

THE TANGLE OF NUCLEAR RECEPTORS THAT CONTROLS XENOBIOTIC METABOLISM AND TRANSPORT: CROSSTALK AND CONSEQUENCES

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Abstract

The expression of many genes involved in xenobiotic/drug metabolism and transport is regulated by at least three nuclear receptors or xenosensors: aryl hydrocarbon receptor, constitutive androstane receptor and pregnane X receptor. These receptors establish crosstalk with other nuclear receptors or transcription factors controlling signaling pathways which regulate the homeostasis of bile acids, lipids, hormones, glucose, inflammation, vitamins, hormones and others. These crosstalks are expected to modify profoundly our vision of xenobiotic/drug disposition and toxicity. They provide molecular mechanisms to explain how physiopathological stimuli affect xenobiotic/drug disposition, and how xenobiotics/drugs may affect physiological functions and generate toxic responses. In addition, the possibility that xenosensors may control other signaling pathways open the way to new pharmacological opportunities.

Abbreviation list

AMPK: AMP-activated protein kinase; AhR : aryl hydrocarbon receptor ; ARNT : aryl hydrocarbon receptor nuclear translocator; CAR : constitutive androstane receptor ; ER : estrogen receptor ; FoxA2 : Forkhead box A2 ; FoxO1 : Forkhead box O1 ; FXR : farnesoyl X receptor ; GR : glucocorticoid receptor ; HIF : hypoxia inducible factor ; HNF-4 : hepatocyte nuclear factor-4 ; LKB1: AMPK upstream kinase; LXR : liver X receptor ; PGC-1 α : peroxisome proliferator activated receptor- γ -coactivator-1 α ; PPAR γ : peroxisome proliferator activated receptor- γ ; PXR : pregnane X receptor ; SHP : small heterodimer partner ; TR : thyroid hormone receptor; VDR : vitamin D receptor ; XMTS : xenobiotic metabolising and transporter systems.

INTRODUCTION

During species evolution, living organisms have developed a general strategy to protect themselves from the threat represented by xenobiotics. This strategy emerged with two major groups of players: i) nuclear receptors or xenosensors and ii) xenobiotic metabolising and transporter systems (XMTS). The role of xenosensors such as AhR (aryl hydrocarbon receptor) (1, 2), PXR (pregnane X receptor) (3-5), and CAR (constitutive androstane receptor) (6-8) is to detect xenobiotics in the cells and coordinate the expression of genes encoding the most appropriate series of XMTS to inactivate and/or eliminate these compounds. XMTS include cytochromes P450 (CYP), conjugation enzymes and transporters (9-11).

Toxic effects of xenobiotics have been primarily ascribed to the chemical reactivity of metabolites generated through biotransformation, leading to covalent binding to DNA, proteins and lipids (12, 13). A further aspect of undesirable effects of xenobiotics concerns drug-drug interactions (14, 15). However, many toxic responses to xenobiotics/drugs remain unexplained. Recent developments have revealed that xenosensors are able to establish crosstalk with many signaling pathways, so that XMTS expression appears to be dependent on many physiopathological parameters that exert positive and/or negative modulation (16-18). In addition, since most of these crosstalks are reciprocal, xenobiotics/drugs may affect other biological functions either positively or negatively. The aim of this chapter is to review some versions of nuclear receptor crosstalk that appear to be important for xenobiotic/drug disposition and toxicity.

NUCLEAR RECEPTORS AND CORECEPTORS THAT CONTROL XENOBIOTIC METABOLISM AND TRANSPORT

The reader is referred to the following excellent reviews on xenosensors AhR, CAR and PXR (2, 19-27).

Coreceptors

Nuclear receptors, and more generally transcription factors, are essential for gene regulation but lack the enzymatic activities necessary for modulating chromatin structure. These activities are catalysed by coreceptors which are recruited in response to different signals including ligand-receptor binding (28). Coregulators may be classified as coactivators or corepressors (29, 30). Coactivators are histone acetyltransferases (HAT) or methyltransferases, or serve as docking-partners for such enzymes, and are accordingly involved in chromatin relaxation, and recruitment of and association with the basic transcriptional machinery. Coactivators known to interact with xenosensors include SRC-1, TIF2, GRIP, ACTR, p/CIP, and CBP/p300 (19, 31). Corepressors (such as NcoR and SMRT) preferentially bind to unactivated receptors (ligand-free, antagonist-bound, inverse agonist-bound) and recruit various forms of histone deacetylases (HDAC), thus leading to chromatin condensation and repression of gene expression. Coactivators and corepressors thus appear to be integral parts of signaling pathways (32).

PPAR γ -coactivator-1 α (PGC-1 α)

Recent developments have revealed that, among coactivators which affect xenobiotic signaling pathways, PGC-1 α plays a critical role. The PGC-1 family has three members: PGC-1 α , β , and PRC (PGC-1 related coactivator), which may generate various isoforms by alternative splicing. PGC-1s have no intrinsic HAT activity but, upon docking to transcription factors or nuclear receptors, provide a platform for recruiting coactivators possessing HAT activity such as SRC-1, p300 and TRAP/DRIP (33, 34). PGC-1s are expressed in various tissues including heart, muscle, liver, brain and kidney where they play critical roles in the control of cellular energy metabolism by coordinating transcriptional programs of

mitochondrial biogenesis, adaptive thermogenesis and fatty acid beta-oxidation (35). PGC-1 α plays a role in the intermediary metabolism by coactivating key transcription factors of hepatic gluconeogenesis and glucose uptake in muscles. In addition, these coactivators are able to displace repressor proteins such as HDAC and SHP (see below) from their target promoters, leading to increased transcription. In the liver, PGC-1 α and 1 β expression is induced by fasting, cAMP and glucocorticoids (36-38), and *PGC-1A* promoter appears to be transactivated by CREB and FoxO1 (39). PGC-1 isoforms appear to affect the activity of numerous transcription factors such as FoxO1, SREBP, MEF-2 (40, 41, 42) and nuclear receptors such as PPAR γ , TR, RXR, GR, ER, FXR, LXR, HNF4 α (33, 34) and notably PXR and CAR (43-45). Thus, PGC-1s appear to represent a convergence point of crosstalk between xenobiotic and other signaling pathways as will be seen below.

