The current understanding of hepatic drug induction in humans has an enormous impact on the drug-discovery process to an extent that safer drugs can be designed more rationally and unsafe compounds can be eliminated at a very early stage.

In Silico Approaches, and *In Vitro* and *In Vivo* Experiments to Predict Induction of Drug Metabolism

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Summary
Despite being described more than 40 years ago, the molecular mechanism that regulates hepatic induction of cytochromes P450 and other drug-metabolizing enzymes and drug transporters by xenobiotics has remained enigmatic until recently. A major breakthrough was the discovery of the orphan nuclear receptors pregnane X receptor and constitutive androstane receptor playing key roles as species-specific xenosensors in this induction response. Using this newly acquired knowledge, the human induction response can now be more accurately predicted. This is of considerable clinical importance, since induction of cytochrome P450s and other enzymes can lead to unwanted drug–drug interactions, adverse drug reactions and drug toxicity. In this review, *in vitro*, *in vivo* and *in silico* techniques are discussed that can identify troublesome compounds at an early stage and that can help to design new, safer medicines faster. © 2003 Prous Science. All rights reserved.

It was observed more than 40 years ago that many drugs and other xenobiotics can increase their own metabolism and clearance, a phenomenon called induction.[1] The major drug-metabolizing enzymes in the liver are members of the gene superfamily of cytochrome P450 (CYP) monooxygenases that in conjunction with conjugating phase II enzymes and transporter proteins (also called phase III enzymes) increase clearance of lipophilic substances.[2,3] The best studied human hepatic drug-metabolizing CYPs, their prototypical inducer compounds and the corresponding major regulatory transcription factors, if applicable, are listed in Table I. Induction of genes by xenobiotics has been recognized as an important part of how patients react to drugs. In fact, induction influences the pharmacokinetic and pharmacodynamic properties of the inducer itself but also of other compounds metabolized by the same CYPs. Therefore, drug-mediated increase of these enzymes can lead to unwanted drug–drug interactions and adverse drug reactions.[4] Lack of knowledge of the inductive capability of a compound can result in therapeutic failure or drug toxicity due to generation of inactive or active metabolites in patients. Much effort has therefore been invested in the study of this phenomenon, but despite its early discovery, little progress in elucidating the molecular mechanism underlying transcriptional activation of drug-metabolizing genes by foreign compounds has been made until recent years. Breakthroughs have been hampered by several peculiarities of drug-induced expression of CYPs. First, inducing compounds have different effects in

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TABLE I. THE MAJOR HUMAN HEPATIC DRUG-METABOLIZING CYTOCHROMES P450

GENE	PROTOTYPICAL INDUCER	RECEPTOR
CYP1A1	PAH, dioxin, cigarette smoke	AhR
CYP1A2	PAH, dioxin, omeprazole	AhR
CYP1B1	PAH, dioxin	AhR
CYP2A6	Phenobarbital, rifampicin, dexamethasone	?
CYP2B6	Phenobarbital, rifampicin, dexamethasone	CAR, PXR
CYP2C8	Phenobarbital	?
CYP2C9	Phenobarbital, rifampicin, dexamethasone	CAR
CYP2C19	Rifampicin, dexamethasone, phenobarbital	?
CYP2D6	Not inducible	--
CYP2E1	Ethanol, acetone	--
CYP3A4	Rifampicin, dexamethasone, phenobarbital	PXR, CAR
CYP3A5	Rifampicin, dexamethasone, phenobarbital	?
CYP3A7	Rifampicin, dexamethasone, phenobarbital	PXR
CYP4A11?	Clofibrate	PPAR α

For details, see refs. 4 and 12. Note that CYP2E1 is induced by a nonreceptor-mediated, posttranscriptional mechanism involving protein stabilization. Furthermore, CYP3A7 is the predominant CYP3A isoform in fetal liver, whereas CYP3A4 and to a lesser extent CYP3A5 are expressed in adult liver. Finally, although human peroxisome proliferator-activated receptor α is activated by fibrate drugs, drug-responsive elements in the 5′-flanking region of the human clofibrate-inducible CYP4A11 have not been reported so far. Abbreviations: PAH, polyaromatic hydrocarbons; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; PPAR, peroxisome proliferator-activated receptor.

