abnormal liver enzymes in the western world and it is defined as a condition caused by fatty infiltration of the liver, in the absence of large alcohol consumption (Croke & Sampson 2012). The advanced form of NAFLD with inflammation and hepatocellular damage is termed non-alcoholic steatohepatitis (NASH) (Zhu et al. 2011) and progressively may result in fibrosis, cirrhosis (possibly complicated by hepatocellular carcinoma) and liver failure (Adams & Angulo 2006).

It is clear that the development of steatosis can be attributed to many different causes, and it is also clear that chemicals, such as alcohol or drugs can cause or influence the development of steatosis. Interaction of exogenous chemicals with nuclear receptors (NRs) stands prominent among the molecular events that may initiate adverse outcomes (IPCS/WHO 2002). One potential MoA could be via interference with those nuclear receptors involved in the homeostasis of fatty acid metabolism. The nuclear receptor superfamily describes a related but diverse array of transcription factors which act as receptors for thyroid and steroid hormones, retinoids and vitamin D, as well as different "orphan" receptors of unknown ligand. Ligands for some of these receptors have been recently identified, showing that products of lipid metabolism such as fatty acids, prostaglandins, or cholesterol derivatives can regulate gene expression by binding to nuclear receptors. Unlike ligands (hormones) for cell surface receptors, lipophilic ligands can traverse the plasma membrane to the cell interiors, the nucleus or cytoplasm, where NRs are located. The nuclear receptor family has 48 functionally distinct members in humans. In addition to the receptors involved in estrogen, androgen and thyroid hormone (EAT) signalling, hormone-activated nuclear receptors in vertebrates include the Liver X receptor, the corticosteroid receptors (e.g., mineralocorticoid, glucocorticoid), retinoic acid receptor (RAR), retinoid X receptor (RXR), vitamin D receptor (VDR), and peroxisome proliferator activated receptors (PPARs) (Le Blanc et al. 2011). Given the widespread relevance of the superfamily of nuclear receptors to almost all aspects of normal human physiology, the role of these receptors in the etiology of many human diseases, and their importance as therapeutic targets for pharmaceuticals, it is obvious that a detailed understanding of these systems has major implications, not only for human biology but also for the understanding and development of new drug treatments (Olefsky 2001) as well as an understanding of not only potential therapeutic effects but also toxic effects of chemicals.

According to LeBlanc et al 2011 in the OECD's Detailed Review Paper, substances which act via the NRs leading to a perturbation of normal homeostasis of fatty acid metabolism may be considered as endocrine-disrupting chemicals (EDCs). Certain chemicals acting via the NRs (or Nuclear Hormone Receptors as they have also been described) may be responsible for inducing alterations as those encountered in steatosis and other NAFLD either directly through a hepatotoxic effect and/or indirectly by triggering hepatic and systemic insulin resistance (IR). There is concern that an increase in exposure to synthetic chemicals in consumer products and the environment acting via such MoAs may be contributing to the current epidemic of obesity and type 2 diabetes mellitus (T2DM). Such chemicals may also

play a significant role in the pathogenesis of fatty liver, thereby increasing the prevalence of NAFLD worldwide (Polyzos et al. 2012). Consequently it would seem a particularly relevant MoA.

Nuclear receptors that could play a role in the formation of steatosis are the PXR, AhR, PPARα, PPARγ and ER. In the present report a MoA from LXR activation to steatosis is presented. This provides just part of the picture and it could be anticipated that this exercise could be expanded at a later stage to encompass other MoAs arising from interaction with other NRs.

5.2. Summary of the events

5.2.1 Identification of the molecular initiating event

Binding to the LXR and activation by appropriate ligands.

5.2.2 Identification of the site of action

The molecular initiating event takes place in the nucleus of the hepatocytes where the LXR is located.

5.2.3 Identification of the responses (Intermediate Effects) at the macromolecular level

A number of intermediate effects at the macromolecular level occur along the pathway as summarized below:

- Binding to the Liver X Receptor Elements (LXREs) and target genes transcription leading to:
    a. Auto-regulation of the LXRα (up regulation, positive feedback)
    b. Increase in expression and activity of the carbohydrate response element binding protein (ChREBP)
    c. Increase in expression of the sterol response element binding protein 1c (SREBP-1c) from LXR activation and from the carbohydrate response element-binding protein (ChREBP)
    d. Induction of lipogenic enzymes from the SREBP-1c
    e. Up-regulation of the free fatty acid uptake transporter FAT/CD36
    f. Induction of the fatty acid synthase (FAS)
    g. Induction of the stearoyl-CoA desaturase 1 (SCD1)

Events a - g lead to an increase in

- De novo fatty acids and triglycerides synthesis
  or
- Fat influx from the peripheral tissues to liver

5.2.4 Identification of the responses (Intermediate Effects) on the organelle/ cellular/ tissue level

Intermediate effects on the organelle level include cytoplasm and nucleus displacement and possibly mitochondrial toxicity. Cell in total could be unaffected in simple case of steatosis but in severe cases the cell could burst or become apoptotic or necrotic. In severe steatosis other tissue cells could be activated leading to inflammation and fibrosis.

5.2.5 Identification of the responses (Intermediate Effects) on the organ level

Mild steatosis does not affect liver function. However, in severe cases steatosis could lead further to steatohepatitis, fibrosis, cirrhosis or even liver failure.

5.3. Scientific evidence in support of the MoA

5.3.1 The LXR receptor

Liver X receptors are ligand-activated transcription factors of the nuclear receptor superfamily first identified in 1994 in rat liver (Apfel et al. 1994, Song 1994). There are two LXR isoforms termed α and ß (NR1H3 and NR1H2) which upon activation form heterodimers with retinoid X receptor (RXR) and bind to the LXR response element found in the promoter region of the target genes (Baranowski 2008). LXRs were shown to function as sterol sensors protecting the cells from cholesterol overload by stimulating reverse cholesterol transport and activating its conversion to bile acids in the liver (Baranowski 2008).