NUCLEAR RECEPTOR CROSTALK AND XENOBIOTIC METABOLISM

Basically, a nuclear receptor signaling pathway comprises a linear series of steps including: i) a stimulus (ligand/activator, others), ii) nuclear receptor and partners, iii) coreceptors (including corepressors and coactivators), iv) battery of target genes which harbor specific response element(s) to the nuclear receptor, and v) biological functions carried out by the gene products (proteins). In textbook models, signaling pathways are generally “boxed” implicitly suggesting a lack of interaction with other pathways. This oversimplification masks the fact that any signaling pathway is actually connected with other pathways *via* multiple possibilities of crosstalk. Crosstalk may involve any step of the pathways. In addition, crosstalk may result from ligand/activator biotransformation or from receptor/coreceptor regulation and post-translational modification via another pathway. Therefore, because most crosstalks are reciprocal, the expression of a particular battery of genes expected to be

regulated by a particular signaling pathway is in fact dependent on, and affects the functioning of other signaling pathways (**Table 1**).

AhR and hypoxia inducible factor (HIF)

HIF-1 α is a member of the PAS family of transcription factors (46). Its transcriptional activity is controlled by oxygen-dependent hydroxylation of key residues which affect the stability of the protein (47). The fact that both AhR and HIF-1 α form heterodimers with ARNT suggests possible crosstalk. This possibility was investigated with controversial results. Poellinger's group (48) showed that ARNT exhibits a greater affinity for HIF-1 α as compared to AhR, so that once activated, HIF-1 α negatively affects the expression of AhR target genes. A similar conclusion was reached by Bradfield and coworkers (49). In contrast, Pollenz and coworkers (50) did not observe any effect due to hypoxia on AhR signaling. More recently, Allen et al. (51) using primary cultures of mouse hepatocytes from wild-type and transgenic (conditional knockdown of HIF1 α) animals concluded that HIF1 α does not interfere with AhR activation and vice versa, although the expression of AhR-target genes decreases during hypoxia. The mechanism of this effect is not yet elucidated. Whether or not functional crosstalk between AhR and HIF occurs in normal human cells (notably hepatocytes) is currently unknown and should be evaluated in the future.

AhR and CAR, and estrogen receptor (ER)

Estrogens control a number of physiological functions including ontogenesis, proliferation and differentiation of mammary glands, reproductive organs, and other tissues through ER α (NR3A1) and ER β (NR3A2) (52). Many reports have focussed on functional interferences between AhR ligands such as dioxin and estrogen-dependent processes including tumor development (53-55).

Polycyclic aromatic hydrocarbons and halogenated derivatives negatively affect estrogen-induced responses in the breast and female reproductive tract (56), pointing to possible crosstalk between AhR and ER. Several mechanisms have been proposed (54). Inhibitory xenobiotic responsive elements have been identified in the promoter of several ER-target genes. In addition, AhR and ER share transcriptional partners including nuclear factor-1, coactivators and corepressors, the level of which may become limiting upon activation of both receptors (57, 58). Moreover, it has been shown that ER degradation by the proteasome pathway is enhanced upon coexposition of cells to AhR activators and E2 (59). Interestingly, Ohtake et al (60) demonstrated that upon activation by agonists the AhR-ARNT heterodimer associates directly with ER in the absence of estrogen, generating a fully competent transcriptional unit which recruits coactivator CBP/P300 and transactivates ER, so that, genes normally controlled by estrogens become activated by AhR agonists. Finally, it was recently demonstrated that the cofactor RIP140 is positively regulated by AhR (61). Since RIP140 is recruited by several nuclear receptors including ER in a ligand dependent fashion and competes with coactivators, AhR-mediated induction of this factor could lead to a transrepression of ER and thus also contribute to the antiestrogenic effects of AhR ligands.

Since CAR and PXR are activated by estrogens and estrogen precursor DHEA (62-64) and control many genes involved in estrogen and DHEA metabolism, it is likely that these xenosensors regulate estrogen homeostasis (65) and thus indirectly modulate the expression of ER target genes. Indeed, several lines of evidence suggest that CAR may be involved in the regulation of an integrated pathway mediating an inhibition of the ER pathway in the liver. In addition, Min et al. (66) reported on a direct crosstalk between CAR and ER in HepG2 cells. These authors showed that CAR (and to a much lesser extent PXR) binds to ER *in vitro*, and inhibits its transcriptional activity in a cotransfection assay. Heterodimer CAR/RXR binds to ERE sequences although with a very low affinity in comparison to ER, so that this cannot

account for the crosstalk. Both CAR and ER share p160 coactivators such as GRIP-1 and SRC-1. Transfection analysis demonstrated that CAR interacts with these coactivators in a ligand (TCPOBOP)-dependent manner. This leads to the inhibition of ER-mediated transcriptional activity by squelching of p160 coactivators on ER. It is therefore possible that agents which are able to activate CAR may influence the expression of ER target genes.

PXR and CAR

Many xenobiotics interact with PXR and CAR either as agonists, activators or inverse agonists (67, 68). For example, phenobarbital and 5 β -pregnane-3,20 dione are activators of both CAR and PXR while clotrimazole and androstanol are activators of PXR but inverse agonists of CAR. Similarly, bile acids such as cholic acid, 12-ketolithocholic acid or derivatives such as 7-ketodeoxycholic acid methyl ester, which are primary ligands of FXR and/or LXR, are activators of PXR and suppressors of CAR transcriptional activity. In addition, both PXR and LXR are activated by the widely used synthetic LXR agonist T0901317, suggesting that we should re-examine the role of LXR concerning the effects of this compound, ascribed initially to LXR alone (69).

Since PXR and CAR activate overlapping series of genes, the actual effect of such compounds should be dependent on the respective levels of these receptors. Another example of this crosstalk has been described. Guggulsterone, the active ingredient of guggulipids, is an FXR antagonist and appears to be a PXR activator (70) and a CAR inverse agonist (71). Thus, *Pxr*^{-/-} mice treated with TCPOBOP and guggulsterone exhibit decreased Cyp2b10 expression while wild-type animals do not. The mechanism proposed is that guggulsterone favors the binding of coactivator SRC-1 to PXR and displaces SRC-1 from CAR. Thus, induction of drug metabolism depends on the ratio PXR to CAR. Interestingly, CITCO has been characterized as a specific activator of hCAR with minor activating effect on hPXR (72). This

compound is therefore expected to help in discriminating between CAR- and PXR-mediated inductions.

