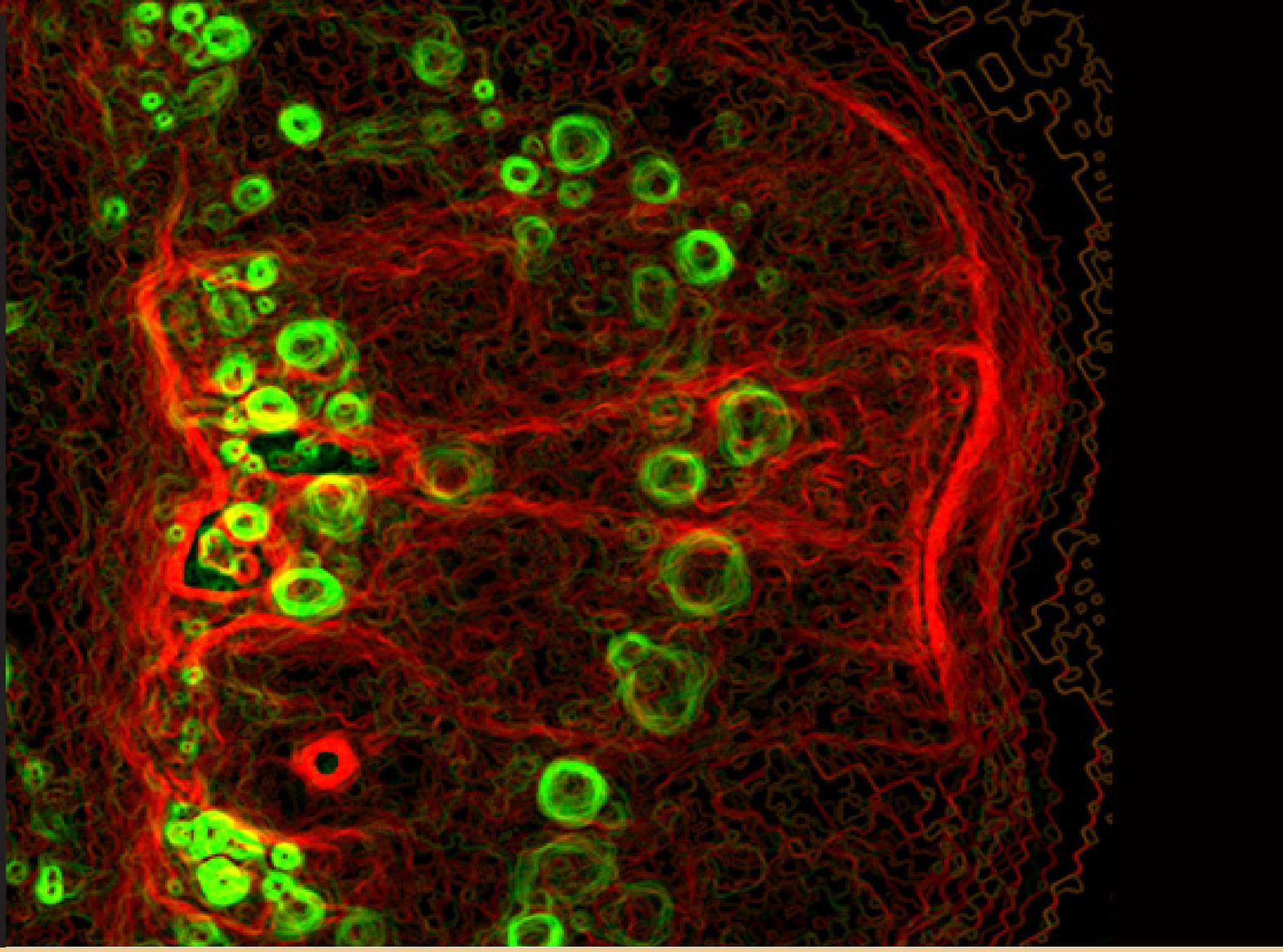


Filipe Emanuel Hasse Velez Furtado

Liver X Receptor alpha
A target for non-alcoholic fatty liver disease therapy

Monografia realizada no âmbito da unidade Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Março 2016

· U C ·

Universidade de Coimbra

Filipe Emanuel Hasse Velez Furtado

Liver X Receptor alpha
A target for non-alcoholic fatty liver disease therapy

Monografia realizada no âmbito da unidade Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Março 2016

· U C ·

Universidade de Coimbra

Eu, Filipe Emanuel Hasse Velez Furtado, estudante do Mestrado Integrado em Ciências Farmacêuticas, com o nº 2009009298, declaro assumir toda a responsabilidade pelo conteúdo da Monografia apresentada à Faculdade de Farmácia da Universidade de Coimbra, no âmbito da unidade Estágio Curricular.

Mais declaro que este é um trabalho original e que toda e qualquer afirmação ou expressão, por mim utilizada, está referenciada na Bibliografia desta Monografia, segundo os critérios bibliográficos legalmente estabelecidos, salvaguardando sempre os Direitos de Autor, à exceção das minhas opiniões pessoais.

Coimbra,11 de Março de 2016.

_____

Assinatura do Aluno

(Filipe Emanuel Furtado)

_____

A Tutora

(Professora Doutora Maria Manuel Silva)

_____

O Aluno

(Filipe Emanuel Furtado)

I hereby wish to express my gratitude to Dr. Maria Manuel Silva for her continuous support, guidance and availability and for encouragement towards choosing this subject.

To my Grandfather and Father, for whom I reserve my deepest respect and admiration I thank for being determinant in choosing this subject.

I wish to thank my family for all the love and support they have always shown me. I am most grateful to be lucky enough to have two wonderful women in my life, my mother and my girlfriend who I know will never disappoint me and will always be present.

Abreviations

ABC *ATP-binding Casset Transporter*

ACC *Acetyl-CoA Carboxylase*

AD *Alzheimer's Disease*

ApoE *Apo-lipoprotein E*

ALP *Alkaline Phosphatase*

ALT *Alanine Transaminase*

AST *Aspartate Aminotransferase*

CD36/FAT *Cluster of Differentiation 36, also known as Fatty Acid Translocase*

Chrebp *Carbohydrate-responsive element-binding protein*

ChIP *Chromatin Immunoprecipitation Assay*

COX2 *Ciclo-Oxygenase 2*

CVD *Cardiovascular Disease*

Cyp7a1 *Cholesterol 7 alpha-hydroxylase*

DBD *DNA Binding Domain*

DDP4 *Dipeptidyl Peptidase-4*

Fasn/FAS *Fatty Acid Synthase or Fatty Acid Synthase encoding gene*

FXR *Farnesoid X Receptor*

GPAT *Glycerol-3-Phosphate Acyltransferase*

GR *Glucocorticoid Receptors*

HDF *High Fat Diet*

HDL *High Density Lipoprotein*

Il1b *Interleukin-1 beta*

Il6 *Interleukin 6*

Il12 *Interleukin 12*

INOS *Nitric Oxide Synthase*

LDB *Ligand Binding Domain*

LDL *Low Density Lipoprotein*

LicA *Licochalcone A*

LXR *Liver X Receptor*

LXRα *Liver X Receptor alpha*

LXRβ *Liver X Receptor beta*

LXRE *Liver X Receptor Response Element*

MDGA *Meso-Dihydroguaiaretic Acid*

MMP-9 *Matrix Metallopeptidase 9*

NAFL *Non-alcoholic Fatty Liver*

NAFLD *Non-alcoholic Fatty Liver Disease*

NASH *Non-alcoholic Steatohepatitis*

NRs *Nuclear Receptors*

OHC *Oxysterol*

PD *Parkinson's Disease*

PPAR Peroxisome *Proliferator Activated Receptor*

PPD *20(S)-Protopanaxadiol*

PPT 20*(S)-Protopanaxatriol*

PR *Progesterone Receptor*

PXR *Pregnane X Receptor*

RCT *Reverse Cholesterol Transport*

RIP140 *Receptor-interacting Protein 1*

RT-PCR *Reverse Transcription Polymerization Chain Reaction*

RXR *Retinoid X Receptor*

SDC1 *Stearoyl-CoA Desaturase-1*

SREBP *Sterol Regulatory Element-binding Protein*

TG *Triglyceride*

TNF-α *Tumor Necrosis Factor alpha*

List of tables and figures

Abstract

Non-alcoholic fatty liver disease is a growing concern continuously observed at earlier ages and in a growing incidence among western populations. Moreover it is expected to keep growing while sedentary lifestyle and harmful nutritional patterns prevail, eventually leading to higher prevalence of obesity and metabolic diseases which strongly co-relate to non-alcoholic fatty liver disease's development.

The Liver X Receptor emerged in the recent years as an interesting pharmacological target for the treatment of a wide variety of pathologies. The Liver X Receptor interference in cholesterol and lipid homeostasis as well as its anti-inflammatory actions make it a very promising target for the treatment of metabolic diseases and in particular non-alcoholic fatty liver disease.

To date, two isoforms are known, the alpha and the beta, both are expressed in the liver. However, it's the alpha's isoform role that is of particular interest in the search for reversing early stage non-alcoholic fatty liver disease and limiting the later stages of non-alcoholic fatty liver disease progression. The antagonism of Liver X Receptor could prove crucial in the treatment of non-alcoholic fatty liver disease by reducing liver lipid content, inflammation and lipogenesis.

The Liver X Receptor alpha's involvement in molecular physiological mechanisms is complex, therefore the path to find viable drugs will present various obstacles to overcome and needs to be tread carefully.

Although the Liver X Receptor alpha proves difficult to safely target, recently discovered compounds have shown promising results and may pave the way to the development of a new class of drugs.

Keywords: Non-alcoholic fatty liver disease, Liver X Receptor, homeostasis, cholesterol, inflammation, lipogenesis.

Resumo

O fígado gordo não alcoólico é um factor de preocupação crescente e é observado um progresso considerável em indivíduos cada vez mais jovens. A tendência é que continue a aumentar, tendo em conta o estilo de vida cada vez mais sedentário e os maus hábitos alimentares consequentemente levando a uma maior prevalência de obesidade e doenças metabólicas que, por sua, vez constituem factores propícios ao desenvolvimento do fígado gordo não alcoólico.

Nos últimos anos, o Receptor X do fígado tem surgido como um alvo terapêutico com interesse no tratamento de diversas patologias. A sua clara influência na homeostase lipídica, regulação do transporte de colesterol e acção anti-inflamatória apontam-no como um alvo promissor no tratamento de doenças metabólicas e em particular no tratamento do fígado gordo não alcoólico.