LXRα expression is restricted to liver, kidney, intestine, fat tissue, macrophages, lung, and spleen and is highest in liver, hence the name liver X receptor α (LXRα). LXRβ is expressed in almost all tissues and organs, hence the early name UR (ubiquitous receptor) (Ory 2004). The different pattern of expression suggests that LXRα and LXRβ have different roles in regulating physiological function. This is also supported from the observation that LXRα deficient mice do not develop hepatic steatosis when treated with LXR agonist that activates both types (Lund et al. 2006) and consequently the role of the two isoforms in relation to adverse effects could be different.

5.3.2 The molecular initiating event

Generally speaking chemicals that are able to act through NRs are usually specific ligands. These chemicals are mainly lipophilic and they mimic the action of natural hormones. However, in some cases hydrophilic chemicals (like phthalates) are also capable to act as ligands in NRs due to the molecular structure of the proteins and the pocket sites of the receptors.

The molecular initiating event in the presented MoA is the binding to the LXR or the permissive RXR of the LXR-RXR dimer leading to activation. LXR activation can be achieved via a wide range of endogenous neutral and acidic ligands as shown by crystallographic analysis (Williams et al. 2003). There are known endogenous but also synthetic ligands that can act as agonists. Endogenous agonists for this receptor are the oxysterols (oxidized cholesterol derivatives like 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, and cholestenoic acid) mainly with similar

affinity for the two isoforms (Baranowski 2008). Oxysterols bind directly to the typical hydrophobic pocket in the C-terminal domain (Williams et al. 2003). Other endogenous ligands are the D-glucose and D-Glucose-6-phosphate (Mitro 2007). However, the hydrophilic nature of glucose and its low affinity for LXR present a challenge to the central dogma about the nature of the NR-ligand interaction (Lazar & Wilson 2007). Unsaturated fatty acids have also been shown to bind and regulate LXRα activity in cells. However, in contrast to the role of oxysterols, the biological relevance of this observation has not been established in vivo (Pawar et al. 2003). The function of LXRs is also modulated by many currently used drugs such as statins, fibrates, and thazolidinedione derivatives (Jamroz-Wiśniewska et al. 2007). Some synthetic LXR agonists have been developed like the non-steroidal agonists T0901317 and GW3965 (Schultz et al 2000, Collins et al. 2002). LXR forms a permissive dimer with the RXR which means that chemicals that can activate this receptor can trigger the same pathway as the LXR agonists. The endogenous RXR agonist is 9-cis-retinoic acid (Heyman et al. 1992) while synthetic agonists include LGD1069 and LG100268 (Boehm et al. 1994 and 1995).

In addition to the agonist binding in the LXR there are other mechanisms for its control. LXRα gene promoter contains also functional peroxisome proliferator response element (PPRE) and peroxisome proliferator-activated receptor (PPAR) α and γ agonists were shown to stimulate LXRα expression in human and rodent (Baranowski 2008). Control of the LXRα expression is also dependent on insulin and post-translationally by protein kinase A that phosphorylates receptor protein at two sites thereby impairing its dimerization and DNA-binding (Baranowski 2008).

5.3.3 Identification of the site of action

As already mentioned above LXR isoforms are expressed in various tissues but in relation to the presented MoA we refer to LXRs that are expressed in the hepatocytes.

Nuclear receptors may be classified into two broad classes according to their sub-cellular distribution in the absence of ligand. Type I NRs (like ER and AhR) are located in the cytosol (and they are translocated into the nucleus after ligand binding) while type II NRs like LXRs (but also PXR, PPARα and PPARγ) are located in the nucleus of the cell.

The specific site of binding and the affinity of a ligand for the LXRs depend on the structure of the ligand.

5.3.4 Identification of the responses (intermediate effects) at the macromolecular level

5.3.4.i Binding in the LXREs and target genes transcription

Upon ligand-induced activation both isoforms form obligate heterodimers with the retinoid X receptor (RXR) and regulate gene expression through binding to LXR response elements (LXREs) in the promoter regions of the target genes (*Fig. 1*). The LXRE consists of two idealized hexanucleotide sequences (AGGTCA) separated by four bases (DR-4 element).

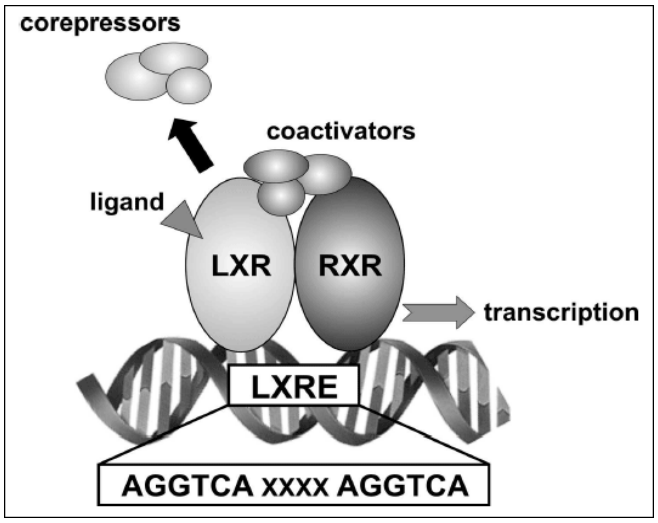


Figure 1. Mechanism of transcriptional regulation mediated by LXRs. RXR - retinoid X receptor, LXRE - LXR response element (Baranowski 2008)

Target genes of LXRs are involved in cholesterol and lipid metabolism regulation (Peet 1998, Edwardsa et al. 2002) including:

- ABC - ATP Binding Cassette transporter isoforms A1, G1, G5, and G8
- ApoE - Apolipoprotein E
- CETP - Cholesterylester Transfer Protein
- CYP7A1 - Cytochrome P450 isoform 7A1 - cholesterol 7a-hydroxylase
- FAS - Fatty Acid Synthase
- LPL - Lipoprotein Lipase
- LXR-α - Liver X Receptor-α
- SREBP-1c - Sterol Response Element Binding Protein 1c
- ChREBP - Carbohydrate Response Element Binding Protein
- FAT/CD36 – Fatty acid uptake transporter (liver)

5.3.4.ii Auto-regulation of the LXRα

Human specific auto-regulated expression specifically of the LXRα has been demonstrated from several studies (Laffitte et al. 2001, Whitney et al. 2001, Li et al. 2002, Kase et al. 2007). Human LXRα gene promoter has a functional LXRE activated by both LXRα and β. In addition human liver LXRα expression is induced by both natural and synthetic LXR agonists.