different species.[5] For instance, the synthetic steroid pregnenolone 16 α-carbonitrile (PCN) is a strong activator of rodent but not human CYP3A isoforms. Vice versa, the antibiotic rifampicin is a potent activator of human CYP3A4 and has no effect on rodent CYP3As.[6] Because of this species-specificity, it has been unclear whether the same molecular mechanism is responsible for drug induction of CYPs in different species.[7] Furthermore, this discrepancy compromised the predictive value of animal studies concerning pharmacodynamic and pharmacokinetic properties of novel compounds in regard to humans. This concept was seemingly supported when the first drug-responsive DNA-enhancer elements in the 5′-flanking regions of different CYPs were isolated because they exhibited no obvious common feature.[8] To confound matters further, the compounds that are known to cause induction comprise a huge variety of divergent chemical structures, and it was difficult to envisage how a single receptor should be able to accommodate all of these diverse ligand structures.[1]

In 1998, two members of the gene superfamily of nuclear receptors,[9] namely the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), were identified in rodents and humans as key transcription factors in hepatic drug induction.[10–13] More recently, orthologs of these receptors have also been isolated in the chicken, pig, dog, monkey and fish, suggesting a mechanism that is evolutionarily conserved in all of these species.[14–18] These receptors along with a number of other members of the nuclear receptor family bind to DNA as heterodimers with the retinoid X receptor (RXR). Moreover, they bind to dimeric repeats of core hexamers in different arrangements such as found in the drug-inducible DNA-enhancer elements of CYPs.[19] Whereas PXR is predominantly activated by dexamethasone/rifampicin-type inducers and affects preferentially CYP3A transcription, CAR activity is more influenced by the phenobarbital-type class of compounds and increases transcription of CYP2B and CYP2C genes.[20–22] However, recent studies revealed a considerable overlap of these two receptors both in terms of their activator spectrum as well as in their affinity to DNA-response elements.[23] The importance of PXR and CAR in mediating hepatic drug induction was further underlined by the generation of the respective knockout mouse lines that are characterized by severely impaired CYP induction.[24,25] Whereas PXR is located in the nucleus and is directly activated by binding of a number of compounds to its ligand-binding domain, the activation mechanism of CAR is less clear. Apparently, CAR is a constitutively active transcription factor even in the absence of ligand, and thus regulation of CAR activity has to be achieved in a different manner. In unchallenged hepatocytes, CAR resides in the cytoplasm until drugs trigger a cytoplasmic-nuclear translocation of this receptor.[26,27] Then, phosphorylation events in the cytoplasm as well as the nucleus modulate CAR activity both positively and negatively.[28] Finally, androstanes have been found to act as inverse agonists on mouse CAR. Androstane repression of CAR can be relieved by inducer compounds, such as the potent mouse Cyp2b activator TCPOBOP.[29] Again, it is not know if this reversal of inhibition is a direct or an indirect effect of the inducers.

The mechanism by which CAR is activated is reminiscent of the basic helix-loop-helix aryl hydrocarbon receptor, which likewise resides in the cytoplasm until exposure to aromatic hydrocarbons results in a translocation into the nucleus and subsequent transcriptional activation of CYP1A genes.[30] The peroxisome proliferator-activated receptor (PPAR) is another receptor that can be activated by xenobiotics and increases transcription of hepatic CYP4A genes. PPAR is also involved in peroxisomal fatty acid hydroxylations and thus in basic energy metabolism.[31] A summary scheme of the activation pathway mediated by these four xenobiotic-sensing receptors is depicted in Figure 1A. A large variety of drugs used for the treatment of different clinical conditions have been found to affect these receptors, and some examples are listed in Figure 1B.[25,32–45] Obviously, this list represents just the tip of the iceberg and will be expanded in the coming years. A more comprehensive listing of the