Até à data são conhecidas duas isoformas, a isoforma alfa e a beta, ambas são expressas no fígado. Contudo, é o papel isoforma alfa no desenvolvimento do fígado gordo não alcoólico que suscita maior interesse e promete abrir novas opções terapêuticas no sentido de reverter a patologia em fases iniciais ou limitar a sua progressão em fases mais tardias. O antagonismo do Receptor X do fígado tem grande potencial terapêutico no tratamento do fígado gordo não alcoólico, através da redução de acumulação excessiva de gordura no fígado, redução da lipogénese nos hepatócitos e supressão de consequentes processos inflamatórios.

O envolvimento da isoforma alfa do Receptor X do fígado a nível molecular é bastante complexo e não totalmente compreendido pelo que a procura de novos compostos que o tenham como alvo terapêutico deve ser feita tendo em conta todas as consequências negativas que podem advir da sua desregulação.

Embora o Receptor alfa X do fígado apresente bastantes desafios para a modulação da sua acção de uma forma segura e eficaz. Recentemente foram descobertos compostos que apresentam resultados promissores e podem abrir o caminho a uma nova classe de fármacos.

Palavras Chave: fígado gordo não alcoólico, Receptor X do fígado, homeostase, colesterol, inflamação, lipogénese.

Index

1 –Non-alcoholic fatty liver disease (NAFLD)

1.1 - Etiology and definition of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease, and its worldwide prevalence continues to increase with the growing obesity epidemic through all age groups.[1]

Non-alcoholic fatty liver disease (NAFLD) is defined by existing hepatic steatosis, before evidence provided either by imaging or histology or in the absence of secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders.[2] NAFLD is a lipotoxic disease where lipid molecules accumulate in the liver eventually altering the organ's function. A model of "two-hit hypothesis" is generally accepted to describe NAFLD's progression. The "first-hit" consists of lipid accumulation in the hepatocytes which will in turn increase the organ's vulnerability to many other factors that constitute the "second-hit" and lead to hepatic injury, inflammation and fibrosis.[3]

In the majority of patients, NAFLD is associated with metabolic risk factors such as obesity, diabetes mellitus, and dyslipidemia (Table 1.). Few studies suggest that type 1 diabetes have increased prevalence of hepatic steatosis based on imaging, however, there is little histological evidence[2] and therefore type 2 diabetes mellitus is generally considered as the main risk condition. In addition to the classic risk factors, there are data supporting that polycystic ovary syndrome, hypothyroidism, obstructive sleep apnea, hypopituitarism, hypogonadism and pancreato-duodenal resection independently of obesity[4] are important risk factors for the development of NAFLD. As an independent risk factor for cardiovascular disease (CVD), NAFLD allows the prediction of future events, regardless other risk factors such as gender, low density lipoprotein (LDL) cholesterol, smoking habits, age or other metabolic risk factors. NAFLD is also associated with all-cause mortality, contributing both by liver related deaths and non-liver related causes i.e. malignancy, diabetes and coronary artery disease.[5] NAFLD comprehends a wide range of liver diseases ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) and cirrhosis. NAFL is characterized by the presence of hepatic steatosis with no hepatocellular injury. Non-alcoholic steatohepatitis is defined by hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis[2]. Approximately 20% of the patients with NASH progress into fibrosis and cirrhosis in a 15 years' time period.[6] Table 1. illustrates the progression of NAFLD.

Conditions with established association	Conditions with emerging association
• Obesity • Type 2 diabetes mellitus • Dyslipidemia • Metabolic syndrome	• Polycystic ovary syndrome • Hypothyroidism • Obstructive Sleep apnea • Hypopituitarism • Hypogonadism • Pancreato-duodenal resection

Table 1. Risk factors for NAFLD (Table adapted from Chalasani *et al.,* American Gastroenterological, A. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *American Journal of Gastroenterology.* **2012**, *107*, 811-826.)[2]

1.2 -Prevalence of NAFLD

As show by the discrepancies among different studies, the prevalence of NAFLD varies widely depending on the targeted population and the definition considered. Two Japanese studies[7, 8], reported an incidence rate of 31 and 86 cases of suspected NAFLD per 1,000 person-years respectively, whereas a study from England showed a much lower incidence rate of 29 cases per 100,000 person-years.[9] Global estimates of NAFLD prevalence range from 6.3%-33% with a median of 20% in general population.[2] More studies are needed to better understand the incidence of NAFLD across different age, ethnic, and geographic groups. In patients with diabetes the prevalence of NAFLD is estimated to range from 43-60%[10,11], obese patients undergoing bariatric surgery 91%[12], and in patients with hyperlipidemia 90 %.[13, 14] Individuals under the age of 20 have an estimated prevalence of less than 20% whereas at and over the age of 60 it is estimated to be around 40 %.[15] Both age and the male gender are risk factors for further progression into NASH and fibrosis.[16]

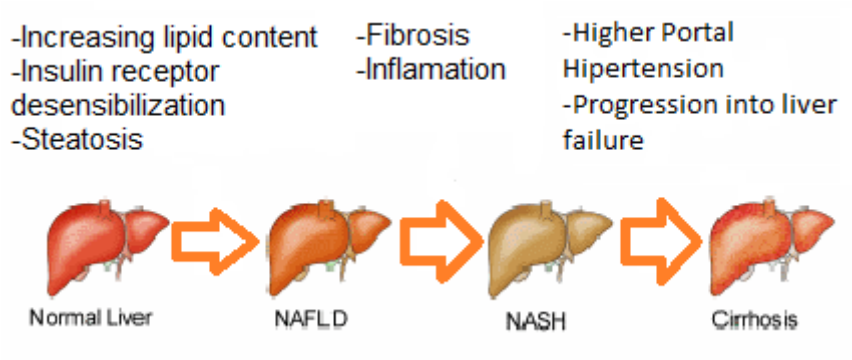


Fig.1. Progression of NAFLD into later stages and consequent pathologies. Figure adapted from Phenex Pharmaceuticals AG[17]

1.3 -Current NAFLD treatment approaches.

Current therapy of NAFLD includes both pharmacological and non-pharmacological methods. Non-pharmacological methods are still the mainstay in the management of patients with NAFLD. These consist of life style interventions with diet and exercise, therefore reducing caloric intake and eventually weight.[2]

Surgical approach is also a non-pharmacological strategy often considered, in particular bariatric surgery, indicated for weight loss, consequently increasing liver, muscle, and adipose tissue sensitivity to insulin.[2]

Weight loss can also be obtained pharmacologically, through orlistat and sibutramine.

As for pharmacological NAFLD management, there are currently a wide variety of strategies:

- Insulin sensitizers, metformin, thiazolidinedione, incretin based therapies(e.g. liraglutide, exenatide), dipeptidyl peptidase-4 (DDP4) inhibitors (e.g. sitagliptin);
- Lipid lowering drugs such as statins, fibrates and omega-3 fatty acids;
- Hypertension management through antiotensin II receptor blockers (e.g. losartan);
- Anti-oxidant and cytoprotective therapies;
- Probiotics.

As a last resource, patients that develop into an end-stage liver disease, due to NFLD require liver transplantation as the only definitive treatment.[18]

2 –Liver X receptors

2.1-Nuclear Receptors

Nuclear receptors (NRs) encompass a wide superfamily of transcription factors that modulate and interfere with a variety of important physiological functions (i.eg. cell differentiation, homeostasis, lipid metabolism, embryonic development, among others.). NRs have also been identified to play key roles in various pathological processes such as diabetes, cancer, asthma, hormone resistance syndromes and others. NRs are soluble proteins with the ability of binding to specific DNA-regulatory elements and acting as cell-type and promoter specific regulators. The activity of NRs has been shown to be modulated by binding of the corresponding ligands.[19]

Numerous drugs that target NRs are currently marketed, such as glucocorticoids (i.eg. prednisolone, dexamethasone) targeting glucocorticoid receptors (GR); estrogen receptor agonists and antagonists (i.eg. ethinylestradiol, clomiphene, tamoxifen), progesterone receptor (PR) ligands (i.eg. levonogestrel and drospirenone) among others. (Fig. 2.)

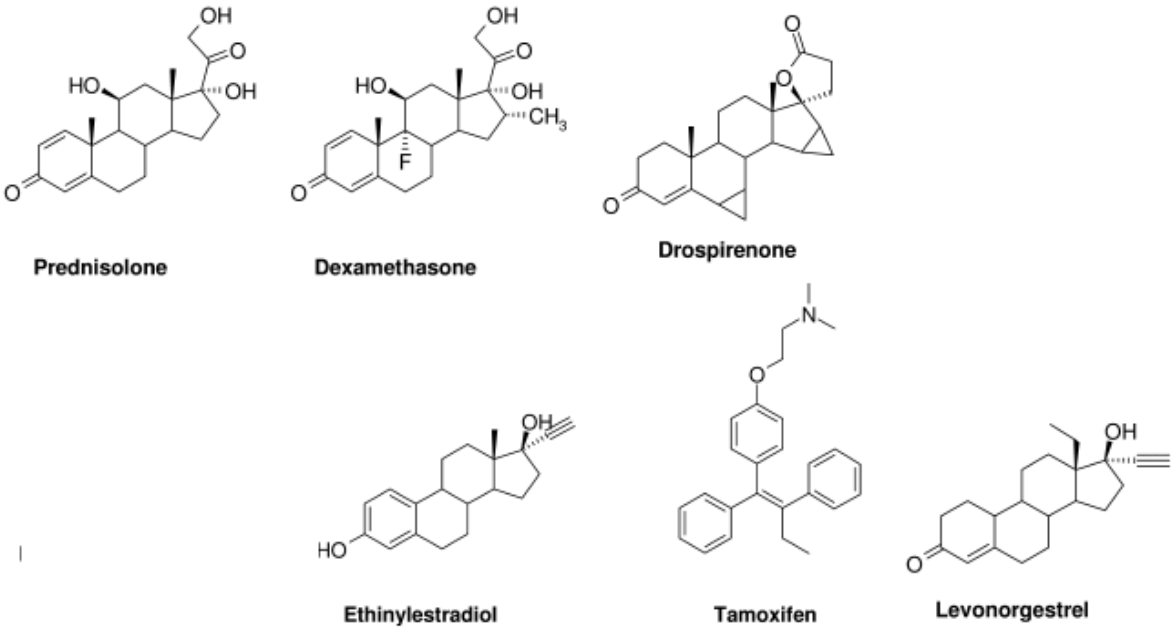


Fig. 2. Examples of nuclear receptors ligands. Glucocorticoid receptors agonists, prednisolone and dexamethasone. Progesterone receptor agonists, drospirenone and levonogestrel. Estrogen receptor agonist ethinylestradiol and antagonist by selective modulative inhibition tamoxifen. (Adapted from Soares *et al*., Quantitative approach to glucocorticosteroids analysis in human urine using LC-MS/MS. *Journal of the Brazilian Chemical Society*. **2012,** *23***,** 21949-900)[20]