5.3.4.iii Increase in expression and activity of the carbohydrate response element-binding protein (ChREBP)

ChREBP is an LXR target that independently enhances the up-regulation of select lipogenic genes. The up-regulation of the ChREBP target gene is through liver-type pyruvate kinase (L-PK). Therefore, activation of LXR not only increases ChREBP mRNA via enhanced transcription but also modulates its activity (Cha & Repa 2007). In the liver, ChREBP mediates activation of several regulatory enzymes of glycolysis and lipogenesis including L-PK, acetyl CoA carboxylase (ACC), and fatty acid synthase (FAS). However, according to the study of Denechaud increase in the glucose flux in the cell is a prerequisite for ChREBP activation from T0901317 in mice (Denechaud et al. 2008).

5.3.4.iv Increase in expression of the SREBP-1c from LXR activation and from the ChREBP

SREBP transcription factors are synthesized as inactive precursors bound to the endoplasmatic reticulum membranes. SREBP-1c is one of the SREBP family and it is expressed in most of the tissues of mice and humans with especially high levels in the liver, white adipose tissue, adrenal gland and brain (Shimomura et al. 1997). SREBP-1c is also expressed in various muscles in adult rats and humans at appreciable levels (Ducluzeau et al. 2001, Guillet-Deniau et al. 2002). Its activation requires cleavage to release the NH2-terminal active domain (Eberle et al. 2004). LXR agonist binding leads to enhanced expression of the SREBP-1c (Schultz et al. 2000, Horton et al. 2002). Animals lacking LXRα exhibit reduced basal expression of SREBP-1c, FAS, ACC and SCD-1 (Peet 1998, Repa 2000). In contrast, animals fed synthetic LXR agonists demonstrate a selective increase in SREBP-1c mRNA and nuclear protein induced expression of lipogenic target genes and elevated rates of lipogenesis (Repa 2000, Schultz et al. 2000, Laffitte et al. 2003). However, there are many studies supporting a different behaviour between LXRα and LXRβ, suggesting that SREBP-1c up-regulation is only due to LXRα (Lund et al. 2006, Baranowski 2008). Finally, SREBP-1c is also induced from the ChREBP (Ferré & Foufelle 2010).

5.3.4.v Induction of lipogenic enzymes from the SREBP-1c

An increase on the mRNA of the SREBP-1c is responsible for an increase of the mRNA of lipogenic enzymes like acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Foretz et al. 1999, Foretz et al. 2000). This finding is demonstrated from the absence of triglyceride accumulation on SREBP-1c (-/-) mice (Liang et al. 2002, Schultz et al. 2000, Horton et al. 2002, Shimano et al. 1999). However there is evidence that this effect is not induced in the embryonic state indicating a different role of the SREBP-1c between embryonic and adult life (Liang et al. 2002). It is also suggested that for lipogenic genes, SREBP-1c acts together with ChREBP (Ishii et al. 2004). In addition, in STZ diabetic mice, adenovirus-mediated over-expression of SREBP-1c in the liver resulted in an increase of lipogenic enzyme expression with an increase of the triglyceride hepatic content and a marked decrease in the hyperglycaemia of diabetic mice mimicking perfectly the effect of an insulin injection (Bécard et al. 2001).

Finally there are a number of studies that demonstrated that SREBP-1c is essential for glucokinase (GK) expression and that it is a mediator of insulin action (Ferre 2007, Fleischmann 1999).

5.3.4.vi Up-regulation of the free fatty acid uptake transporter FAT/CD36

Fatty acid translocase CD36 (FAT/CD36) is a scavenger protein mediating uptake and intracellular transport of long-chain fatty acids (FA) in diverse cell types (Su & Abumrad 2009, He et al. 2011). In addition, CD36 can bind a variety of molecules including acetylated low density lipoproteins (LDL), collagen and phospholipids (Krammer 2011). CD36 has been shown to be expressed in liver tissue (Pohl et al. 2005, Cheung et al. 2007). It is located in lipid rafts and non-raft domains of the cellular plasma membrane and most likely facilitates LCFA transport by accumulating LCFA on the outer surface (Ehehalt et al. 2008, Pohl et al. 2005, Krammer 2011).

FAT/CD36 gene is a liver specific target of LXR activation (Zhou 2008). Studies have confirmed that the lipogenic effect of LXR and activation of FAT/CD36 was not a simple association, since the effect of LXR agonists on increasing hepatic and circulating levels of triglycerides and free fatty acids (FFAs) was largely abolished in FAT/CD36 knockout mice suggesting that intact expression and/or activation of FAT/CD36 is required for the steatotic effect of LXR agonists (Febbraio et al. 1999, Lee et al. 2008). In addition to the well-defined pathogenic role of FAT/CD36 in hepatic steatosis in rodents the human up-regulation of the FAT/CD36 in NASH patients is confirmed (Zhu et al. 2011). There are now findings that can accelerate the translation of FAT/CD36 metabolic functions determined in rodents to humans (Love-Gregory et al. 2011) and suggest that the translocation of this fatty acid transporter to the plasma membrane of hepatocytes may contribute to liver fat accumulation in patients with NAFLD and HCV (Miquilena-Colina et al. 2011). In addition, hepatic FAT/CD36 up-regulation is significantly associated with insulin resistance, hyperinsulinaemia and increased steatosis in patients with NASH and HCV G1 (Hepatitis C Virus Genotype1) with fatty liver. Recent data show that CD36 is also increased in the liver of morbidly obese patients and correlated to free FA levels (Bechmann et al. 2010).

5.3.4.vii Induction of the fatty acid synthase (FAS)

LXR agonist treatment has been shown to induce the genes encoding fatty acid synthase (FAS) in SREBP-1c-deficient mice (Oisterveer et al. 2010, Liang et al. 2002, Schultz et al. 2000). This finding shows that in parallel with the increase of FAS expression from the SREBP-1c (Liang et al. 2002, Schultz et al. 2000, Horton et al. 2002) and the ChREBP the enzyme is also directly induced from the LXR.