The PXR-response elements are recognized and transactivated by CAR and vice versa (73-82). This suggests, in theory, a symmetrical crossregulation of target genes by both xenosensors. However, results from a recent study show that this may not be the case (83). Indeed, although PXR and CAR bind to and transactivate the same response elements in CYP2B6 and 3A4, their efficacies in gene transcription may not be identical. In fact, hPXR regulates both CYP3A4 and CYP2B6 genes without selectivity, while hCAR exhibits a pronounced selectivity for CYP2B6 as compared to CYP3A4. This finding contrasts with observations made in rodents, and emphasises the fact that human and rodent xenoreceptors CAR and PXR not only exhibit marked differences in ligand binding and activation but also in regulation of target genes.

In summary, the response of XMTS to PXR and CAR may originate from either of these receptors or from both, depending on their relative abundance, and affinity for and ability to transactivate the response elements. In addition, these receptors compete for a common partner, RXR α , and coactivators such as SCR-1 and PGC-1 α , and ligands. It is possible that this crosstalk has resulted from an adaptative advantage for living organisms to increase their ability to detect and respond to a wide variety of xenobiotics.

CAR/PXR and farnesoyl X receptor (FXR), small heterodimer partner (SHP), liver X receptor (LXR) and HNF-4

Bile acids are essential for solubilisation of dietary fat and vitamins, and for cholesterol absorption. In addition, they represent a pathway of cholesterol elimination and play a critical role in the control of bile flow. Conversion of cholesterol to bile acids may be divided in two main branches (84, 85). The classical pathway (first step catalysed by CYP7A1) is highly

regulated by bile acids and is the major one in man. In this pathway sterol nucleus is biotransformed before oxidative side chain cleavage to generate cholic acid (CA). The alternative pathway (first step catalysed by CYP27A1) is not significantly regulated and appears to contribute little under normal conditions in man. In this pathway oxidative side chain oxidation precedes sterol nucleus biotransformation to generate chenodeoxycholic acid (CDCA). In addition, hydroxylation of cholesterol or bile acid precursors in positions 25 and 26, normally catalysed by CYP27A1, may be catalysed by CYP3A4 (86). CA and CDCA are converted to deoxycholic acid (DCA) and lithocholic acid (LCA) by 7 α -dehydrogenase in the gut flora. Bile acids are reabsorbed in the ileum and transported back to the liver before excretion into the bile.

Some bile acids appear to be highly toxic, so that their homeostasis is tightly controlled. Indeed, CYP7A1, the rate-limiting enzyme in bile acid-biosynthesis, is the convergence point of a number of nuclear receptors and transcription factors including FXR (NR1H4), SHP (NR0B2), FTF/LRH-1 (NR5A2), LXR (NR1H3), HNF-4 (NR2A1) (84, 85), and PXR/CAR (43, 87, 88). In man, CYP7A1 expression is constitutively regulated by HNF-4 α and PGC-1 α . Feedback repression of CYP7A1 is mediated by bile acids which activate FXR; this nuclear receptor activates the expression of SHP which inhibits HNF-4 and FTF/LRH-1, another positive regulator of CYP7A1 (89, 90). However in contrast to what is observed in rodents, this gene is sensitive neither to LXR (lack of binding site) nor to cholesterol/oxysterols in man (91). In addition, bile acids are being oxidized, conjugated and transported by PXR/CAR-target genes such as CYP3As, UGTs, STs, MRPs and OATP2 (84-86). Bile acid and xenobiotic detoxication pathways appear therefore to be closely linked and indeed many levels of crosstalk exist between both pathways.

PXR and FXR share ligands. LCA and its 3-keto derivative and ursodeoxycholic acid are agonists of rodent and human PXR at physiologically relevant concentrations (92, 93).

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Interestingly, LCA which is highly toxic, induces PXR target genes such as CYP3A and OATP2 involved in the 6 α and 6 β -hydroxylation and transport of bile acids, respectively, and represses CYP7A1 (94, 95). Similar observations have been made on CXR in a chicken hepatoma cell line (96). This suggests that activation of PXR by LCA has represented a way for protection of the liver. The use of transgenic and *pxr*-null animals support this conclusion (97). This is consistent with the long standing observation that rifampicin protects from cholestasis associated pruritus in man (98). PXR appears therefore as bile acid sensors in many species (birds to mammals), in spite of the wide inter-species differences in bile acid composition. This suggests that during species evolution bile acids have represented sentinels for liver metabolism and toxicity (99).

FXR transactivates CAR/PXR target genes. The finding that LCA induces Cyp3a in PXR-null mice and that FXR activates CYP3A4 promoter in vitro, suggests that CYP3A gene expression is controlled by FXR (93, 100). In recent work, Gnerre et al. (101) identified two functional FXR responsive elements (ER8 and IR1/DR3) in the XREM of CYP3A4, one of which (the distal DR3) is known to bind PXR (102). These observations support the finding that CYP3A expression is elevated during cholestasis and might explain the variability of drug responsiveness and/or toxicity between individuals. These results are consistent with previous reports in which several sequences known to be responsive elements of PXR/CAR are shown to be targets of FXR in *SULT2A1* and *MRP2* (79, 80).

FXR controls PXR gene expression. In recent work, Jung et al. (103) observed that feeding wild-type mice with cholic acid or the synthetic FXR agonist GW4064 resulted in marked PXR and PXR-target gene induction, while no induction was observed in *Fxr*-null mice. A region containing four FXR binding-sites (IR1) was identified in the mouse *Pxr* gene. Two of these elements exhibited binding to and transcriptional activation by FXR protein. These results strongly suggest that PXR is positively regulated by FXR. The combination of

FXR-mediated activation of CYP3A and induction of PXR provides an amplification mechanism towards efficient protection of the liver against bile acid-induced toxicity.

SHP negatively regulates CAR and PXR transcriptional activity. SHP is a member of the nuclear receptor superfamily which lacks the conventional DNA-binding domain. It is mostly expressed in the liver under the control of FXR by primary bile acids (104, 105). SHP represses CYP7A1 primarily by inhibiting the activity of FTF/LRH-1. SHP has been shown to bind to and inhibit the transcriptional activity of CAR and other nuclear receptors (104, 106), LXR (107), FoxO1 and HNF-4 (108, 109). We recently showed that SHP interacts also with PXR in a ligand-dependent manner and inhibits its transcriptional activity (110). This is likely to generate functional interference between bile acid homeostasis and xenobiotic detoxication pathways. In addition, the fact that SHP represses other nuclear receptors and transcription factors suggests that bile acids control several signaling pathways.