2.2 -Liver X receptor

2.2.1 -Liver X receptor Structure

Liver X receptors (LXR) belong to the NR superfamily and act as oxysterol (OHC) sensors playing a crucial role in the regulation of genes involved in cholesterol and lipid metabolism. There are two know isoforms of LXR, the alpha (LXRα) and the beta (LXRβ). LXRα and LXβ are formed by 447[21] and 461[22] amino acids, respectively. LXR's have a number of common domains with other NRs, namely: an N-terminal ligand-dependent activation domain, a zinc finger DNA binding domain (DBD), a hinge region, a C-terminal domain and a ligand binding domain (LBD). The DBD and LDB regions of LXRα and LXRβ share 75, 6% and 74% identity respectively.[23] The LBD's structure is an α–helical sandwich which is well conserved across the NR superfamily. LXR's LDB contains 10 α-helices where helix 2 is missing and helices 10-11 are merged as seen in Fig. 3.[24] In vivo, LXR form heterodimers with retinoid X receptors (RXR) (Fig. 4). This heterodimer is present in the nucleus and bound to co-repressor proteins whilst not bound to any ligands.[25] Upon binding of an agonist to the LXR, the co-repressors are released and helix 12 changes its conformation, closing the ligand binding pocket and forming a groove where co-activator proteins can bind, this way allowing gene transcription.[24]

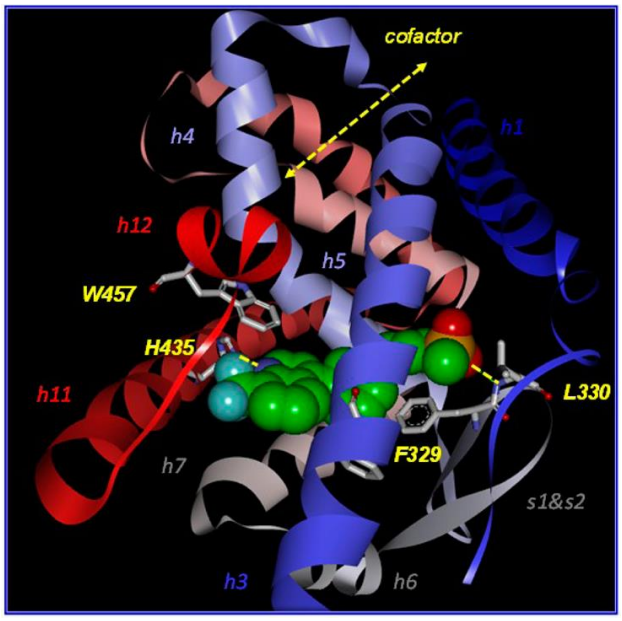


Fig. 3. Schematic diagram of LXR-β LBD based on PDB ID 3KFC. The LXRβ protein is depicted in ribbon format, and the agonist ligand is shown in CPK representation.[24]

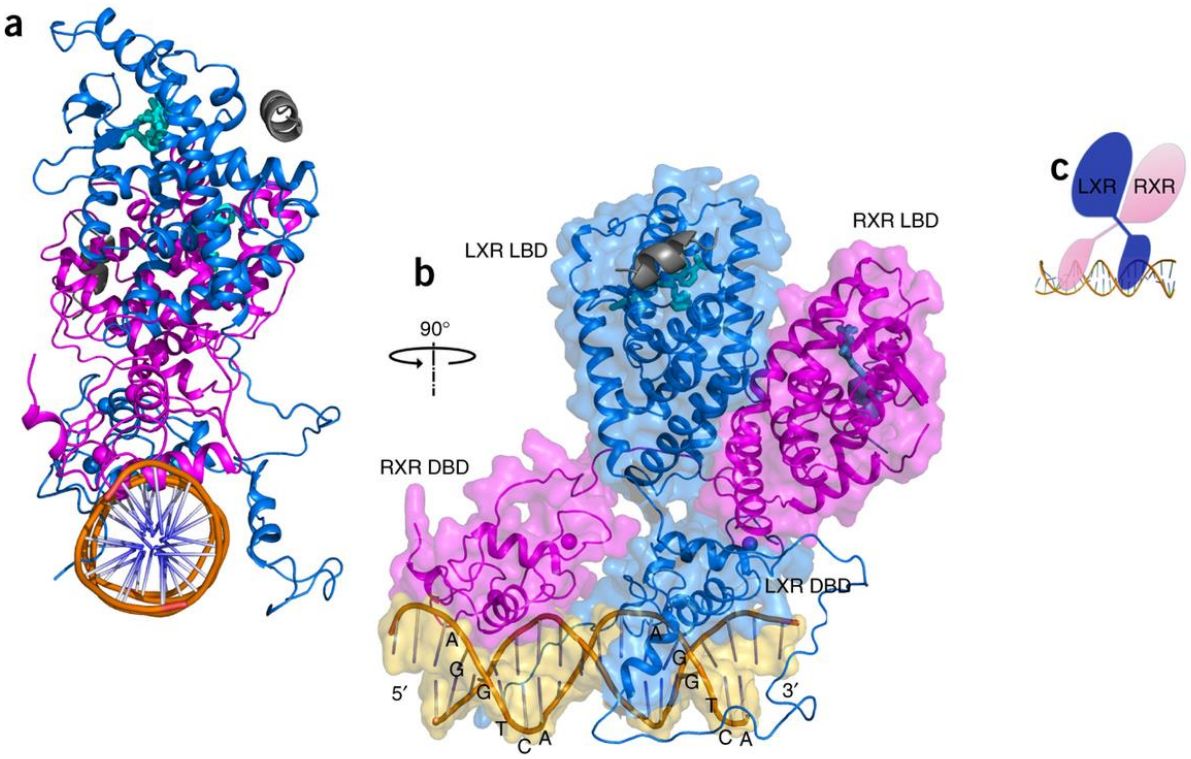


Fig. 4. (a,b) Ribbon-diagram overviews of the complex with LXRβ(blue)-RXR-α (magenta), ligands (cyan) and cofactor peptide (gray) on the DR-4 element. (a) View along the direction of DNA (5' to 3'). (b) Side view of the heterodimer with surfaces represented. (c) Schematic representation of subdomain positions within the complex. (Image adapted from Lou *et al*., Structure of the retinoid X receptor alpha-liver X receptor beta (RXRalpha-LXRbeta) heterodimer on DNA. *Nature Structural & Molecular Biology*. **2014**, *21*, 277-281).[25]

2.2.2 -Liver X receptor's Physiology and Pathological processes

LXR general acting mechanism:

LXR's activation is the consequence of high intracellular cholesterol and/or oxysterols (OHCs) levels. The LXR/RXR heterodimer then bind to LXR response elements (LXREs) present within the promoters of several genes, via transactivation. In the absence of LXR ligands, the heterodimer is associated with co-repressors and constitutively bound to the promoter of the LXR's target genes[26,27], in what is called a basal gene repression state. In the presence of agonist ligands, nuclear co-repressors dissociate from the LXR's target genes promoters, inducing recruitment of co-activators by the LXRs and ultimately leading to the

initiation of gene transcription[28,29] (Fig. 5). LXR also contributes to the gene expression regulation via transrepression, a mechanism by which LXR inhibits inflammatory pathways switching off the expression of pro-inflammatory genes.[30]

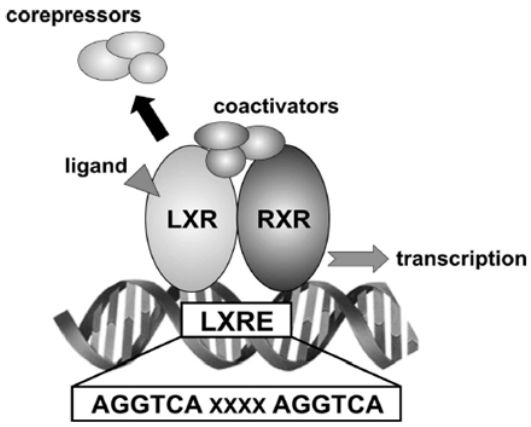


Fig. 5. Initiation of Gene transcription after co-activation.[31]

2.2.3 -LXR's involvement in pathological processes

LXR's isoforms are not evenly distributed across the organism, LXRα is mainly expressed in liver, intestine, adipose tissue and macrophages while LXRβ is present in all tissues and organs.

2.2.3.1 -Antibacterial response

LXR's are thought to initially prepare macrophages for antibacterial response and in a later phase, exert anti-inflammatory actions to restore normal cell conditions.[23]

2.2.3.2 -Neurodegenerative processes. (Alzheimer and Parkinson's disease)

LXR's modulation has been proposed as a strategy to treat Alzheimer's Disease (AD).[32,33] Its anti-inflammatory properties, the recently suggested influence in increase of Apo-lipoprotein E (ApoE) levels[34,35] (mostly by LXR-β) and modulation of microganglia-mediated neuronal toxicity[36] set LXRs as potential targets for both Alzheimer and Parkinson's Disease treatment. The development of central nervous system-penetrant LXR

modulators might prove beneficial in the setting of neurological disorders, either alone or associated with other agents.[36]

2.2.3.3 -Cancer

The LXR-β's upregulation of ApoE have been show to effectively suppress metastic invasion and angiogenesis. In addition to this, LXR-β leads to the suppression of gene networks involved in tumor progression such as those regulated by E2F genes.[37] Therefore associating LXR-β activity with the reduction of melanoma growth.[38]

Moreover, anti-proliferative and pro-apoptotic effects were also reported in hormone-dependent cancers like breast and prostate cancers.[37,39] LXR knockout studies have shown the protective role of LXRs in prostate cancer development.[40] Activation of LXR via treatment of human prostate cancer cells leads to induced apoptosis and blockage of prostate tumor progression.[40, 41]

2.2.3.4 -Atopic dermatitis

LXRs activation by natural or synthetically ligands resulted in reduced expression of pro-inflammatory cytokines, prevented keratinocyte differentiation and increased lipid production with a barrier function improvement which, in turn, stimulated epidermal development.[42, 43]

2.2.3.5 -Asthma and chronic obstructive pulmonary Disease (COPD)

The modulation of cytokine and pro-inflammatory factors by LXR suggest that LXR targeting could have a therapeutic application in respiratory inflammatory diseases.[44]