5.3.4.viii Induction of the stearoyl-CoA desaturase 1 (SCD1)

In addition to the FAS gene induction LXR activation leads to the direct induction of the stearoyl-CoA desaturase 1 (SCD1) in SREBP-1c-deficient mice (Oisterveer et al. 2010, Liang et al. 2002, Schultz et al. 2000). The role of SCD-1 could be crucial for the lipogenic activity of LXRs as there are data supporting that SCD-1 deficient mice are completely protected against hypertriglyceridemia and TG accumulation in liver is decreased after treatment with T0901317 (Chu et al. 2006).

5.3.4.ix De novo fatty acids and triglyceride synthesis

A number of pathways and a great number of enzymes like GK, L-PK, ACC, FAS and SCD-1 are involved in the de novo FA synthesis (Fig. 2 from Postic & Girard 2008). As it is already discussed above these enzymes are induced by LXR agonists (FAS, SCD1), the SREBP-1c

(GK, ACC, FAS) and the ChREBP (L-PK, ACC, FAS) leading to enhancement of the de novo FA synthesis.

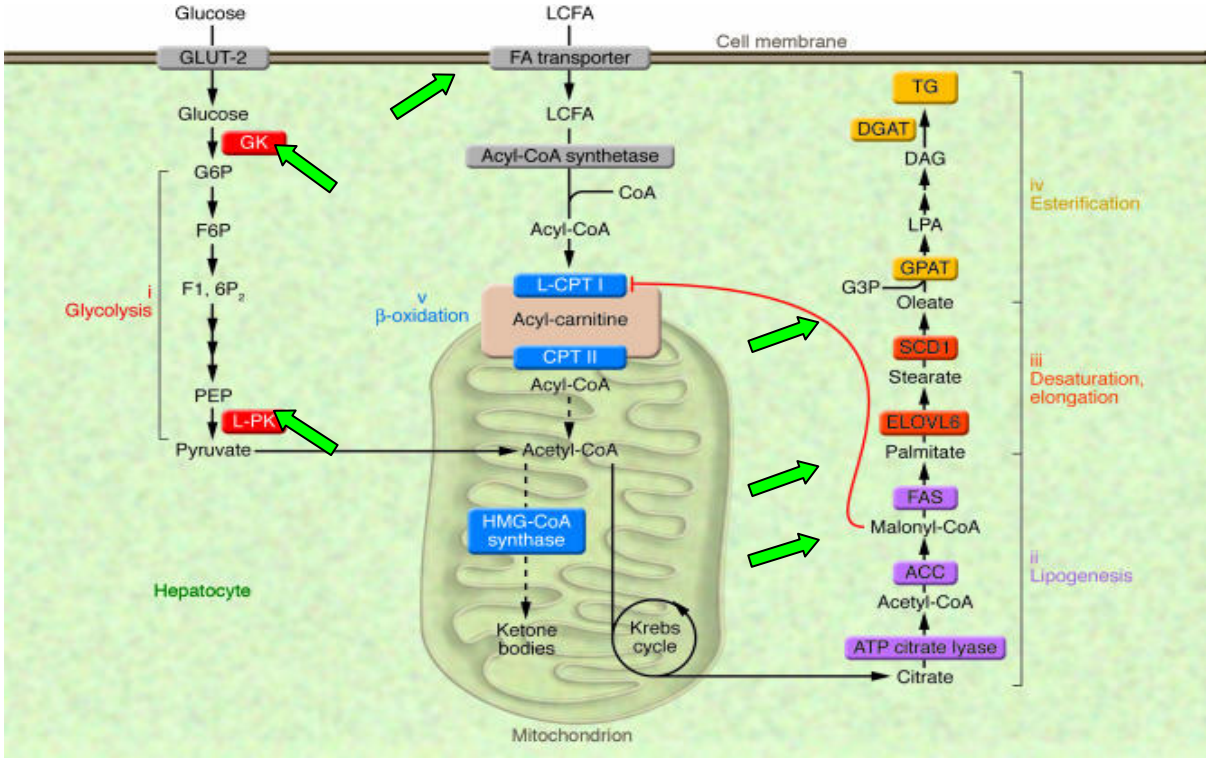


Figure 2. Metabolic pathway for de novo FA synthesis and TG formation (Postic & Girard 2008)

As proposed from Diraison et al 1997 the de novo FA synthesis contributes maximum 5% to the synthesis of FA and TG under normal conditions. Conditions associated with high rates of lipogenesis, such as low fat - high carbohydrate (LF/HC) diet, hyperglycemia, and hyperinsulinemia are associated with a shift in cellular metabolism from lipid oxidation to TG esterification, thereby increasing the availability of TGs derived from VLDL synthesis and secretion.

5.3.4.x Fat influx from the peripheral tissues

Fat influx to the liver is usually increased under condition like obesity. Free fatty acids (FFA) increase in blood leads to an increase of FFA uptake in the liver. Especially the long chain fatty acids (LCFAs) are translocated across the plasma membrane, reassembled to triglycerides and stored in lipid droplets causing hepatic steatosis (Amacher 2011).
As mentioned above CD36 has consistently been shown to be expressed at the plasma membrane and to enhance LCFA uptake upon over-expression (Baranowski 2008, Su & Abumrad 2009).

5.3.5 Identification of the responses (intermediate effects) on the organelle level

Lipid accumulation in the hepatocytes can cause cytoplasm displacement, nucleus distortion and mitochondrial toxicity. While the first two effects are mechanical and not considered to lead to malfunctions, mitochondrial toxicity is a crucial effect for cell function and viability.

Mitochondrial toxicity is commonly considered to be caused due to impairment of β-oxidation or/and oxidative phosphorylation (Fromenty & Pessayre 1997, Pessayre 1999, Labbe 2008, Begriche 2011). However, steatosis in adolescents with NASH seems to be mainly due to increased fatty acid uptake and the de novo synthesis of FAs (Zhu et al. 2011). The development of steatohepatitis and other adverse consequences of steatosis may well be due to steatosis-induced mitochondrial toxicity (Zhu et al. 2011) which in this case should be mainly attributed to increased lipid peroxidation and reactive oxygen species (ROS) overproduction. Endoplasmic reticulum stress activation has been reported in rodents and humans with steatosis. Endoplasmic Reticulum stress is documented to affect liver fat deposition but there are also studies which support Endoplasmic Reticulum stress as a consequence of increased hepatic lipids (Amacher 2011).