PXR controls SHP expression. Another possibility of crosstalk involving PXR and SHP has been discovered through an *in silico* approach and then confirmed by direct observation in HepG2 cells. Frank et al. (111) created a position weight matrix for the screening of heterodimer PXR-RXR responsive elements. When used to screen the promoter of OATP2 and SHP genes, this strategy identified 17 putative PXREs of which 7 bound PXR-RXR *in vitro*, suggesting that both genes are regulated by PXR. Although this was known for OATP2 (112), induction of SHP expression by PXR in the presence of rifampicin was confirmed (although moderate : 1.5-fold) in HepG2 cells. On one hand, the finding that SHP is a target of PXR and that PXR is activated by bile acids suggests another way of amplifying cellular response to elevated levels of bile acids by decreasing CYP7A1 expression. On the other hand, however, this is particularly intriguing because the repressive effect of SHP on PXR and CAR (104, 110) should circumvent this effect. It should be noted however that we

observed no significant induction of SHP mRNA by rifampicin in primary human hepatocytes (110).

LXR negatively regulates CAR and PXR transcriptional activity. In rodents (but not in man) LXR enhances CYP7A1 expression in response to cholesterol or oxysterols (91). Hydroxylated bile acids, generated in part by CYP3A4, CYP2B and CYP2C, are also agonists of LXR. All these compounds were shown to inhibit xenobiotic metabolism in the liver and phenobarbital-mediated induction of CYP2H1 in a chicken cell line (96). LXR/RXR heterodimer was shown to bind to the same response elements as CXR in the chicken and CAR/PXR in man. In addition, cotransfection experiments revealed that LXR inhibits the transcriptional activity of CXR and of hCAR and hPXR. This is consistent with the observations that statins (inhibitors of cholesterol biosynthesis) induce CYP2B1/2 in rat liver (113), while rats fed a high cholesterol diet or hyperlipidemic rats with increased cholesterol levels have lower basal and inducible expression of CYP genes compared with control animals (114).

CAR/PXR and HNF-4 α . Li et al. (43) and Bhalla et al. (87) demonstrated that ligand-activated PXR interferes with HNF-4 α signaling by targeting the common coactivator PGC-1 α , via the so-called “squenching effect”, through an increase of PXR/PGC-1 α complex formation and a concomitant decrease of HNF4 α /PGC-1 α complex formation, leading to the down-regulation of CYP7A1 and CYP8B1 gene expression. More recently Miao et al. (88) demonstrated that CAR inhibits HNF-4 α transactivation activity by two possible mechanisms: i) competitive binding to DR1 motifs in CYP7A1, CYP8B1 and PEPCK promoters ; ii) competition with HNF-4 α for the recruitment of coactivators GRIP-1 and PGC-1 α . Since HNF-4 α and PGC-1 α are jointly involved in the control of many genes involved in gluconeogenesis and fatty acid homeostasis (115), it is likely that this crosstalk applies to these genes. Interestingly, phenobarbital was shown to repress CYP7A expression

in a chicken cell line, suggesting that this crosstalk was maintained through species evolution (96). Together, these observations are consistent with the finding that in epileptic children treated with phenobarbital or carbamazepine plasma cholesterol and lipoprotein levels are increased (116), and that in AIDS patients the human immunodeficiency virus-protease inhibitor ritonavir (a PXR agonist (117)) often leads to elevated cholesterol and triglyceride levels (118).

Other crosstalk. AhR also appears to interfere with bile acid pathway. By performing a transcriptome analysis of rat liver after dioxin treatment, Fletcher et al observed a significant decrease (> 2-fold) of the expression of CYP7A1, FXR, SHP, NTCP, and OATP2. Although the molecular mechanism of this effect was not investigated, these alterations provide objective arguments to explain the dioxin-induced hepatotoxicity and hypercholesterolemia (119).

In conclusion, the various levels of crosstalk described above reveal tight connexions between the xenobiotic and the cholesterol/bile acid pathways. The sharing of regulators (CAR/PXR, SHP, FXR, LXR), enzymes (CYP3A, UGTs, STs) and transporters (MRPs, OATPs) suggests that both pathways have established long-standing cooperation during species evolution, favoring mutual efficacy. Thus, on one hand xenobiotic pathway provides bile acid pathway with regulatory control (PXR/CAR) and functional assistance (enzymes, transporters), while on the other hand the bile acid pathway provides xenobiotic pathway with a major route of excretion, the bile.

CAR/PXR and FoxO1 and FoxA2

Fox (Forkhead box) genes encode a subgroup of helix–turn–helix class of remarkably conserved transcription factors (>50 genes in man), controlling numerous biological processes including development, organogenesis, cell differentiation, cell cycle control, apoptosis, and

functions as diverse as speech and language development, gluconeogenesis and lipid metabolism (120). Hepatic gluconeogenesis and lipid metabolism are tightly controlled by insulin and glucagon and play a major role for survival during fasting or starvation.

Genes involved in gluconeogenesis include notably phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) (40). In the liver, gluconeogenesis is controlled positively by glucocorticoids, cAMP and glucagon, and negatively by insulin and glucose. Two proteins have been shown to be critical in this respect, FoxO1 and PGC-1 α . PGC-1 α is induced upon fasting and is elevated in diabetes or during deficiency in insulin signaling (36-38). PGC-1 α turns on the process of gluconeogenesis and functions as a coactivator of FoxO1 (40). When insulin level is low, FoxO1 positively controls the expression of genes involved in gluconeogenesis. Upon binding to its receptor, insulin triggers the phosphorylation of FoxO1 *via* phosphatidylinositol 3-kinase and Akt (121). Phosphorylated FoxO1 is no longer retained in the nucleus and is unable to bind PGC-1 α , and thus becomes transcriptionally inactive. This is the basis of the negative control of gluconeogenesis by insulin. In contrast, insulin has no effect on PGC-1 α expression (40).