2.2.3.6 -Stroke

Ischemia-related inflammatory markers such as INOS, MMP-9, COX-2 and TNF-α have been shown to decrease upon treatment with LXRs agonists.[45] This means that, in

cases of cerebral ischemia, LXRs modulation may be an important neuroprotective mechanism reducing neurological deficits and cerebral inflammation.[46]

2.2.4 -Overview of LXR in lipid homeostasis and NAFDL

The LXR's ability to sense oxysterols derivatives of cholesterol make LXR an essential transcription factor for cholesterol clearance. Most of the existing evidence for LXR's role was obtained in transgenic mice, allowing the study of the roles of both LXR isoforms together and individually. The lack of LXR-α in transgenic mice led to an augmented accumulation of cholesterol esters in the liver, which was even more marked under a high cholesterol diet.[47,48] These data emphasize the importance of LXR-α on the clearance of cholesterol either from dietary sources or the *de novo* synthesis (controlled by SREBP-2 and SREBP-1c). In mice the deficient cholesterol clearance has been shown to correlate to reduced expression of cholesterol 7 alpha-hydroxylase (Cyp7a1), caused by LXRs absence. Cyp7a1 encodes a rate-limiting enzyme for cholesterol clearance, a key enzyme in bile acid synthesis. LXR also plays an important role in cholesterol reverse transport, since LXRs arbitrate the regulation of the genes involved. LXR regulates ABC transporters *Abca1, Abcg1, Abcg5 and Abcg8.* The regulation of ABC transporters allows the modulation of cholesterol efflux since these transporters are responsible for the intracellular cholesterol's transport to high density lipoproteins (HDL) which are then transported to the liver, and will be eliminated. *Abcg5 and Abcg8* act as dimers in cholesterol transport and are mainly present in liver and bowel cells where they limit the absorption rate of cholesterol. LXR-α's extra-hepatic activity in particular its activity in the bowel, is thought to be crucial. Moreover, LXR-α's activity in the bowel has been shown to decrease cholesterol absorption and induce reverse cholesterol transport (RCT), while hepatic LXR-α's activity does not seem to contribute to increase RCT as much. The overexpression of LXR-α in the intestinal epithelium contributes to a clear improvement in atherogenic markers and hepatic lipid content. The overexpression of LXR-α in the bowel induced a reduced level of hepatic triglyceride (TG) and cholesterol accumulation on a high fat disease.[49] Therefore the LXR-α's roles above mentioned suggest that its specific activity in the bowel could positively influence NAFLD by modulation of the lipid profile in liver. LXR-α activation improves cholesterol clearance and has a clear anti-inflammatory action which are key factors in the progression of NAFLD and its subsequent complications.[47] On the other hand, LXR is a direct regulator (by activation) of the expression of critical genes for lipogenesis, however the effect on this

regulation is thought to be tissue-specific in the adipose[50] and bowel tissues[51] and especially important in the hepatic tissue.[49]

A study with transgenic mice lacking LXR-α but not LXR-β showed reduced lipogenic gene expression when compared to wild-type mice on a high cholesterol diet[47], this justifies why LXR-α is considered to have a more significant impact in hepatic lipogenesis than LXR-β. However, the latter one is still important since findings with mice lacking the β isoform show a reduced effect of LXR-α's agonists such as T0901317 in lipogenic gene expression.[51]

In the liver, lipid accumulation, inflammation and fibrosis are key for NAFLD's development and progression. In this tissue, LXR is strongly involved in steatosis development. LXR controls the expression of *Abcg5* and *Abcg8* genes as well as Cyp7a1 gene involved in cholesterol excretion and degradation into bile acids[49], LXR also controls Abca1 expression and consequently excretion of cholesterol into HDL particles. This contributes to reducing and eliminating lipid content in the liver.

LXR, particularly LXR-α as shown by a recent study[47], controls lipogenesis by modulating the expression of lipogenic genes such as Scd1, Acetyl-CoA carboxylase (Acc), Fas as well as transcription factors Srebp-1c and Carbohydrate-responsive element-binding protein (Chrebp).[49] Free cholesterol in liver is known to trigger inflammation and inverse agonists of LXR-α such as SR9238 have been shown to decrease the number of kupffer cells[52] which contributes to the pathogenesis by secreting proinflammatory cytokines.[53] This suggests a strong involvement of LXR-α in kupffer cells activation. Additionally the same inverse agonist SR9238 led to the reduced expression of inflammatory markers Cd68, Tnfa, IL6, IL1b and IL12.[52] Finally, collagen deposition which is crucial in the development of fibrosis was also reduced with the LXR-α agonist above.[52]

Considering the information gathered, it is clear that LXR can represent an important therapeutic target in NAFLD. This is due to its potential on preventing the "first-hit" as well as in attenuating or relieving of the "second-hit" phase progression. This happens by altering the organ's lipid profile (either by promoting RCT or lipogenesis modulation) and later with its anti-inflammatory and anti-fibrotic actions. However, the wide array of functions that LXRs displays, turn its targeting without causing deleterious effects a difficult task to achieve.

3 –LXR as a therapeutic target for NAFLD

LXR presents a set of physiological functions that make it a very attractive and interesting therapeutic target with potential benefits for NAFLD. To this date there are no marketed drugs targeting it, as far as NAFLD is concerned. Therefore, in this section, compounds whose activity towards LXRs (in particular LXR-α) was recently found and deemed relevant for NAFLD's progression will be presented and discussed. Few compounds with an interesting profile for NAFLD treatment through LXR modulation have been discovered and developed.

3.1 -SR9238

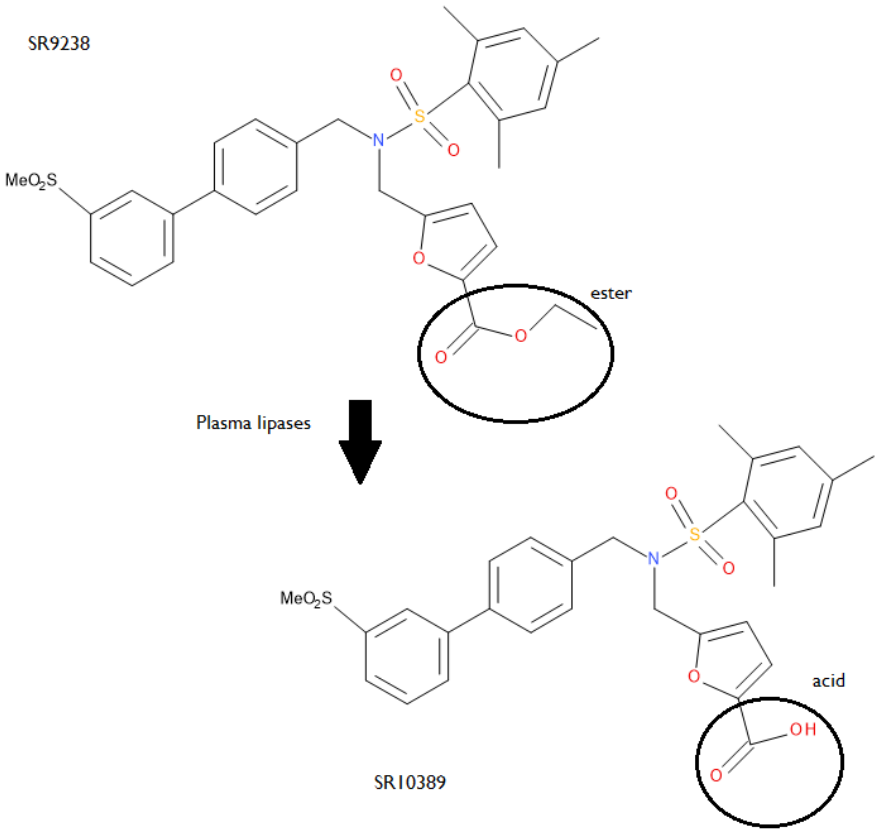


Fig. 6. LXR inverse agonist SR9238 suffers cleavage of the ester group by plasma lipases resulting in its acid analogue SR10389 which has no LXR-α or β activity.

SR9238 (Fig. 6) is an LXR inverse agonist. Using a tertiary sulfonamide, LXR antagonist shown in chemical scaffold as a point of initiation, compounds that would effectively recruit co-repressor proteins in biochemical assays were screened. Among the compounds identified, SR9238 displayed a potent inverse agonist activity with high potency both for LXR-α and LXR-β and liver specificity.

Two studies by Griffett et al.[52,54], were made to better understand and provide evidence of how SR9238 as an LXR agonist could positively influence NAFLD progression.

The first study[54] focused on describing and demonstrating its hepatic selectivity and effective suppression of hepatic lipogenesis, inflammation and lipid accumulation in a model of NASH mice.

In this study[54] evidence of hepatic selectivity of SR9238 is provided, and it was concluded that SR9328 is not detectable on brain, skeletal muscle or plasma after administration, being entirely confined to the liver and intestine in oral and IP administration. SR9238 contains an ester group that is metabolized by plasma lipases into an acid analogue SR10389 that shows no LXR-α or β activity, this reaction is illustrated in Fig. 6.

The treatment of mice suffering induced hepatic steatosis with SR9238 resulted in a substantial repression of lipogenic gene expression. Hepatic fatty acid synthase gene (FASN/FAS) and SREBP-1c expression was suppressed by 60 and 80% respectively, stearoyl-CoA desaturase-1 (SCD1) was reduced up to 90% following the treatment. Visualization of lipid in liver section stained by bodipy displayed remarkable decrease of lipid content in the liver.

SR9238 impacted inflammation in hepatocytes by reduction of TNFα and Interleukin-1 beta (Il1b) expression. Kupffer cells (the first cells that respond to hepatocyte injury increasing TNFα secretion and playing a critical role in inflammation) were also influenced, and markedly reduced in the immunohistochemical studies. Alkaline phosphatase (ALP), alanine transaminase (AST) and aspartate aminotransferase (AST) levels were also assessed given their importance as liver injury markers. These markers were all substantially reduced.

The second study[52] sought to provide more evidence that a therapy with SR9238 would prove valuable in reducing inflammation and fibrosis in a mouse model of NASH.

For the second study six-week-old B6 V- Lep^{ob} /J (ob/ob) mice (Annex1) were used and kept on D09100301 diet (Annex1) for six weeks prior to treatment, inducing NASH. In week 6, treatment with 30mg/kg of SR9238 started and continued for 30 days (D09100301 diet continued until the end of the study). During this time bodyweight and food intake were monitored daily and blood glucose weekly. The study analyzed gene and protein expression as well as liver weight.