5.3.6 Identification of the responses (intermediate effects) on the cellular level

In mild cases fat accumulation is not per se particularly detrimental to the cell. However, large vacuoles may coalesce and produce fatty cysts which are irreversible lesions and in severe cases of fat accumulation the cell may even burst. In addition, fat progressively causes oxidative stress due to the overproduction of ROS, energy depletion, cytokine release, mitochondrial toxicity and eventually necrosis and/or apoptosis.

Fatty hepatocytes may undergo cell cycle arrest due to (1) an inability to replenish ATP caused by over-expressed uncoupling protein-2 (UCP-2) or (2) induction of growth inhibitor p21 leading to G1/S phase arrest. Thus, fatty hepatocytes may fail to undergo compensatory cell division, rendering the liver susceptible to progression of liver injury (i.e. sensitization to injury).

5.3.7 Identification of the responses (intermediate effects) on the tissue level

As already mentioned steatosis is characterized by the accumulation of lipid droplets in the hepatocytes which can be identified histologically as either microvesicular or macrovesicular accumulation. However, the progression of this condition can lead to tissue inflammation (steatohepatitis) and fibrosis with the involvement of other cells of the hepatic tissue like the Kupffer (inflammation) and the stellate (fibrosis) cells.

5.3.8 Identification of the responses (intermediate effects) on the organ level

Liver steatosis as mentioned above is not per se an adverse condition for the organ but it is possible that the progressive development of steatosis may eventually lead to serious adverse effects like steatohepatitis, fibrosis and cirrhosis with liver dysfunction or even liver failure.

5.4. Flow diagram

The following flow diagram of the reported MoA facilitates the understanding of the interaction between the events on different levels of biological organization. The grey areas correspond to other MoAs (AOPs)s via binding to different NRs leading to steatosis.

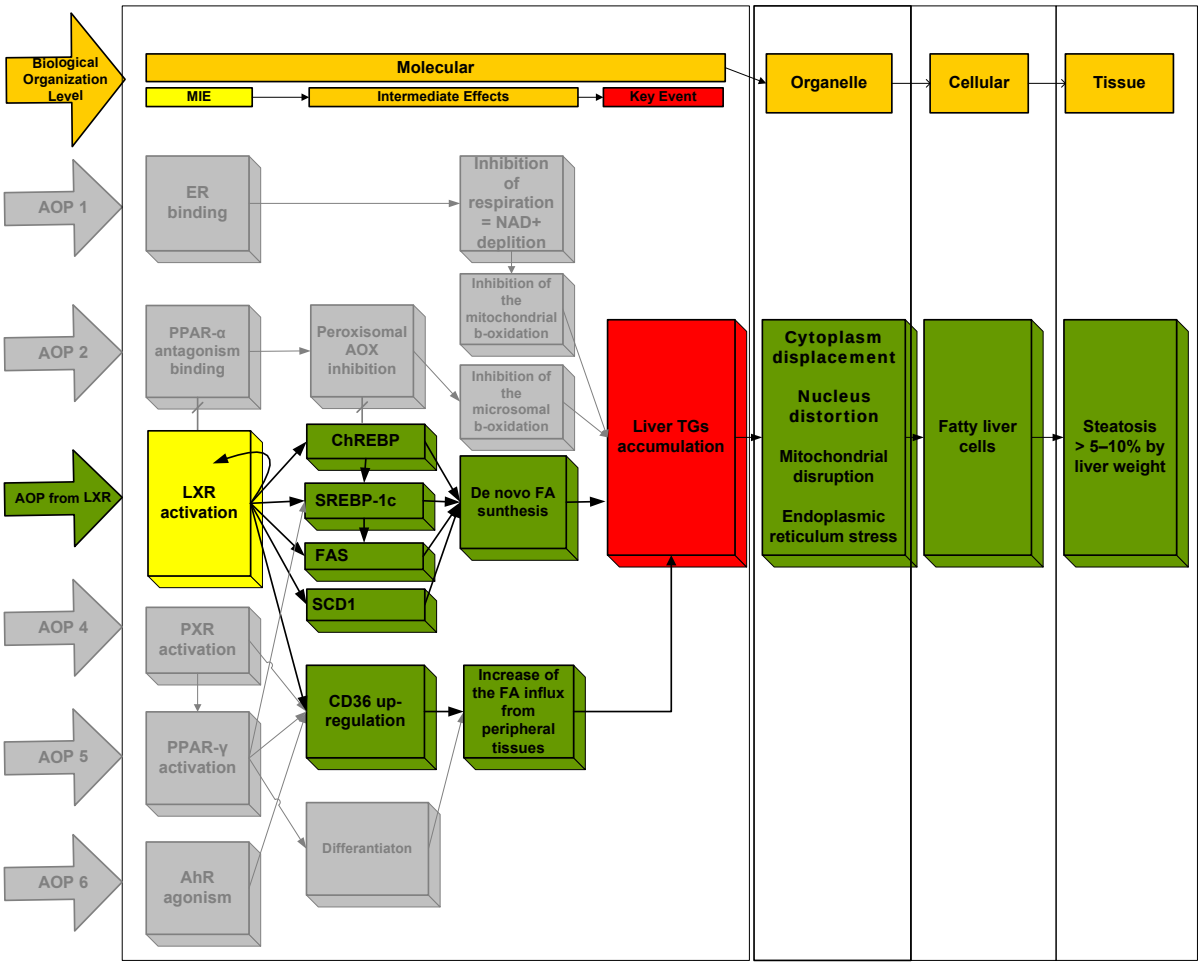


Figure 3. Flow diagram of the MoA from LXR activation to liver steatosis

5.5. Assessment of the MoA

The following assessment is based on the proposed methodology presented in the OECD AOP template (OECD, 2012)

5.5.1 Assessment of the Weight-of-Evidence supporting the MoA (Answer the Hill criteria)

5.5.1.i Concordance of dose-response relationships

OECD template suggestion: *Report any reference/study giving evidence of dose-response relationship.*

The existing studies do not provide dose-response curves. However it may be possible in some cases to construct curves from the given numerical data and to relate the dose response for LXR activation with the dose response for TG accumulation in vitro and in vivo in a second more quantitative iteration as the next step of this AOP development.