FoxO1 has been shown to interact with several nuclear receptors in a ligand-dependent (ER) or independent (TR or RAR) manner, and behaves either as corepressor (HNF4, GR, PR, ER) or coactivator (RAR, TR) (108, 122). Kodama et al. reported on the crosstalk between FoxO1 and CAR/PXR (123). By performing a yeast two-hybrid screening, these authors identified FoxO1 as a CAR-binding protein. Further experiments demonstrated that FoxO1 binds CAR in a ligand-dependent manner and activates its transcriptional activity, thus behaving as a coactivator. In addition, these authors demonstrated that FoxO1 similarly binds to and activates PXR suggesting that this crosstalk may be of general significance for xenobiotic metabolism. Consistent with these observations, insulin represses the induction of Cyp2b10 mRNA by TCPOBOP in mouse hepatocytes. Interestingly, this crosstalk was shown

to be reciprocal. Both CAR and PXR inactivate FoxO1 transcriptional activity by preventing its binding to its responsive element in target genes such as PEPCK. These results indicate that drug metabolism and gluconeogenesis are reciprocally coregulated in response to insulin and/or xenobiotics. This is consistent with previous observations revealing functional links between insulin and xenobiotic-mediated pathways (124). It has been known for many years that diabetes enhances hepatic drug metabolism, while phenobarbital or expression of activated PXR in mice reduce hepatic level of PEPCK and G6P (125, 126). In addition, phenobarbital has been shown to decrease plasma glucose in diabetic patients and animals (127, 128, 129). It seems therefore that this crosstalk has clinical implications. Whether the physiological objective of this crosstalk is NADPH homeostasis remains to be evaluated. NADPH is essential for cytochrome P450-dependent monooxygenase activities and glutathion recycling. In the liver, the pentose phosphate pathway converts glucose 6-phosphate to ribose 5-phosphate by glucose 6-phosphate dehydrogenase (G6PDH) and generates NADPH. In the gluconeogenesis pathway, glucose 6-phosphate is the last intermediate on the way to glucose, a reaction catalysed by G6P. Thus, repression of gluconeogenesis by xenobiotic-activated CAR/PXR might be required to maintain sufficient NADPH levels for xenobiotic metabolism. Alternatively, the decrease in CAR/PXR activity by insulin is consistent with decreased NADPH production through repression of gluconeogenesis. Interestingly, patients deficient in G6PDH exhibit reduced detoxication ability (130).

During fasting or starvation, or after exercise when glucose blood level is low, the liver metabolise fatty acids to provide extrahepatic tissues and other organs with energy sources such as ketone bodies through ketogenesis and β -oxidation (131, 132). At low insulin levels, FoxA2 has been shown to regulate positively these functions by controlling the transcription of target genes including mitochondrial hydroxy-methylglutarate CoA synthase 2 (HMGCS2) and carnitine palmitoyltransferase 1A (CPT1A) (133, 134). Activation of the

phosphatidylinositol 3-kinase and Akt pathway by insulin induces FoxA2 phosphorylation which leads to nuclear exclusion and eventually to the inhibition of FoxA2 target gene transcription (133, 134). Using *Pxr*^{-/-} and wild type starving mice, Nakamura et al. (135) recently reported that activated PXR and FoxA2 interact through their ligand and DNA binding domains, respectively. This interaction prevents the binding of FoxA2 to its DNA response elements and eventually leads to the repression of target gene. Thus, activated PXR appears to repress hepatic energy metabolism by decreasing both ketogenesis and β -oxidation and accordingly increasing hepatic triglycerides in the fasting mice. This is consistent with previous observations that repeated treatment of rats with phenobarbital down-regulates a number of genes involved in energy metabolism, including HMGCS and CPT1 (136).

PXR and PPAR γ

Peroxisome proliferator-activated receptor γ (PPAR γ), is a member of the nuclear receptor superfamily and a key regulator of adipogenesis and lipid storage (137). While PPAR γ 2 expression is limited exclusively to adipose tissue, PPAR γ 1 is expressed at relatively low levels in many tissues including the liver. In a recent paper, using transgenic mice expressing activated *Pxr* and pharmacological (PXR agonist) approaches, Zhou et al. showed that in mice, PXR activation causes hepatic steatosis characterized by a marked accumulation of triglycerides. Further analysis revealed that a number of genes involved in lipid metabolism including PPAR γ 1, CD36, stearyl CoA desaturase and fatty acid elongase were induced in a PXR-dependent manner. Finally similar results were obtained with rifampicin-treated hPXR humanized mice (126). Although the mechanism of induction of PPAR γ by PXR is not yet known, the finding that expression of this receptor is dependent on PXR suggests a potential crosstalk between both pathways, the consequence of which is hepatic steatosis. Interestingly, Yu et al. reported that overexpression of PPAR γ in mice induced hepatic steatosis (138).

Northern blotting and gene expression profiling results showed that adipocyte-specific genes and lipogenesis-related genes including adipisin, adiponectin, aP2, caveolin-1, fasting-induced adipose factor, fat-specific gene 27 (FSP27), CD36, 9 desaturase, and malic enzyme among others, were markedly induced. These results suggest that a high level of PPAR γ in mouse liver is sufficient for the induction of adipogenic transformation of hepatocytes with adipose tissue-specific gene expression and lipid accumulation.

LKB1-AMPK cascade and CAR activation

AMP-dependent protein kinase (AMPK) is the down-stream player of a protein kinase cascade which senses increased AMP/ATP ratio, and helps the cell to adapt in such a way as to maintain its energy balance (139). Thus, among other functions, AMPK turns-off ATP-consuming pathways (such as glycogen, fatty acid, and sterol synthesis) and turns-on ATP-generating pathways (such as glycolysis, fatty acid oxidation, glucose and fatty acid transport) by phosphorylating various target proteins. Since several of the crosstalks described in the previous sections concern some of these pathways, it is not surprising that AMPK has been recently shown to be involved, at least in part, in the control of XMTS induction. Using hepatoma cell lines, primary cultures of human and mouse hepatocytes and transgenic animals (deficient for AMPK α 1/ α 2 subunits), Rencurel et al. demonstrated that AMPK plays an essential role in the induction of CYP2B and CYP3A forms by phenobarbital, and that among several CAR and PXR agonists (including notably rifampicin, CITCO and TCPOBOP), only phenobarbital increased the AMP/ATP ratio and activated AMPK (140, 141). Likewise, AICAR and metformin, two known AMPK activators induced CYP2B and CYP3As. However, whether these compounds induce CYPs in *Car* (-/-) or *Pxr* (-/-) mice is unknown. AMPK was not necessary for controlling the nuclear translocation of CAR, nor did it phosphorylate CAR, although CAR concentrated in nuclear speckles in the hepatocytes