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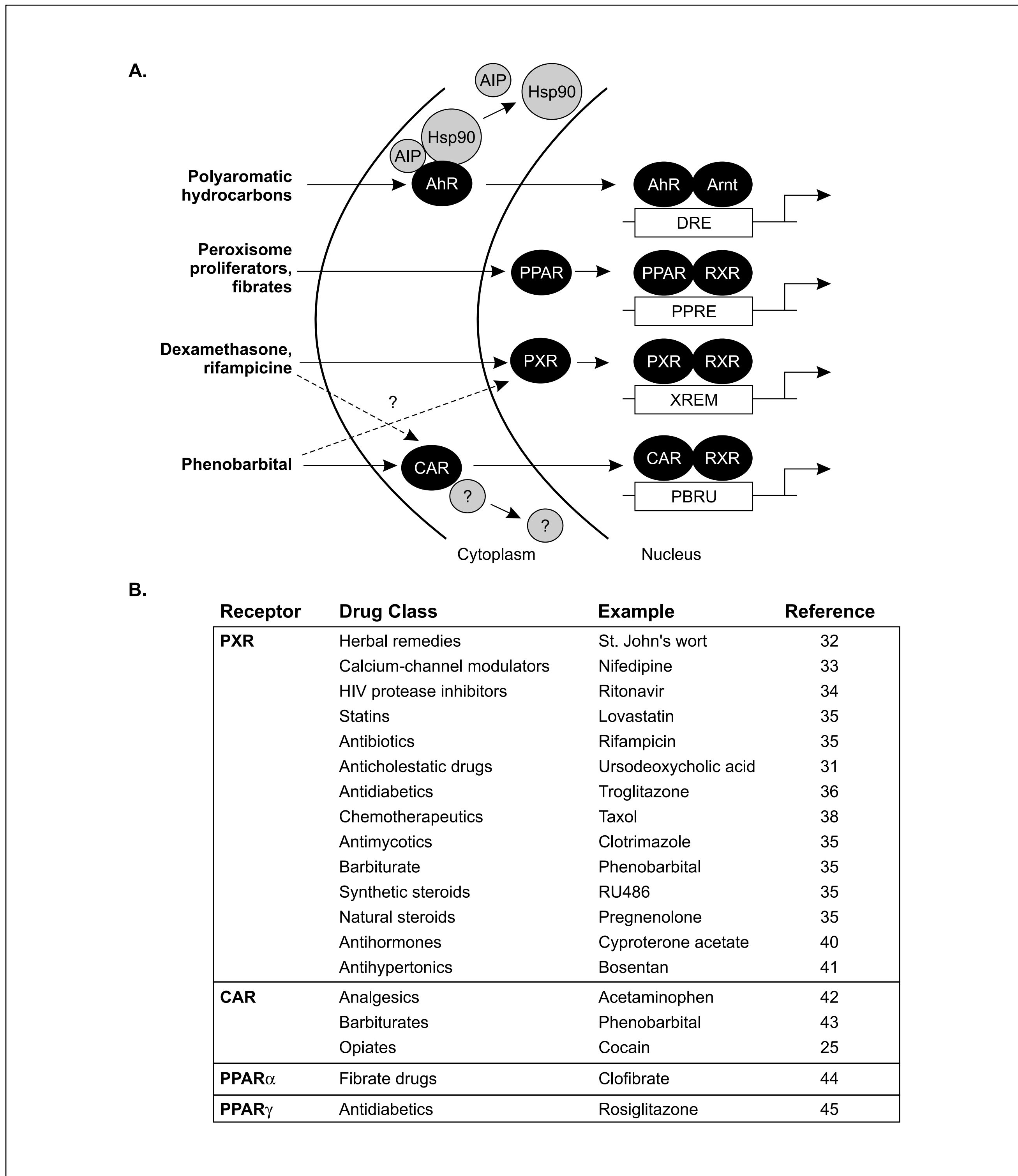