5.5.1.ii Temporal concordance among the key events and adverse outcome

OECD template suggestion: *State the agreement between the sequences of biochemical and physiological events leading to the adverse outcome together with the evidence in the literature.*

According to the available information the sequence of the events is in strong agreement and consequently the presented MoA could be considered as qualitatively accurate.

5.5.1.iii Strength, consistency, and specificity of association of adverse outcome and initiating event

OECD template suggestion: *Give the scientific evidence on the linkage between initiating event and adverse outcome.*

The scientific evidence is presented in Chapter 3. Scientific Evidence in support of the MoA.

5.5.1.iv Biological plausibility, coherence, and consistency of the experimental evidence

OECD template suggestion: *Explain the logic, coherence and consistency along with the experimental data supporting the AOP. Describe how the experimental evidence is logical and consistent with the mechanistic plausibility proposed by the theory explaining the initiation of the adverse outcome. If possible, describe the coherence of experimental results for multiple chemicals across different species.*

The steatogenic effect of chemicals like LXR ligands is well established in the literature (Peet 1998, Schultz et al. 2000, Horton et al. 2002) and it is well correlated with the expression of the receptor (Moya et al. 2010) the binding to it. In addition it is believed that LXR acts as a cholesterol sensor. Consistent with this role, it has been proposed that LXR induces SREBP-1c in order to generate fatty acids needed for the formation of cholesterol esters, which buffer the free cholesterol concentration (Ferré & Foufelle 2007). Further analysis of the logic, coherence and consistency along with the experimental data has already been presented in *Chapter 3. Scientific Evidence in support of the MoA.*

5.5.1.v Alternative mechanism(s) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanism(s) of action, if supported, require a separate AOP.

OECD template suggestion: *Report other possible mechanisms that can lead to the adverse outcome and state if they can be covered by this AOP.*

As already mentioned in the introduction and *Chapter 3: Scientific Evidence in support of the MoA* there are many other possible MoAs of a chemical in the development of steatosis including MoAs involving the inhibition of β-oxidation, the inhibition of oxidative phosphorylation (leading to a decrease of ATP needed for β-oxidation) and the malfunction of

the mechanisms of the excretion of TG from the cell. These pathways are not covered in the presented MoA as they are not directly linked with the activation of LXR. Furthermore, as already explained LXR is not the only receptor which has been identified to be involved in fatty acid metabolism and steatogenesis. Exogenous chemicals acting as ligands for any of the following Nuclear Receptors (AhR, PXR, PPARα, PPARγ and ER) may play a role in the development of steatosis (grey elements on the AOP flow diagram, Fig. 3). There also known interactions or cross-talk between the NRs. Examples of possible interactions are related with fact that LXR is also regulated by the PPARα, the FAT/36 up-regulation from the AhR, PXR and PPARγ, the inhibition of β-oxidation from PPARα and indirectly from the ER.
It may be possible from existing literature, or further experimental work to develop MoAs taking binding to each of these receptors as the molecular initiating event and describing the converging pathways leading to steatosis. In fact this work is in progress and indicated as the grey elements on the AOP flow diagram, Fig. 3.

The biology of LXR function has been studied using the high affinity synthetic ligands T0901317. According to the study of Mitro (2007), T0901317 binds and activates hPXR and hLXRα with similar affinity, and can regulate multiple PXR target genes in human cells and mice (like CD36) with similar efficacy to established PXR ligands, but significantly greater potency (Mitro 2007). The author suggested that some of the effects observed with T0901317 such as the more deleterious increase in lipogenesis and hepatic lipid accumulation (in comparison to the LXR-selective GW3965) that have been ascribed to LXR activation maybe the result of simultaneous stimulation of PXR and LXR activity and that the assumption that T0901317 behaves as an LXR-selective agonist may have led to some inaccurate conclusions regarding the effects of LXR activation in vivo.

From the data of this study it is evident that SREBP-1c, FAS and SCD-1, which are LXR but not PXR regulated genes, were significantly up-regulated by T0901317. In contrast GW3965 up-regulates less effectively the SREBP-1c, marginally the SCD-1 and not at all the FAS despite the fact that it is considered as a selective LXR agonist (Mitro 2007). The CD36 gene is considered also as a liver specific target of LXR activation (Zhou 2008). However, in the study of Mitro (2007), GW3965 did not up-regulate CD36. These findings could be explained by the lower affinity of this synthetic LXR agonist (EC50 of 0.19 and 0.03 μM for hLXRα and hLXRβ) in relation to the T0901317 (EC50 of 0.02-0.05 μM for both isoforms). Interestingly and despite the low up-regulating activity, GW3965 increases FA and TG accumulation in rat and primary human hepatocytes (Kotokorpi et al. 2007). Based on this information, it could be possible that T0901317 binding on PXR could enhance its steatogenic activity with the proposed MoA still being plausible. This plausibility, however, is clearly related with quantitative aspects.

In conclusion, the MoA described can be considered very well supported by the available scientific evidence and it is biologically plausible.

5.5.1.vi Uncertainties, inconsistencies and data gaps

OECD template suggestion: *Include any uncertainties about the experimental details, such as uncertainties regarding the differences in sensitivity of different biological targets (e.g. cysteine versus lysine, Type I pyrethroid versus Type II), the measurements of biological activity in different assays. Describe inconsistencies within the reported data, such as differences between in vivo responses for very similar chemicals, and report any data gap that causes the weakness of the AOP.*

The information used for the development of the present pathway is based on in-vitro and in-vivo studies. In the in-vitro studies several cell lines have been used. The expression of the LXR, the SREBP-1c and other elements on these cell lines is a key factor for the plausibility of the pathway in human. According to the study from Moya et al 2010, LXR expression (as measured from mRNA using RT-PCR) in human hepatocytes, HepG2 and HeLa cells was approximately 70%, 70% and 50% in relation to the level of expression in human liver. In addition the expression of SREBP-1c was significantly down-regulated (to less than 25% of normal levels of expression in the liver) in all 3 cell lines. Consequently positive results in relation to fat accumulation after LXR activation from studies using these cell lines may under-estimate the magnitude of effect on human liver while negative results could be interpreted as inconclusive. The assessment of the relative expression of these receptors in other cell lines would be of great importance in order to evaluate the relevance of each in vitro study result.