After the treatment with SR9238 no changes in the eating habits were noticed. In the beginning of second week of treatment significant weight loss was observed and was

consistent until the end. SR9238 was shown to cause a significant reduction in hepatic lipids and lipid droplet size (determination by bodipy 493/503 lipid staining (Annex1)) which in turn translates as a reduction on hepatic steatosis. A reduction in liver weight was observed. As observed previously by Griffett et al., SR9238 decreased expression of genes regulating lipogenesis and steatosis (SREBF1, SCD1, CD36), and the hepatic injury markers, Kupffer cells were reduced as well. Furthermore, the hepatic gene expression of inflammatory markers (Cd68, Tnfα, Il6, Il1b and Il12) was reduced in SR9238 treated mice. To evaluate the impact on fibrosis, the level of collagen deposition was analyzed and there was 90% less deposition on mice treated with SR9238 then vehicle treated mice. The levels of LDL cholesterol were reduced but there was no significant reduction on plasma triglyceride levels.

In conclusion, SR9238 shows high liver selectivity, modulating LXR and effectively leading to the reduction of hepatic steatosis (by lipogenesis modulation), inflammation and fibrosis. This represents an attractive set of properties towards limiting the progression of NAFLD, especially in the ''second-hit'' phase of the disease since it focus mainly on inflammation, fibrosis and steatosis regression, which are more prominent in a later stage of the disease.

3.2 -*Meso*-dihydroguaiaretic acid (MDGA)

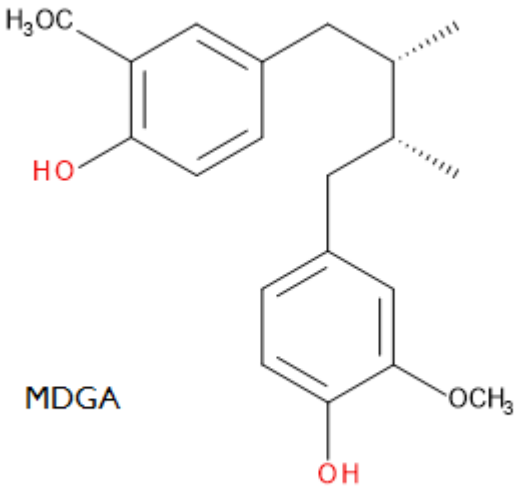


Fig.7. Structure of *meso*-dihydroguaiaretic acid.

Meso-dihydroguaiaretic acid (MDGA) (Fig. 7) is a dibenzylbutane lignan isolated from *Machilus Thunbergii* and possesses anti-oxidative, anti-inflammatory and anti-neurotoxic properties[55-57] without significant toxicity. Its use in traditional medicine is well known in a variety of pathologies. A study[58] was able to show how MDGA is an LXR-α antagonist, inhibiting ligand activated LXR-α co-activation and transcriptional expression of its downstream genes involved in fatty acid synthesis and ultimately reducing lipid accumulation in the liver. The same study provides evidence of MDGA's selectivity towards LXR-α and of no interference with other nuclear receptors involved in steatosis such as pregnane X receptor (PXR) and farnesoid X receptor (FXR). In this study[58] the mechanism by which MDGA antagonizes LXR-α was proposed. MDGA did not affect the co-activator binding by itself but, instead, it inhibits LBD (ligand binding domain) activation therefore inhibiting ligand binding to the LXR-α. This inhibition is achieved by either specific or non-specific binding to the LBD which will result in a conformational change. A potent selective agonist for LXR was used concomitantly, T0901317 and docking using the X-ray crystallographic structure of LXR-α of the complexed with T0901317 was performed. Using the Surflex-Dock (Annex1) program, MDGA was docked in the binding site for T0901317, showing that MDGA fits well in the T0901317's binding pocket. The results from the concomitant administration of

MDGA and T0901317 led to the conclusion that MDGA inhibits the LXR-α LBD by competitively binding to the pocket of T0901317.

An analysis of how MDGA affected a high fat diet (HFD) induced fatty liver model was made. Mice fed with HFD for 6 weeks had a 48,5% increase in body weight and when treated with MDGA from 4 weeks until sacrifice, the mice had a complete reverse in hepatic lipid accumulation, despite no significant changes in body weight was observed. Hepatic TG, total cholesterol, plasma TG and ALT levels were decreased in the mice treated with MDGA. In addition to this, western blot analysis and real-time PCR indicated a decrease in gene and protein expression of sterol regulatory element binding protein (SREBP), SCD-1 and FAS. The HFD-induced fatty liver was completely reversed even in low dosage.[58] The evidence provided suggests MDGA's potent anti-steatotic effect.

Reverse cholesterol transport and MDGA

LXR-α has key role regulating reverse cholesterol transport. Therefore antagonism of LXR-α's activation potentially causes defects in this processes. MDGA inhibition of transporters in various cell types and liver was studied.[58] The results indicated that MDGA does not significantly interfere with the expression of cholesterol transporters. This is thought to be due to selective action of MDGA, leading to results where serum total and HDL cholesterol levels are not affected by MDGA in HFD-fed mice.

MDGA's influence in receptor-interacting protein 1 (RIP140)

RIP140 is a nuclear co-regulator controlling lipid and glucose metabolism. Its specific function in hepatocytes is acting as a co-activator of LXR-α to induce SREBP-1c and FAS resulting in TG synthesis. The study by Sim *et al.*[58] on the interaction between MDGA and RIP140, showed that, although the mechanisms by which they interact were not clearly presented, MDGA markedly inhibited the expression of RIP140. This RIP140 down regulation by MDGA in hepatocytes emerges as an important aspect for the treatment of NAFLD and other metabolic syndromes with LXR-α antagonists.

In order to further prove the selectiveness of MDGA towards LXR-α and RIP140, the expression of peroxisome proliferator activated receptor (PPAR)-γ and α, which play important roles in hepatic lipid metabolism, was evaluated. No significant changes in the expression of either PPAR-γ, PPAR-α or their target genes were found.

In summary MDGA is a selective anti-steatogenic antagonist of LXR-α, inhibiting its activation and gene expression associated with fatty acid synthesis. MDGA seems to have little or no impact in the expression of genes associated with peripheral cholesterol clearance. MDGA binds directly to the LBD inhibiting native ligand binding and receptor activation. Given the wide variety of molecular targets associated with LXR, specific inhibition is crucial to avoid side effects and MDGA proves to be valuable in this perspective. However the effect of MDGA in other tissues was not studied and given the tissue specificity of LXR's function MDGA cannot be considered yet for direct therapeutic use. MDGA, by its selectiveness and high efficacy reversing liver lipid accumulation, may represent an important and attractive model to the development of new antagonists.

3.3 -*20(S)*-Protopanaxatriol (PPT)

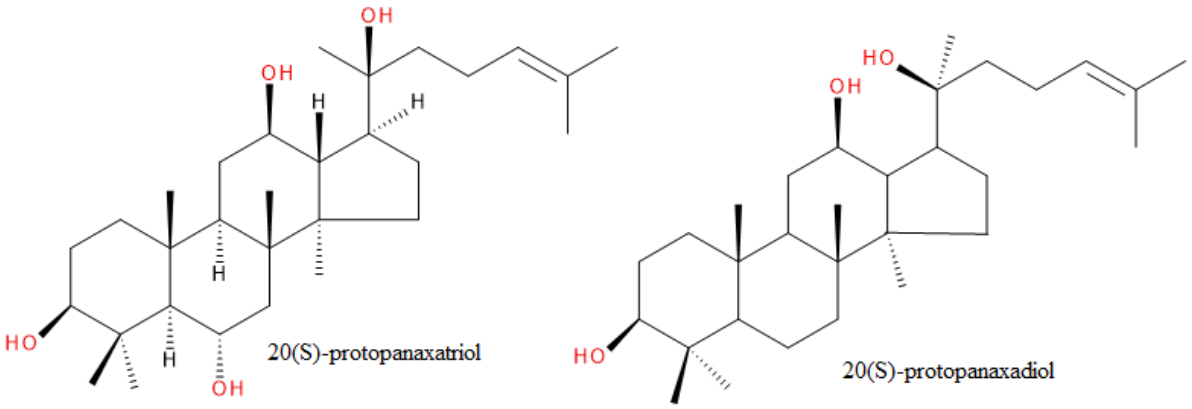


Fig. 8. Two types of ginsenosides, *20(S)*-protopanaxatriol (PPT-type) and *20(S)*-protopanaxadiol (PPD-type).

20(S)-Protopanaxatriol (PPT) (Fig. 8.) is part of a special group of triterpenoid saponins found exclusively in ginseng. These can be classified based on their aglycones into two groups: *20(S)*-protopanaxatriol (PPT-type) and *20(S)*-protopanaxadiol (PPD-type). Ginsenosides have been shown to have beneficial therapeutic effects in diabetes mellitus and obesity[59-66] as well as in diabetic patients.[67] However the mechanisms by which many of their specific constituents exert their action remains unclear.

In a study by Oh *et al.*[68] the aglycone PPT was identified as an inhibitor of autonomous LXR-α transactivation. For this purpose, a Gal4-UAS screening system (Annex1) was used. The data obtained in this study shows that PPT is a novel LXR-α inhibitor with selective effects on the lipogenic process while not inhibiting the transcription of LXR-α target genes related to RCT.

Reverse transcription polymerization chain reaction (RT-PCR) analysis showed that PPT markedly suppresses the LXR-α-dependent transcription of SREBP-1c. The study elucidates in detail the transcription inhibition mechanism by which PPT inhibits SREBP-1c.

PPT inhibition of T0901317-dependent recruitment of RNA polymerase II

An assessment of the T0901317-dependent recruitment of RNA polymerase II to the endogenous LXRE of the SREBP-1c gene was made recurring to a ChIP assay (Annex1) and the results pointed to an annulment of this recruitment by PPT. These results suggest that PPT directly regulates the transcription of SREBP-1c by interfering in the recruitment of RNA polymerase II.

Assessment of T0901317-dependent transcription of LXR-α target genes related to lipogenesis by PPT

The study tested whether PPT influenced the expression of other lipogenic genes (other than SREBP-1c) such as SCD1, FAS and glycerol-3-phosphate acyltransferase (GPAT). It was previously known that SCD1, FAS and SREBP-1c are LXR-α's target genes but, FAS, SCD1 and GPAT are also SREBP-1c target genes. RT-PCR analysis showed that all of these protein's expression was decreased by PPT in mice hepatocytes. TG accumulation induced by T0901317 in mice hepatocytes was also reduced by PPT. Both of these observations allow the conclusion that PPT effectively reduces lipogenic genes expression and suppresses TG accumulation in hepatocytes.