from AMPK α 1/ α 2 (-/-) mice while its accumulation was homogeneous in cell nuclei from wild type animals. The nature of the protein phosphorylated by AMPK and its role in CYP induction by phenobarbital are presently unknown. More recently, using a chicken cell line (LMH) the same group demonstrated that phenobarbital induces the formation of a complex between LKB1 (a previously known upstream activator of AMPK) and AMPK, and, as previously shown for metformin, triggers mitochondrial ROS formation resulting in the phosphorylation of LKB1 and the subsequent phosphorylation of AMPK by LKB1 (142). The LKB1-AMPK cascade appears therefore to be necessary for induction of CYP genes by phenobarbital. Whether this is to be generalized to mammalian species and other phenobarbital-like inducers is presently unknown. The finding that phenobarbital activates AMPK is consistent with the previously reported observation that this drug lowers blood glucose in patients and animals with non-insulin-dependent diabetes (128, 129), as discussed above for the CAR-FoxO1 crosstalk.

CAR/PXR and glucocorticoid receptor (GR)

In primary human hepatocytes, PXR and CAR expression is glucocorticoid-dependent (143-145). A functional glucocorticoid-responsive element (GRE) has been identified in the hCAR gene promoter (146). A functional GRE in the PXR regulatory region remains to be identified. These observations suggest the existence of a cascade of signal transmission GR-CAR/PXR-XMTS which may have potential consequences for xenobiotic/drug metabolism pathways. Indeed, any process that affects the expression and/or transcriptional activity of GR is expected to reduce expression of PXR/CAR and XMTS genes regulated by these xenoreceptors (17, 18). Several recent observations support this hypothesis.

First, nuclear receptors, including GR, are thought to need the microtubule network for nuclear translocation (147). Dvorak et al. (148) investigated the consequence of a treatment of

human hepatocytes by colchicine, a microtubule disrupting agent (149), on the GR-CAR/PXR-XMTS cascade. Colchicine strongly inhibited the transcriptional activity of GR, but affected neither GR mRNA expression nor dexamethasone-GR binding. This was accompanied by a marked decrease of TAT, CAR, PXR and CYP2B/2C/3A mRNA expression.

Second, inflammation and/or infection are frequently associated with a reduced capacity of liver to metabolise xenobiotics/drugs (150). Cytokines and bacterial endotoxins are likely to be responsible for this effect (150, 151), and were shown to reduce PXR and CAR gene expression and subsequently XMTS expression in primary human hepatocytes (152, 153). Cytokines, oxidative stress, and chemicals are known to trigger NF κ B activation (154) and this factor has been shown to bind and inactivate GR (155). Thus, activation of NF κ B by IL-1 β in human hepatocytes leads to inhibition of GR transcriptional activity, followed by downregulation of PXR, CAR, CYP2/3A, UGT1A1, and SLC26A1 (153). The mechanism by which NF κ B inhibits CAR transcription is unknown. A likely possibility is competition for common coactivators CBP/P300 or SRC-1 (156), as recently demonstrated on the transrepression of AhR-mediated Cyp1a1 expression by TNF α and LPS (157). Recently Zhou et al. (158) showed that the activated PXR inhibits the activity of NF- κ B and that in Pxr (-/-) mice NF- κ B target genes are upregulated and small bowel inflammation significantly increased, thereby demonstrating a direct link between PXR and drug-mediated antagonism of NF- κ B. Interestingly, NF- κ B activation reciprocally inhibited PXR and its target genes whereas inhibition of NF- κ B enhanced PXR activity. This PXR–NF- κ B crosstalk provides a further molecular explanation for the suppression of hepatic CYP mRNAs by inflammatory stimuli as well as the immunosuppressant effects of xenobiotics and PXR-responsive drugs.

Third, ketoconazole and miconazole were recently shown to be antagonists of hGR (159). Indeed, treatments of hepatocytes by these compounds down-regulate TAT, PXR, CAR

and numerous XMTS gene expression. Thus, in addition to their well know inhibitory effect on CYP enzyme activities (15), azole derivatives are expected to down-regulate gene expression through their antagonist effect on hGR. Together, these results are consistent with the existence of the GR-CAR/PXR-XMTS cascade.

CAR, PXR and thyroid hormone receptor (TR)

Thyroid hormone homeostasis is controlled through the hypothalamus-pituitary-thyroid axis, liver and peripheric metabolism, and thyroid receptor signaling pathway (160). The hypothalamus synthesizes and secretes the TRH releasing hormone (TRH) which, in turn, stimulates the pituitary gland. The thyroid stimulating hormone (TSH) secreted by the pituitary gland then stimulates the thyroid gland which secretes thyroid hormone T4 (3,5,3',5'-tetraiodothyronine), a precursor of T3 (3,3',5-triiodothyronine) the bioactive hormone. Thyroid hormone receptors TR α and TR β (NR1A1 and NR1A2) form heterodimers with RXR and control a number of physiological functions including skeleton development and growth, skeletal muscle development, bile acid synthesis, and basal metabolism. Notably, expression of both TRH and TSH is controlled negatively by TR, so that T3 controls its own level *via* this negative feedback mechanism. To summarize, increased levels of T3 reduce thyroid hormone synthesis, while decreased levels of T3 enhance TRH and TSH synthesis and stimulation of the thyroid gland.

Deiodinases (DIO) convert T4 to T3 in the liver and peripheric tissues and also degrade both hormones by inner ring deiodination. *DIO-1* has been shown to be a target of TR (161). We recently observed that rifampicin and phenobarbital increase *DIO-1* mRNA expression in cultured human hepatocytes. Similarly, phenobarbital-mediated induction of *Dio-1* gene expression observed in mice after partial hepatectomy has been shown to be CAR-dependent (162). Interestingly, we observed the CAR/RXR and PXR/RXR are able to bind to

the same TRE of *hDIO1* as TR/RXR, suggesting that TR and CAR and PXR could share DNA response element and target genes (JM Pascussi et al., unpublished data).