In relation to the in vivo studies which have be made mainly (if not exclusively) in rodents the relevance for humans should be addressed. LXR expression is considered adequately conserved from rodents to humans. In addition it is well known that all the other elements of the pathway are present in human liver. A good example of this is that the well-defined pathogenic role of FAT/CD36 in hepatic steatosis in rodents is also confirmed by the up-regulation in humans of the FAT/CD36 in cases of NASH, NAFLD, insulin resistance, hyperinsulinaemia, HCV and morbidly obese patients (Zhu et al. 2011, Love-Gregory & Abmurad 2011, Miquilena-Colina et al. 2011, Bechmann et al. 2010). However, there is some speculation in relation to the extent that adverse side effects observed in rodents will occur in higher species, including humans. These speculations are raised due to the different behaviour of the LXR agonist GW3965 in *in vitro* systems which although markedly stimulating lipogenic gene expression in primary human hepatocytes leading to significant TG accumulation at all 3 dose levels after 48 hr, produced only a very modest increase in the triglyceride content in rat cells (Kotokorpi et al. 2007), demonstrating that the use of this rat cell line could underestimate the effect in humans. FA increase was reported in both cell lines.

Another interesting finding is that in humans, total CD36 deficiency is relatively common (3–5%) in persons of African and Asian descents (Su & Abmurad 2009). Consequently the presented MoA could be affected mainly quantitatively among humans of different origin.

Induction of lipogenic enzymes from the SREBP-1c is evidenced in adult mice but not during the fetal life indicating a different role of the SREBP-1c between these two stages (Liang et al. 2002). This finding gives a strong indication that the presented pathway may be altered in other than adult life stage.

Another finding is that of the study of Hu et al. 2005 according to which administration of T0901317 in PPAR-null mice promoted a dose-dependent increase in the rate of peroxisomal β-oxidation in the liver and in relation only to the LXRα. The author suggests that this induction may serve as a counter regulatory mechanism for responding to the hypertriglyceridemia and liver steatosis that is promoted by potent LXR agonists *in vivo.*

T090137 was shown to up-regulate hepatic expression and plasma activity of PLTP in mice in addition to angiopoietin-like protein 3 (Angptl3), playing a critical role in LXR-induced hypertriglyceridemia. However it should be noted that hypertriglyceridemic effect of LXR agonists is usually transient and limited to the first few days of the treatment likely due to

enhanced VLDL-triglyceride hydrolysis resulting from increased expression of hepatic LPL (Baranowski 2008).

Some studies have demonstrated absence of triglyceride accumulation on SREBP-1c (-/-) mice suggesting that SREBP-1c is a crucial element of the present MoA (Liang et al. 2002, Schultz et al. 2000, Horton et al. 2002, Shimano et al. 1999). In another study in FAT/CD36 knockout mice the effect of LXR agonists on increasing hepatic and circulating levels of triglycerides and free fatty acids (FFAs) was largely abolished suggesting that intact expression and/or activation of FAT/CD36 is required for the steatotic effect of LXR agonists (Febbraio et al. 1999). These two findings together and considering that they are constant and not related with specific experimental conditions could lead one to the hypothesis that both SREBP-1c and CD36 are imperative elements for the cause of steatosis. This hypothesis could be further examined.

The present MoA could also be affected by factors related to the formation of steatosis such as trends in adipose tissue (AT) deposition, the total body fat, the visceral AT and the subcutaneous AT which vary among different life stages such as childhood, puberty and adolescence, between sexes and among humans of different origin (Staiano 2012).

5.5.2 Assessment of the quantitative understanding of the MoA

OECD template suggestion: *Include an evaluation of the experimental data and models to quantify the molecular initiating event and other key events. If possible, describe transparent determination of thresholds and response-to-response relationship to scale in vitro and in chemico effects to in vivo outcomes*.
In the present study only qualitative assessment of the proposed MoA was performed. In the studies used there are numerical data mainly to support the expression and up-regulation of the different elements of the pathway. However, further analysis of these numerical data is suggested in following steps.
Interestingly, the existence of many network motifs along the pathways was noted during the analysis of the literature, e.g. the positive feed forward LXR up-regulation. This information could be used in the future for the quantitative interpretation of dose response curves and the development of quantitative prediction models of the adverse outcome following the activation of the LXR.

5.6. Confidence in the MoA

OECD template suggestion: *Discuss the summary of the scientific evidence supporting the AOP by answering the following questions*:

5.6.1 How well characterised is the MoA?

OECD template suggestion: *Describe how well the adverse outcome is understood qualitatively and quantitatively.*

Liver steatosis is a well understood adverse outcome. A great number of publications from in vitro, in vivo, mechanistic, clinical and epidemiological studies exist for the qualitative assessment of steatosis. However, the quantitative analysis of the role of a specific exogenous chemical in an adverse outcome in human is a very challenging task due to the involvement of a large number of inter-related factors following the MIE. In fact one chemical may bind to

more than one receptor and consequently have different impacts either quantitatively or qualitatively on the downstream events.

5.6.2 How well are the initiating and other key events causally linked to the outcome?

OECD template suggestion: *Give short statement on the relationship between each key event and adverse outcome.*

LXR agonists such T0901317 have been shown to produce LXR activation, as well as triglyceride accumulation, which has been demonstrated in rodent (mouse and rat) and human liver cell lines in vitro. The same chemicals shown to be LXR agonists in the in vitro assays have shown triglyceride accumulation in the liver leading to steatosis in animals and humans through steps of the reported MoA.

5.6.3 What are the limitations in the evidence in support of the MoA?

OECD template suggestion: *Indicate any lack or disagreement in the scientific evidence supporting the AOP.*

Disagreement in the scientific evidence supporting the presented AOP was not found. In relation to data gaps in addition to lack of quantitative information as discussed above there is also lack of specific information in relation to the role of other target genes expressed after the LXR activation.