PPT in reverse cholesterol transport

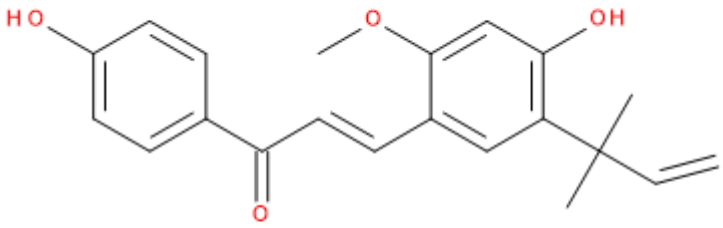
Because of the implications of LXR-α in RCT, it was important to show whether PPT reduced RCT or not. Given this importance, the effect of PPT in ABCA1 transcription was studied. The results point out that PPT does not inhibit the promoter activity of ABCA1, in addition to this, PPT does not inhibit the recruitment of RNA polymerase II to the ABCA1 LXRE region.

PPT interaction with lipogenic co-activator TRAP80

TRAP80 is a lipogenic co-activator in hepatocytes[69] which can stimulate transcription of lipogenic genes such as SREBP-1c but not affecting RCT-related genes. TRAP80 is selectively recruited to the SREBP-1c promoter but not to the ABCA1 promoter. PPT was shown[68] to inhibit the TRAP80 T0901317-dependent recruitment to the LXRE region of the endogenous SREBP-1c promoter.

In summary, PPT is an inhibitor of the ligand-dependent transactivation of LXR-α, effectively inhibiting the expression of LXR-α lipogenic regulated genes, lowering TG accumulation in hepatocytes and with no effect on RCT-related genes expression. The capacity of differentially inhibiting lipogenesis and not RCT pathways makes PPT an exciting model for development of new drugs targeting LXR-α in NAFLD treatment.

3.4 -Licocalcone A



Licochalcone A

Fig. 9. Structure of licochalcone.

Licochalcone A (LicA) is a natural phenol, more specifically a calchonoid related to chalcone. Chalconnes are aromatic ketones with two phenyl rings that are also intermediates in the synthesis of many biological compounds. LicA can be isolated from *Glycyrrhiza inflate* or *Glycyrrhiza glabra.* Licochalcone A shows antimalarial, anticancer, antibacterial and antiviral properties.[70-73]

In a study[74], where the impact of 238 natural chemicals on autonomous transactivity of LXR-α was evaluated by the GAL4-TK-Luciferase reporter system(Annex1), LicA stood out by considerably inhibiting autonomous transactivity of LXR-α and decreasing LXR-α-dependent expression of SREBP-1c. LicA also repressed LXR-α agonist T0901317-stimulated transcription of SREBP-1c.[74]

Impact on lipogenic genes transcription

The transcription level of various lipogenic genes was evaluated in mice primary hepatocytes treated with LicA. LicA substantially repressed the expression of SREBP-1c, FAS SCD1 and GPAT.[74]

Influence in hepatocyte TG accumulation

Cellular lipids were extracted and analyzed determining a significant reduction of the T0901317-stimulated TG accumulation in primary hepatocytes.[74]

LicA and its consequences in the promoter activity and transcription of RCT-related LXR-α target genes

The activity of LicA on LXR-α-dependent activation of ABCA1 and ABCG1 promoters was examined by analyzing the effects of LicA on the transcript levels of ABCA1 and ABCG1 in the presence of the agonist T0901317 by quantitative RT-PCR. The results showed that LicA has no impact on the T0901317-dependent increase of ABCA1 and ABCG1.[74] Therefore, LicA does not impact RCT, which is a major factor for lipid content clearance.

LicA selectivity for reducing recruitment of RNA polymerase II to SREBP-1c LXRE but not ABCA1 LXRE

Using a ChIP assay (Annex1), the recruitment of RNA polymerase II to the LXRE region of the SREBP-1c was compared with that of ABCA1. The recruitment of RNA polymerase II to the LXRE region was inhibited on SREBP-1c gene but not on the ABCA1 gene.[74]

In conclusion, LicA proves to selectively inhibit LXR-α-mediated transcription of lipogenic genes with no impact on the RCT-related genes. The recruitment RNA polymerase II to the LXRE was also differentially regulated by LicA between SREBP-1c and ABCA1 promoters. These findings attest for the selectiveness and differentiated inhibition of ABCA1, much like PPT with no negative effects on RCT but beneficial effects on hepatocytes lipogenesis. Therefore LicA presents enthusiastic features for the treatment of NAFLD.

4 –Conclusion

The Liver X Receptor presents a set of physiological functions that make it a very attractive and interesting therapeutic target with benefits for many pathologies. Its functions and influence in a wide variety of physiological processes and pathologies has been shown and attested by numerous scientific reports, and the mechanisms by which it acts will continue to be further explored and clarified.

The importance of Liver X Receptor alpha in the development of metabolic syndromes and NAFLD in particular is clear. In the future we can expect that with the continuous development and research of novel and selective antagonists, tissue and function (LXR-α's function) specific drugs will emerge targeting this receptor's alpha isoform.

Given the complexity of Liver X Receptor molecular activity and its tissue-dependent expression and functions there is an undeniable potential for serious side effects when modulating its expression. In addition to this not all its interferences at a molecular level are fully understood. Therefore caution is needed in the development of new drugs and probably the main reason why so little has been done towards effective drug development targeting LXR-α for NAFLD treatment.

An ideal drug targeting Liver X Receptor alpha for NAFLD would have to show high liver tissue specificity, reverse cholesterol transport up regulation, decrease in liver lipid accumulation, lipogenic suppressing and anti-inflammatory activity. Using these ideal standards, SR9238 looks likes the most promising of the compounds earlier discussed.

Taking into consideration how NAFLD progresses, a therapeutic strategy that approaches the disease accordingly to its stage could prove valuable in overcoming the duality beneficial/negative effects that Liver X Receptor alpha has in the disease. Drugs that would promote RCT without significantly potentiate lipogenesis could prove useful in an early stage of the disease to limit its progression and reverse lipid accumulation, while drugs like SR9238 could prove valuable limiting the disease in a later stage by its anti-inflammatory and lipogenic suppression actions.

Although there are serious side effects to be considered and these may not be easy to overcome, the potential that Liver X Receptor represents for NAFLD and the recent findings of promising antagonist models will encourage the development of new drug candidates which will effectively and safely prove to be a viable option in NAFLD treatment and progression limiting.

5 –Bibliography

1. Vernon, G.; Baranova, A.; Younossi, Z. M. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Alimentary Pharmacology & Therapeutics.* **2011**, *34*, 274-285.
2. Chalasani, N.; Younossi, Z.; Lavine, J. E.; Diehl, A. M.; Brunt, E. M.; Cusi, K.; Charlton, M.; Sanyal, A. J.; American Association for the Study of Liver, D.; American College of, G.; American Gastroenterological, A. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *American Journal of Gastroenterology.* **2012**, *107*, 811-826.
3. Day, C. P.; James, O. F. Steatohepatitis: a tale of two "hits"? *Gastroenterology.* **1998**, *114*, 842-845.
4. Vuppalanchi, R.; Chalasani, N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology.* **2009**, *49*, 306-317.
5. Armstrong, M. J.; Adams, L. A.; Canbay, A.; Syn, W. K. Extrahepatic complications of nonalcoholic fatty liver disease. *Hepatology.* **2014**, *59*, 1174-1197.
6. Angulo, P. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? *Hepatology.* **2010**, *51*, 373-375.
7. Suzuki, A.; Angulo, P.; Lymp, J.; St Sauver, J.; Muto, A.; Okada, T.; Lindor, K. Chronological development of elevated aminotransferases in a nonalcoholic population. *Hepatology.* **2005**, *41*, 64-71.
8. Hamaguchi, M.; Kojima, T.; Takeda, N.; Nakagawa, T.; Taniguchi, H.; Fujii, K.; Omatsu, T.; Nakajima, T.; Sarui, H.; Shimazaki, M.; Kato, T.; Okuda, J.; Ida, K. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Annals of Internal Medicine.* **2005**, *143*, 722-728.
9. Whalley, S.; Puvanachandra, P.; Desai, A.; Kennedy, H. Hepatology outpatient service provision in secondary care: a study of liver disease incidence and resource costs. *Clinical Medicine (Lond).* **2007**, *7*, 119-124.
10. Williamson, R. M.; Price, J. F.; Glancy, S.; Perry, E.; Nee, L. D.; Hayes, P. C.; Frier, B. M.; Van Look, L. A.; Johnston, G. I.; Reynolds, R. M.; Strachan, M. W.; Edinburgh Type 2 Diabetes Study, I. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care.* **2011**, *34*, 1139-1144.

11. Machado, M.; Marques-Vidal, P.; Cortez-Pinto, H. Hepatic histology in obese patients undergoing bariatric surgery. *Journal of Hepatology.* **2006**, *45*, 600-606.

12. Chang, E.; Park, C. Y.; Park, S. W. Role of thiazolidinediones, insulin sensitizers, in non-alcoholic fatty liver disease. *Journal of Diabetes Investigation* **2013**, *4*, 517-524.

13. Chen, J. D.; Evans, R. M. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature.* **1995**, *377*, 454-457.

14. Gaggini, M.; Morelli, M.; Buzzigoli, E.; DeFronzo, R. A.; Bugianesi, E.; Gastaldelli, A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients.* **2013**, *5*, 1544-1560.

15. Brea, A.; Puzo, J. Non-alcoholic fatty liver disease and cardiovascular risk. *International Journal of Cardiology.* **2013**, *167*, 1109-1117.

16. Attar, B. M.; Van Thiel, D. H. Current concepts and management approaches in nonalcoholic fatty liver disease. *Scientific World Journal.* **2013**, *2013*, 481893.

17. Phenex Pharmaceuticals http://www.phenex-pharma.com/en/r-and-d-programs/fxr/ accessed 10/01/2016

18. Newsome, P. N.; Allison, M. E.; Andrews, P. A.; Auzinger, G.; Day, C. P.; Ferguson, J. W.; Henriksen, P. A.; Hubscher, S. G.; Manley, H.; McKiernan, P. J.; Millson, C.; Mirza, D.; Neuberger, J. M.; Oben, J.; Pollard, S.; Simpson, K. J.; Thorburn, D.; Tomlinson, J. W.; Wyatt, J. S.; British Transplant, S. Guidelines for liver transplantation for patients with non-alcoholic steatohepatitis. *Gut.* **2012**, *61*, 484-500.