T3 and T4 are inactivated in the liver by UDP-glucuronosyltransferase and sulfotransferase to glucuronide- and sulfate-derivatives which are eliminated *via* urine and bile. Two recent studies (163, 164) used wild-type and *Car*^{-/-} mice to demonstrate that CAR mediates the induction of UDP-glucuronosyltransferases (UGT1A1 and 2B1) and sulfotransferases (SULT1A1, 2A1, N) involved in glucuronidation and sulfation of T3 and T4. Enzyme induction coincided with reduced levels of T4 and T3 and increased levels of TSH. These results are consistent with previous observations pointing to numerous thyroid hormone-linked disorders during chronic treatments with drugs such as phenobarbital and phenytoin, including, reduced thyroid hormone levels, thyroid follicular cell proliferation, altered thyroid function, altered pituitary response to TRH, hypothyroidism, thyroid gland tumors (in rat), thyroid gland neoplasia and thyroid goiter (in man) (165, 166). These disorders are thought to be the consequence of xenobiotic-mediated enhancement of metabolism and excretion of thyroid hormones *via* induction of DIO-1, UGT and SULF enzymes through CAR (and presumably PXR) activation.

Interestingly, one important function regulated by thyroid hormones is the basal metabolism. T3-T4 serum levels correlate positively with energy expenditure and caloric loss. For instance, T3 has been shown to be reduced by 33% in subjects following a weight loss program. The concomitant decrease in basal metabolism is believed to represent a mechanism to resist weight loss. Maglish et al. (163) showed that *Cyp2b10*, *Sult1a1*, *SultN* and *Sult2a1*, and *Ugt1a1* gene expression is significantly induced during fasting in wild-type but not in *Car*^{-/-} mice. In parallel experiments, they observed that T3 and T4 levels were greater in *Car*^{-/-} animals than in wild-type littermates during fasting. Most importantly, when placed under caloric restriction for a long period, the weight loss in *Car*^{-/-} mice was twice

that observed in wild-type animals. It is likely that PGC-1 α is responsible for this process since this coactivator of CAR is induced during fasting (36, 38, 44). Thus, CAR agonists may be viewed as having a high therapeutic potential for obesity treatments.

PXR and Vitamin D Receptor (VDR)

VDR (NR1H1) mediates the effect of 1 α ,25-dihydroxyvitamin D₃ on a large battery of genes mediating biological functions such as bone mineralisation (see below). After ligand binding, VDR forms a heterodimer with RXR α which transactivates the vitamin D response elements (VDRE) present in the regulatory region of target genes (167). Although the consensus VDRE is an imperfect DR3, previous investigations identified non-consensual motifs including DR4 and an inverted palindrome IP9. Several studies demonstrated that 1 α ,25-dihydroxyvitamin D₃ induces the expression of CYP3A4, CYP2B6 and CYP2C9 in intestinal cell lines and human hepatocytes (168-170). Since this compound activates neither PXR nor CAR, VDR was suspected and subsequently demonstrated to be responsible for this induction. Indeed, the heterodimer VDR/RXR binds to and transactivates PXR/CAR responsive elements of CYP2B6, CYP2C9, and CYP3A4, (169-171), as well as of rat sulfotransferase 2A1 gene (79). This suggests that in the absence of xenobiotic the basal expression of those XMTS which are controlled by PXR/CAR may be, at least partly, controlled by VDR. Vitamin D is present in our every-day diet (fish, eggs, milk) and is also produced in the skin *via* photoconversion of 7-dehydrocholesterol. It is thus possible that interindividual differences in dietary habits and light exposure contribute to interindividual variations in basal expression of XMTS *via* VDR. Interestingly, it was recently reported that LCA, a hepatotoxic bile acid and possible colon carcinogen, binds to and transactivates VDR and this leads to CYP3A4 and MRP3 induction in the colon (171). Together, these observations are consistent with the anti-cancer effect of Vitamin D (172). Finally, it is expected that in the presence of activators PXR and CAR will

compete efficiently with VDR for their cognate responsive elements and enhance the expression of target genes.

The reciprocal version of this crosstalk, i.e., PXR and CAR control VDR target genes, has also been investigated. The major route of vitamin D degradation is the oxidation of the side chain catalyzed by vitamin D-24 hydroxylase, a mitochondrial enzyme encoded by CYP24, a target gene of VDR (167). CYP24 appears therefore to mediate the negative regulatory feedback process that controls vitamin D homeostasis. Thus, increased CYP24 gene expression over long periods of time is expected to lower $1\alpha,25$ -dihydroxyvitamin D₃ concentration, and eventually, to lower VDR target gene expression. CYP24 was therefore considered as a critical target if transactivated by PXR and/or CAR. In primary human hepatocytes treated with PXR agonists, CYP24 and CYP3A4 mRNAs exhibit similar dose-dependent and kinetic profiles of induction accompanied with an increase in vitamin D-24 hydroxylase. Studies on mice confirmed these observations. Moreover, both human and mice PXR bound to and transactivated the VDR response elements present in CYP24 promoter (173). Further experiments revealed similar crosstalk with CAR (JM Pascussi et al. unpublished data). Together, these results demonstrate that CYP24 (an VDR-target gene) is an PXR-target gene, although this conclusion has been recently questioned (174). Interestingly, it has been recently reported that CYP2D25, the porcine microsomal vitamin D 25-hydroxylase, is downregulated at the transcriptional level by VDR in the presence of vitamin D metabolites and by both CAR and PXR in the presence of phenobarbital (M Ellfolk et al., Abstract. 15th International Conference on Cytochromes P450, Bled Slovenia 2007). This is consistent with the observation by the same group that phenobarbital causes vitamin D deficiency via impairment of vitamin D 25-hydroxylation in the pig liver, as observed in other species (175).

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An alternative view to this crosstalk was recently proposed. Xu et al (176) demonstrated that, in addition to CYP24, CYP3A4 may efficiently contribute to the metabolism of $1\alpha,25$ -dihydroxyvitamin D₃ by generating 23*R*- and 24*S*-hydroxy metabolites. Although CYP3A4 efficiency appears to be approximately one order of magnitude lower than that of CYP24, the relative expression of both enzymes suggests that CYP3A4 may play a dominant role on the metabolism of $1\alpha,25$ -dihydroxyvitamin D₃ in liver and intestine.