5.6.4 Is the AOP specific to certain tissues, life stages / age classes?

OECD template suggestion: *Indicate if there are critical life stages, where exposure must occur, to results in the adverse effect. Or specify if there are key events along the pathway which are dependent on the life stage, although the AOP is known to be initiated regardless of life stage. Indicate also if the AOP is associated also with age- or sex-dependence.*

There is evidence of different levels of expression of CD36 in different ethnic groups which may be expected to alter the sensitivity to development of steatosis. There may also be differences in expression and role of the same proteins/enzymes in foetal life but this has not been fully elucidated. Further information can be found in the *Chapter 5.1.6. Uncertainties, inconsistencies and data gaps*

5.6.5 Are the initiating and key events expected to be conserved across taxa?

OECD template suggestion: *State if the key events for this AOP appear to be conserved across any group of animals (e.g. mammals).* .

From the analysis of the available information from experimental studies using rodents the elements of the MoA appeared to be well conserved between mice and rats. Some concerns in relation to the relevance of the in vivo studies to human are raised mainly due to the different **behaviour of the LXR agonist GW3965 which while stimulating lipogenic gene expression in** human hepatocytes, causes only a slight increase in TGs in rats (Kotokorpi et al. 2007). Some more differences were also reported between hamsters and monkeys in relation to hypertriglyceridemia (Groot et al. 2005).

6. Discussion

The search for drugs to target certain steps or molecular actors in the progression of disease has led to a wealth of mechanistic data being available in the biomedical literature. This has proved a rich source of information in relation to the disease etiology and treatment of fibrosis and steatosis which can inform on toxicological MoAs of exogenous chemicals which may be contributing, or have a potential to contribute, to the development of these conditions. However, the task of sifting through the vast amount of information and extracting the most appropriate evidence is both time consuming and demands an adequate level of expertise

The strategy followed as described was a) to understand the normal biological/physiological processes involved in e.g. response to cell injury, or lipid metabolism, b) to understand and describe how these normal physiological processes can be dysregulated and c) by taking reference chemicals known to induce either steatosis or fibrosis, to describe the chemico-biological interaction of the chemical with the system (i.e. the molecular initiating event) and d) to describe in a qualitative way how such a stimulus could promote a series of events leading to the respective adverse outcomes (i.e. describe a toxicological MoA for these chemicals).

The exercise has demonstrated that there are clearly many factors involved in controlling e.g. fatty acid metabolism or response to injury and hence there are many possible ways in which a chemical may exert an effect, in other words there may be multiple MoAs leading to one adverse outcome. However, the aim here was to describe one toxicological MoA for steatosis and one for fibrosis by taking reference chemicals identified by the SEURAT Gold Compound Working Group and attempting to describe the necessary steps from protein alkylation to fibrosis and from a specific nuclear receptor activation to steatosis in order to identify the key intermediate events that could be the basis for development of suitable models for measuring potential activity of chemicals in such a pathway.

It is clear from the descriptions presented that the process is complex with many feedback and feed-forward loops and complex inter-relations, which is a common feature of any homeostatic process.

Nevertheless, as can be seen from the flow diagrams, it was possible to depict a more or less linear sequence of events also capturing the events that are interrelated to many other intermediate events or which follow and participate at different points along the whole process within a specific MoA, such as oxidative stress and inflammation. It was also possible to identify some very clear pivotal events such as Kupffer and stellate cell activation, TGF-β1 expression together with chronic inflammation, which are prerequisites for liver fibrosis.

In relation to the ultimate goal of safety assessment it will be necessary to not only identify the toxicological MoAs for specific types of chemicals but also understand the dose-response -relationships, i.e. the concentration above which a reactive metabolite of a chemical could, if sustained for long enough, eventually lead to steatosis or fibrosis.

Quantitative data on dose-response-relationships and detailed information on temporal sequences are scarce and often inexistent. However, the available numerical data from the in vivo and in vitro studies could be partially informative and a further in depth analysis of these data could be made in the next steps.

The OECD template and related guidance was followed to differing extents in each of the prototype MoAs described in this report. Overall the template proved useful but in parts it proved difficult to capture essential information in an efficient way. A revised version of the template and guidance is expected to be provided by the OECD after the summer and this will be evaluated in terms of any possible improvements made and the overall relevance for communicating knowledge on MoA within SEURAT-1 in this manner.

7. Conclusions and Next Steps

This report describes two MoAs related to chronic liver toxicity, but it also describes the working process leading to this result including the problems that have been encountered, such as scarcity of quantitative data and the difficulty in capturing and describing complex non-linear processes in a narrative manner. The two substantially different pathways yielded different datasets and the two examples have been tackled in a slightly different way due to the different professional background of the authors; therefore the data presentation slightly differs as well, though the relevant OECD guidance has been followed in general. A multidisciplinary collaboration is probably the best option for capturing and categorising the numerous and various data that are needed for building a MoA.

Each presented MoA was also introduced into the MoA-Wiki. Eventually it could be considered to also depict the events and inter-relationships between the events in the graphical tool *Effectopedia* to proceed in facilitating the interoperability between these various tools for the description and display of an AOP. Currently however *Effectopedia* is still under development and thus will be tested at a later date.

This description of the two MoAs is not a final, but a first step and will feed into a feasibility study for predicting selected types of repeated dose target organ toxicity based on complementary tools and test systems to be developed within the SEURAT cluster. The evaluation and comments by the members of the SEURAT-1 research cluster will be extremely important, and it is hoped that the document will provoke discussions and provide much food for thought. Built on this feedback and in cooperation with the Mode of Action Working Group (MAWG) the work can be adapted and refined and potentially opened up to a larger public for further discussions. The descriptions of these two MoAs will also be analysed and discussed at the next MAWG meeting on MoA/AOP (liver) to be hosted by the JRC in mid-October 2012

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Abstract

This report presents the definition and detailed documentation of chosen toxicological MoAs associated with repeated dose target organ toxicity as a first step in building a "prototype" safety assessment framework. In addition to providing a detailed description of the two chosen MoAs related to chronic liver toxicity, namely "MoA from Protein Alkylation to Liver Fibrosis" and "MoA from Liver X Receptor Activation to Liver Steatosis", the report also describes the working process leading to this result including the problems that have been encountered. The exercise followed as far as possible relevant WHO-IPCS and OECD guidance. The report represents the first deliverable of a contract of work between Cosmetics Europe and the European Commission's Joint Research Centre for supplementing the work of the SEURAT-1 research cluster.

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