19. Novac, N.; Heinzel, T. Nuclear receptors: overview and classification. *Current Drug Targets Inflammatory Allergy.* **2004**, *3*, 335-346.

20. Soares, R.; Araújo, A.; Castro, J.; Gomes, L.; Pereira, H.; Neto, F.; Quantitative approach to glucocorticosteroids analysis in human urine using LC-MS/MS. *Journal of the Brazilian Chemical Society*. **2012,** *23***,** 21949-900.

21. Griffett, K.; Solt, L. A.; El-Gendy Bel, D.; Kamenecka, T. M.; Burris, T. P. A liver-selective LXR inverse agonist that suppresses hepatic steatosis. *ACS Chemestry & Biology.* **2013**, *8*, 559-567.

22. Shinar, D. M.; Endo, N.; Rutledge, S. J.; Vogel, R.; Rodan, G. A.; Schmidt, A. NER, a new member of the gene family encoding the human steroid hormone nuclear receptor. *Gene.* **1994**, *147*, 273-276.

23. Tice, C. M.; Noto, P. B.; Fan, K. Y.; Zhuang, L.; Lala, D. S.; Singh, S. B. The medicinal chemistry of liver X receptor (LXR) modulators. *Journal of Medicinal Chemistry.* **2014**, *57*, 7182-7205.

24. Svensson, S.; Ostberg, T.; Jacobsson, M.; Norstrom, C.; Stefansson, K.; Hallen, D.; Johansson, I. C.; Zachrisson, K.; Ogg, D.; Jendeberg, L. Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. *EMBO Journal.* **2003**, *22*, 4625-4633.
25. Lou, X.; Toresson, G.; Benod, C.; Suh, J. H.; Philips, K. J.; Webb, P.; Gustafsson, J. A. Structure of the retinoid X receptor alpha-liver X receptor beta (RXRalpha-LXRbeta) heterodimer on DNA. *Nature Structural & Molecular Biology.* **2014**, *21*, 277-281.
26. Chen, J. D.; Evans, R. M. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature.* **1995**, *377*, 454-457.
27. Horlein, A. J.; Naar, A. M.; Heinzel, T.; Torchia, J.; Gloss, B.; Kurokawa, R.; Ryan, A.; Kamei, Y.; Soderstrom, M.; Glass, C. K.; et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature.* **1995**, *377*, 397-404.
28. Hu, X.; Li, S.; Wu, J.; Xia, C.; Lala, D. S. Liver X receptors interact with corepressors to regulate gene expression. *Molecular Endocrinology.* **2003**, *17*, 1019-1026.
29. Wagner, B. L.; Valledor, A. F.; Shao, G.; Daige, C. L.; Bischoff, E. D.; Petrowski, M.; Jepsen, K.; Baek, S. H.; Heyman, R. A.; Rosenfeld, M. G.; Schulman, I. G.; Glass, C. K. Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. *Molecular Cell Biology.* **2003**, *23*, 5780-5789.
30. Ghisletti, S.; Huang, W.; Ogawa, S.; Pascual, G.; Lin, M. E.; Willson, T. M.; Rosenfeld, M. G.; Glass, C. K. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Molecular Cell.* **2007**, *25*, 57-70.
31. Hong, C.; Tontonoz, P. Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nature Reviews Drug Discovery.* **2014**, *13*, 433-444.
32. Whitney, K. D.; Watson, M. A.; Collins, J. L.; Benson, W. G.; Stone, T. M.; Numerick, M. J.; Tippin, T. K.; Wilson, J. G.; Winegar, D. A.; Kliewer, S. A. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. *Molecular Endocrinology.* **2002**, *16*, 1378-1385.
33. Zelcer, N.; Khanlou, N.; Clare, R.; Jiang, Q.; Reed-Geaghan, E. G.; Landreth, G. E.; Vinters, H. V.; Tontonoz, P. Attenuation of neuroinflammation and Alzheimer's disease pathology by liver x receptors. *Proceedings of the National Academy of Sciences. U S A* **2007**, *104*, 10601-10606.
34. Mak, P. A.; Laffitte, B. A.; Desrumaux, C.; Joseph, S. B.; Curtiss, L. K.; Mangelsdorf, D. J.; Tontonoz, P.; Edwards, P. A. Regulated expression of the apolipoprotein E/C-I/C-IV/C-II

gene cluster in murine and human macrophages. A critical role for nuclear liver X receptors alpha and beta. *Journal of Biolgy & Chemistry.* **2002**, *277*, 31900-31908.

35. Jiang, X. C.; Beyer, T. P.; Li, Z.; Liu, J.; Quan, W.; Schmidt, R. J.; Zhang, Y.; Bensch, W. R.; Eacho, P. I.; Cao, G. Enlargement of high density lipoprotein in mice via liver X receptor activation requires apolipoprotein E and is abolished by cholesteryl ester transfer protein expression. *Journal of Biolgy & Chemistry.* **2003**, *278*, 49072-49078.

36. Dai, Y. B.; Tan, X. J.; Wu, W. F.; Warner, M.; Gustafsson, J. A. Liver X receptor beta protects dopaminergic neurons in a mouse model of Parkinson disease. *Proceedings of Nationall Academy of Sciences. U S A* **2012**, *109*, 13112-13117.

37. Nguyen-Vu, T.; Vedin, L. L.; Liu, K.; Jonsson, P.; Lin, J. Z.; Candelaria, N. R.; Candelaria, L. P.; Addanki, S.; Williams, C.; Gustafsson, J. A.; Steffensen, K. R.; Lin, C. Y. Liver x receptor ligands disrupt breast cancer cell proliferation through an E2F-mediated mechanism. *Breast Cancer Research.* **2013**, *15*, R51.

38. Pencheva, N.; Buss, C. G.; Posada, J.; Merghoub, T.; Tavazoie, S. F. Broad-spectrum therapeutic suppression of metastatic melanoma through nuclear hormone receptor activation. *Cell.* **2014**, *156*, 986-1001.

39. El Roz, A.; Bard, J. M.; Valin, S.; Huvelin, J. M.; Nazih, H. Macrophage apolipoprotein E and proliferation of MCF-7 breast cancer cells: role of LXR. *Anticancer Research.* **2013**, *33*, 3783-3789.

40. Dufour, J.; Pommier, A.; Alves, G.; De Boussac, H.; Lours-Calet, C.; Volle, D. H.; Lobaccaro, J. M.; Baron, S. Lack of liver X receptors leads to cell proliferation in a model of mouse dorsal prostate epithelial cell. *PLoS One.* **2013**, *8*, e58876.

41. Pommier, A. J.; Alves, G.; Viennois, E.; Bernard, S.; Communal, Y.; Sion, B.; Marceau, G.; Damon, C.; Mouzat, K.; Caira, F.; Baron, S.; Lobaccaro, J. M. Liver X Receptor activation downregulates AKT survival signaling in lipid rafts and induces apoptosis of prostate cancer cells. *Oncogene.* **2010**, *29*, 2712-2723.

42. Fowler, A. J.; Sheu, M. Y.; Schmuth, M.; Kao, J.; Fluhr, J. W.; Rhein, L.; Collins, J. L.; Willson, T. M.; Mangelsdorf, D. J.; Elias, P. M.; Feingold, K. R. Liver X receptor activators display anti-inflammatory activity in irritant and allergic contact dermatitis models: liver-X-receptor-specific inhibition of inflammation and primary cytokine production. *Journal of Investigative Dermatolology.* **2003**, *120*, 246-255.

43. Hatano, Y.; Man, M. Q.; Uchida, Y.; Crumrine, D.; Mauro, T. M.; Feingold, K. R.; Elias, P. M.; Holleran, W. M. Murine atopic dermatitis responds to peroxisome proliferator-

activated receptors alpha and beta/delta (but not gamma) and liver X receptor activators. *Journal of Allergy and Clinical Immunology.* **2010**, *125*, 160-169 e161-165.

44. Delvecchio, C. J.; Bilan, P.; Radford, K.; Stephen, J.; Trigatti, B. L.; Cox, G.; Parameswaran, K.; Capone, J. P. Liver X receptor stimulates cholesterol efflux and inhibits expression of proinflammatory mediators in human airway smooth muscle cells. *Molecular Endocrinology.* **2007**, *21*, 1324-1334.
45. Morales, J. R.; Ballesteros, I.; Deniz, J. M.; Hurtado, O.; Vivancos, J.; Nombela, F.; Lizasoain, I.; Castrillo, A.; Moro, M. A. Activation of liver X receptors promotes neuroprotection and reduces brain inflammation in experimental stroke. *Circulation.* **2008**, *118*, 1450-1459.
46. Sironi, L.; Mitro, N.; Cimino, M.; Gelosa, P.; Guerrini, U.; Tremoli, E.; Saez, E. Treatment with LXR agonists after focal cerebral ischemia prevents brain damage. *FEBS Letter.* **2008**, *582*, 3396-3400.
47. Repa, J. J.; Liang, G.; Ou, J.; Bashmakov, Y.; Lobaccaro, J. M.; Shimomura, I.; Shan, B.; Brown, M. S.; Goldstein, J. L.; Mangelsdorf, D. J. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Developments.* **2000**, *14*, 2819-2830.
48. Ducheix, S.; Podechard, N.; Lasserre, F.; Polizzi, A.; Pommier, A.; Murzilli, S.; Di Lisio, C.; D'Amore, S.; Bertrand-Michel, J.; Montagner, A.; Pineau, T.; Loiseau, N.; Lobaccaro, J. M.; Martin, P. G.; Guillou, H. A systems biology approach to the hepatic role of the oxysterol receptor LXR in the regulation of lipogenesis highlights a cross-talk with PPARalpha. *Biochimie.* **2013**, *95*, 556-567.
49. Ducheix, S.; Montagner, A.; Theodorou, V.; Ferrier, L.; Guillou, H. The liver X receptor: a master regulator of the gut-liver axis and a target for non alcoholic fatty liver disease. *Biochemical Pharmacology.* **2013**, *86*, 96-105.
50. Korach-Andre, M.; Archer, A.; Gabbi, C.; Barros, R. P.; Pedrelli, M.; Steffensen, K. R.; Pettersson, A. T.; Laurencikiene, J.; Parini, P.; Gustafsson, J. A. Liver X receptors regulate de novo lipogenesis in a tissue-specific manner in C57BL/6 female mice. *American Journal of Physiology, Endocrinology and Metabolism.* **2011**, *301*, E210-222.
51. Ducheix, S.; Montagner, A.; Polizzi, A.; Lasserre, F.; Marmugi, A.; Bertrand-Michel, J.; Podechard, N.; Al Saati, T.; Chetiveaux, M.; Baron, S.; Boue, J.; Dietrich, G.; Mselli-Lakhal, L.; Costet, P.; Lobaccaro, J. M.; Pineau, T.; Theodorou, V.; Postic, C.; Martin, P. G.; Guillou, H. Essential fatty acids deficiency promotes lipogenic gene expression and hepatic steatosis through the liver X receptor. *Journal Hepatology.* **2013**, *58*, 984-992.