Many reports in the literature point to deleterious effects of long-term drug treatment on vitamin D blood levels and bone mineralisation (173, 174, 176). In addition, significant bone disorders affect HIV-infected patients receiving antiretroviral therapy (177). Interestingly, protease inhibitors have been suspected to play some role in this process and some of them, ritonavir and saquinavir are PXR agonists (117). Thus, three possible mechanisms which are not mutually exclusive may be proposed to explain, at least in part, these drug-related disorders: i) drug-mediated inhibition of bioactivation of vitamin D₃ by decreased 25-hydroxylation of vitamin D₃ in liver, ii) PXR-VDR crosstalk leading to CYP24 gene up-regulation by PXR agonists, and iii) induction of intestinal CYP3A4 leading to inactivation of $1\alpha,25$ -dihydroxyvitamin D₃.

PXR-CYP3A4 and AhR

Omeprazole (OM), a benzimidazole-derived antiulcer drug (178), has been reported to be an aryl hydrocarbon-like inducer of *CYP1A1/2* in human hepatocytes (179). Further investigations confirmed these conclusions in vivo (180-182). However, in contrast to aryl hydrocarbons, OM does not bind AhR in vitro (183, 184). The mechanism of AhR activation by OM is currently unknown. During the course of a structure-activity relationship study we observed that omeprazole-sulphide (OMS), a minor degradation metabolite of OM-sulfenamide, the active form of the drug, exhibited remarkable biological properties (185). In

hepatoma cell lines including Hepa-1c1c7 and HepG2, OMS was characterised as a pure antagonist of AhR. However, when this compound was tested in highly differentiated primary human hepatocytes, it behaved as an aryl hydrocarbon-like inducer of *CYP1A1* and, in addition, did not affect the induction of this gene in response to dioxin or OM. Since a major difference between hepatoma cell lines and differentiated hepatocytes is their capacity to metabolise xenobiotics/drugs, conversion of OMS to an AhR activator was suspected and subsequently demonstrated. Indeed, OMS is converted to OM (and other derivatives) by CYP3A4 while no biotransformation occurs in hepatoma cell lines. Modulation of expression and activity of CYP3A4 in hepatocytes by induction and inhibition confirmed these findings. These observations reveal a metabolism-mediated crosstalk between PXR/CYP3A4 and AhR. To our knowledge this is the first example of a compound that may be turned from antagonist to agonist and vice versa by modulating the metabolic activity of the host cells.

CONCLUSION

These various examples of crosstalk demonstrate that signaling pathways controlling xenobiotic/drug metabolism and disposition are embedded within a tangle of regulatory networks controlling the homeostasis of glucose, bile acids, lipids, hormones, inflammation and others. These crosstalks are expected to modify profoundly our vision of xenobiotic/drug disposition and toxicity. First, they explain how physiopathological stimuli affect xenobiotic/drug metabolism and disposition. Second, they show that xenobiotics/drugs affect physiological functions with the possibility of discovering new functions for xenosensors, thus opening the way to interesting pharmacological opportunities, notably in the treatment of diabetes.

However, this is just the beginning of the story. Indeed, there are now approximately 50 nuclear receptors working with >200 coactivators, most of which have not yet been fully

characterised (186). The extreme flexibility and versatility of nuclear receptors opens the prospect of regulating their transcriptional activity by ligands, post-translational modifications, partners, coreceptors and promoter context. While nuclear receptors activate batteries of genes, coactivators activate batteries of nuclear receptors and transcription factors. In addition, coactivators exist as multiprotein complexes, are subjected to transcriptional regulation (like PGC-1 α), post-translational modification, controlled degradation, and exhibit polymorphism which are expected to influence the activity of their partners. Similar considerations likely apply to corepressors. We are therefore far from having a wide and clear view of the tangle of regulatory networks in which the signaling pathways controlling xenobiotic/drug metabolism and disposition are embedded.

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MINI-GLOSSARY

1. Nuclear receptors. Multifunction proteins activated by low molecular weight ligands, covalent modification or protein-protein interaction. Nuclear receptors form homo-oligomers or hetero-oligomers with partners, bind specific DNA sequence in target gene promoter, recruit coactivators or corepressors, and eventually enhance or repress target gene transcription.
2. Xenosensors. Nuclear receptors activated by xenobiotics. Upon activation, xenosensors coordinate the expression of the most appropriate genes to metabolise and eliminate xenobiotics.
3. Signaling pathway. A linear series of steps including a triggering stimulus (ligand/activator, others), nuclear receptor and partners, coreceptors (corepressors/coactivators), battery of target genes, and biological functions carried out by the target gene products (proteins).
4. Coactivators. Proteins recruited by activated nuclear receptors (or other DNA binding transcription factors) that elicit enhanced gene expression. Coactivators are diverse, both structurally and in the way they contribute to the transcriptional process, namely through a diverse array of enzymatic activities such as acetylation, methylation, ubiquitination, and phosphorylation or as chromatin remodelers.
5. Corepressors. Proteins that interact with unliganded, antagonist- or inverse agonist-bound nuclear receptors, and repress transcription. Corepressor-associated proteins such as histone deacetylases enforce chromatin compaction, thereby opposing to coactivators to modulate nuclear receptor activity.
6. Crosstalk. Functional interaction between two or more signaling pathways. Crosstalk may involve any step of the pathways.

SUMMARY POINTS

1. Xenosensors AhR, PXR and CAR establish crosstalk with other nuclear receptors or transcription factors involved in other signaling pathways
2. As a consequence of crosstalk, expression of a battery of genes expected to be triggered by a particular stimulus (ie. hormone) is in fact dependent on the functioning of other signaling pathways through different stimuli (ie. xenobiotic)
3. Most of crosstalks between xenosensors and other nuclear receptors or transcription factors are reciprocal
4. Crosstalks explain how various physiopathological stimuli may affect xenobiotic/drug metabolism and transport
5. Crosstalks explain how xenobiotics/drugs may affect various biological functions
6. Crosstalks allow to predict new functions for xenosensors and thus open the way to new pharmacological opportunities

FUTURE DIRECTIONS

1. In many crosstalks (PXR-FoxO1, AhR-ER, GR-NFκB, AMPK-CAR, etc.) the molecular mechanism by which the functional interaction affects xenosensor or partner transcriptional activity remains unknown.
2. Further crosstalks involving xenosensors remain to be discovered.
3. New functions of xenosensors, resulting from crosstalk with other signaling pathways, have to be explored in more detail in order to design new therapeutic approaches, and discover non-invasive biomarkers of the enterohepatic metabolism of patients in response to a treatment.
4. The role of coreceptors (coactivators and corepressors) in crosstalks is likely to be of major importance and will require extensive investigations.