52. Griffett, K.; Welch, R. D.; Flaveny, C. A.; Kolar, G. R.; Neuschwander-Tetri, B. A.; Burris, T. P. The LXR inverse agonist SR9238 suppresses fibrosis in a model of non-alcoholic steatohepatitis. *Molecular Metabolism.* **2015**, *4*, 353-357.
53. Tosello-Trampont, A. C.; Landes, S. G.; Nguyen, V.; Novobrantseva, T. I.; Hahn, Y. S. Kuppfer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production. *Journal Biology Chemical.* **2012**, *287*, 40161-40172.
54. Griffett, K.; Solt, L. A.; El-Gendy Bel, D.; Kamenecka, T. M.; Burris, T. P. A liver-selective LXR inverse agonist that suppresses hepatic steatosis. *ACS Chemical Biology* **2013**, *8*, 559-567.
55. Lee, W. S.; Baek, Y. I.; Kim, J. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. Antioxidant activities of a new lignan and a neolignan from Saururus chinensis. *Bioorganic Medicinal Chemistry Letter.* **2004**, *14*, 5623-5628.
56. Moon, T. C.; Seo, C. S.; Haa, K.; Kim, J. C.; Hwang, N. K.; Hong, T. G.; Kim, J. H.; Kim do, H.; Son, J. K.; Chang, H. W. Meso-dihydroguaiaretic acid isolated from Saururus chinensis inhibits cyclooxygenase-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells. *Archieves Pharmaceutical Research.* **2008**, *31*, 606-610.
57. Ma, C. J.; Lee, M. K.; Kim, Y. C. meso-Dihydroguaiaretic acid attenuates the neurotoxic effect of staurosporine in primary rat cortical cultures. *Neuropharmacology.* **2006**, *50*, 733-740.
58. Sim, W. C.; Park, S.; Lee, K. Y.; Je, Y. T.; Yin, H. Q.; Choi, Y. J.; Sung, S. H.; Park, S. J.; Park, H. J.; Shin, K. J.; Lee, B. H. LXR-alpha antagonist meso-dihydroguaiaretic acid attenuates high-fat diet-induced nonalcoholic fatty liver. *Biochemical Pharmacology.* **2014**, *90*, 414-424.
59. Jeon, W. J.; Oh, J. S.; Park, M. S.; Ji, G. E. Anti-hyperglycemic effect of fermented ginseng in type 2 diabetes mellitus mouse model. *Phytotherapy Research.* **2013**, *27*, 166-172.
60. Attele, A. S.; Zhou, Y. P.; Xie, J. T.; Wu, J. A.; Zhang, L.; Dey, L.; Pugh, W.; Rue, P. A.; Polonsky, K. S.; Yuan, C. S. Antidiabetic effects of Panax ginseng berry extract and the identification of an effective component. *Diabetes.* **2002**, *51*, 1851-1858.
61. Liu, Z.; Li, W.; Li, X.; Zhang, M.; Chen, L.; Zheng, Y. N.; Sun, G. Z.; Ruan, C. C. Antidiabetic effects of malonyl ginsenosides from Panax ginseng on type 2 diabetic rats induced by high-fat diet and streptozotocin. *Journal of Ethnopharmacology.* **2013**, *145*, 233-240.

62. Cho, W. C.; Chung, W. S.; Lee, S. K.; Leung, A. W.; Cheng, C. H.; Yue, K. K. Ginsenoside Re of Panax ginseng possesses significant antioxidant and antihyperlipidemic efficacies in streptozotocin-induced diabetic rats. *European Journal of Pharmacology.* **2006**, *550*, 173-179.

63. Lee, M. R.; Kim, B. C.; Kim, R.; Oh, H. I.; Kim, H. K.; Choi, K. J.; Sung, C. K. Anti-obesity effects of black ginseng extract in high fat diet-fed mice. *Journal of Ginseng Research.* **2013**, *37*, 308-349.

64. Gu, W.; Kim, K. A.; Kim, D. H. Ginsenoside Rh1 ameliorates high fat diet-induced obesity in mice by inhibiting adipocyte differentiation. *Biological and Pharmaceutical Bulletin.* **2013**, *36*, 102-107.

65. Lee, H.; Park, D.; Yoon, M. Korean red ginseng (Panax ginseng) prevents obesity by inhibiting angiogenesis in high fat diet-induced obese C57BL/6J mice. *Food Chemical Toxicology.* **2013**, *53*, 402-408.

66. Hwang, J. T.; Lee, M. S.; Kim, H. J.; Sung, M. J.; Kim, H. Y.; Kim, M. S.; Kwon, D. Y. Antiobesity effect of ginsenoside Rg3 involves the AMPK and PPAR-gamma signal pathways. *Phytotherapy Research.* **2009**, *23*, 262-266.

67. Sotaniemi, E. A.; Haapakoski, E.; Rautio, A. Ginseng therapy in non-insulin-dependent diabetic patients. *Diabetes Care.* **1995**, *18*, 1373-1375.

68. Oh, G. S.; Yoon, J.; Lee, G. G.; Oh, W. K.; Kim, S. W. 20(S)-protopanaxatriol inhibits liver X receptor alpha-mediated expression of lipogenic genes in hepatocytes. *Journal of Pharmacological Sciences.* **2015**, *128*, 71-77.

69. Kim, G. H.; Oh, G. S.; Yoon, J.; Lee, G. G.; Lee, K. U.; Kim, S. W. Hepatic TRAP80 selectively regulates lipogenic activity of liver X receptor. *Journal of Clinical Investigation.* **2015**, *125*, 183-193.

70. Fu, Y.; Hsieh, T. C.; Guo, J.; Kunicki, J.; Lee, M. Y.; Darzynkiewicz, Z.; Wu, J. M. Licochalcone-A, a novel flavonoid isolated from licorice root (Glycyrrhiza glabra), causes G2 and late-G1 arrests in androgen-independent PC-3 prostate cancer cells. *Biochemical and Biophysical Research Communications.* **2004**, *322*, 263-270.

71. Friis-Moller, A.; Chen, M.; Fuursted, K.; Christensen, S. B.; Kharazmi, A. In vitro antimycobacterial and antilegionella activity of licochalcone A from Chinese licorice roots. *Planta Medica.* **2002**, *68*, 416-419.

72. Chen, M.; Theander, T. G.; Christensen, S. B.; Hviid, L.; Zhai, L.; Kharazmi, A. Licochalcone A, a new antimalarial agent, inhibits in vitro growth of the human malaria

parasite Plasmodium falciparum and protects mice from P. yoelii infection. *Antimicrobial Agents Chemotherapy.* **1994**, *38*, 1470-1475.

73. Dao, T. T.; Nguyen, P. H.; Lee, H. S.; Kim, E.; Park, J.; Lim, S. I.; Oh, W. K. Chalcones as novel influenza A (H1N1) neuraminidase inhibitors from Glycyrrhiza inflata. *Bioorganic & Medicinal Chemistry Letters.* **2011**, *21*, 294-298.
74. Oh, G. S.; Lee, G. G.; Yoon, J.; Oh, W. K.; Kim, S. W. Selective inhibition of liver X receptor alpha-mediated lipogenesis in primary hepatocytes by licochalcone A. *Chinese Medicine Journal.* **2015**, *10*, 8.
75. Research Diets, D09100301 NASH diet. Accessed 02/02/2016.
76. Thermofisher scientific, BODIPY® 493/503. https://www.thermofisher.com/pt/en/home/life-science/cell analysis/fluorophores/bodipy-fl.html. Accessed 14/02/2016.
77. The Jackson Laboratory, B6.Cg-Lepob/J mice. https://www.jax.org/strain/000632. Accessed 10/02/16.
78. Thermofisher scientific, ChIP assay procedures and essential tools. https://www.thermofisher.com/pt/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chromatin-ip-chip-assays.html. Accessed 14/02/2016.
79. Brand, A. H.; Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* **1993**, *118*, 401-415.
80. Duffy, J. B. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis.* **2002**, *34*, 1-15.
81. Tripos,Surflex-Dock™, http://www.tripos.com/index.php?family=modules,SimplePage,,,&page=surflex_dock&s=0. Accessed 14/02/2016.

6 –ANNEX I

- **D09100301 (NASH DIET):** 40 kcal% Fat (Vegetable Shortening), 20 kcal% Fructose, and 2% Cholesterol for NASH Models.[75]
- **Bodipy 493/503 lipid staining:** With its nonpolar structure and long-wavelength absorption and fluorescence, BODIPY® 493/503 can be used as a stain for netural lipds and as a tracer for oil and other nonpolar lipids.[76]
- **B6 V- Lep^{ob} /J (ob/ob) mice:** Mice homozygous for the obese spontaneous mutation, (Lepob commonly referred to as ob or ob/ob), exhibit obesity, hyperphagia, a diabetes-like syndrome of hyperglycemia, glucose intolerance, elevated plasma insulin, subfertility, impaired wound healing and an increase in hormone production from both pituitary and adrenal glands.[77]
- **Surflex-Dock: Surflex-Dock™** offers unparalleled enrichments in virtual high-throughput screening combined with state-of-the-art speed, accuracy and usability. It uses an updated and re-parameterized empirical scoring function (based on the Hammerhead docking system) with additional negative training data and a search engine that relies on a surface-based molecular similarity method.[78]
- **The GAL4-UAS** system is a biochemical method used to study gene expression and function in organisms, its early use focused in organisms such as fruit fly but it has been adapted to study receptor chemical-binding functions in in vitro in cell cultures.[79,80]
- **ChIP assays:** Chromatin Immunoprecipitation assays identify links between the genome and the proteome by monitoring transcription regulation through histone modification (epigenetics) or transcription factor: DNA binding interactions. The strength of ChIP assays is their ability to capture a snapshot of specific protein: DNA interactions occurring in a system and to quantitate the interactions using quantitative polymerase chain reaction (qPCR). Chromatin IP experiments require a variety of proteomics and molecular biology methods including crosslinking, cell lysis (protein-DNA extraction), nucleic acid shearing, antibody-based immunoprecipitation, DNA sample clean-up and PCR. Additional techniques such as gel electrophoresis are usually used during optimization experiments to validate specific steps.[81]