Receptor	Drug Class	Example	Reference
PXR	Herbal remedies	St. John's wort	32
	Calcium-channel modulators	Nifedipine	33
	HIV protease inhibitors	Ritonavir	34
	Statins	Lovastatin	35
	Antibiotics	Rifampicin	35
	Anticholestatic drugs	Ursodeoxycholic acid	31
	Antidiabetics	Troglitazone	36
	Chemotherapeutics	Taxol	38
	Antimycotics	Clotrimazole	35
	Barbiturate	Phenobarbital	35
	Synthetic steroids	RU486	35
	Natural steroids	Pregnenolone	35
	Antihormones	Cyproterone acetate	40
	Antihypertonics	Bosentan	41
CAR	Analgesics	Acetaminophen	42
	Barbiturates	Phenobarbital	43
	Opiates	Cocain	25
PPARα	Fibrate drugs	Clofibrate	44
PPARγ	Antidiabetics	Rosiglitazone	45

Fig. 1. Transcription factors involved in the induction of cytochromes P450 (CYPs). **A.** Induction of CYPs by the aromatic hydrocarbon receptor (AhR), the peroxisome proliferator-activated receptor (PPAR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR). Prototypical inducer-compounds activate the respective receptor and, in the case of AhR and CAR, lead to a release of the receptor from heat shock protein 90 (hsp90) and the immunophilin chaperone AIP or from so far unknown proteins, respectively. Subsequently, these receptors translocate into the cell nucleus. The AhR dimerizes to the AhR-nuclear translocator (Arnt), then binds to dioxin response elements (DRE) and increases transcription of target genes. The nuclear receptors PPAR, PXR and CAR heterodimerize with the retinoid X receptor (RXR) and activate PPAR-responsive elements (PPRE), xenobiotic-responsive enhancer modules (XREM) and phenobarbital-responsive enhancer units (PBRU), respectively. In the case of PXR and CAR, considerable overlap in both ligand spectrum and binding to each others response elements are suspected. **B.** Activation of xenobiotic-sensing nuclear receptors by different types of drugs. Different compounds are listed that have been shown to interact with either PXR, CAR or PPAR. See text and references for details.

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most important compounds involved in evoking drug–drug interactions is provided in the cytochrome P450 drug interaction table (http://medicine.iupui.edu/flockhart/). The modulation of PXR and CAR by such a variety of different compound classes underlines the importance of these xenosensors in drug induction, drug–drug interactions and adverse drug reactions.

As mentioned above, prediction of the human induction response to candidate therapeutic compounds has been precarious because of species-specific effects and has limited the transferability of animal studies in regard to humans. Today, the discovery of the xenobiotic-sensing nuclear receptors PXR and CAR has provided us with novel approaches on how to predict drug induction and the associated drug–drug interactions and adverse drug reactions. Currently, *in vitro* assays can highlight substances with an associated risk of unwanted effects at a very early phase of drug development. The possibility to either eliminate such compounds or design derivatives that preserve therapeutic action but lack induction potential helps to decrease the time between lead compound identification and its use as a therapeutic agent in patients.[46] In this review, established and novel *in vitro*, *in vivo* and *in silico* approaches are compared, and their advantages and disadvantages are discussed.

In vitro assays

The discovery of xenobiotic-sensing nuclear receptors to play a key role in the regulation of hepatic drug induction has allowed the development of a range of *in vitro* assays in order to screen promising compounds for their potential to interact with these receptors. In the case of PXR, the correlation between ligand binding, receptor activation and target gene induction has been well established.[47–50] Thus, high-throughput screening of large compound libraries using different *in vitro* approaches helps to identify PXR-activating compounds at a very early stage. This can be achieved by ligand-binding assays using purified PXR ligand-binding domain and measuring displacement of a radiolabeled ligand such as tritiated SR12813, a very potent ligand of human PXR, as shown in Figure 2A.[37,39] Alternatively, fluorescent and bioluminescent resonance energy transfer assays or co-activator-dependent receptor-ligand assays measure the ligand-mediated association or dissociation of receptor proteins with co-factors, as depicted in Figure 2B and Figure 2C, respectively.[37,51–54] In order to look at biological activation, expression plasmids for PXR and a reporter gene plasmid under the control

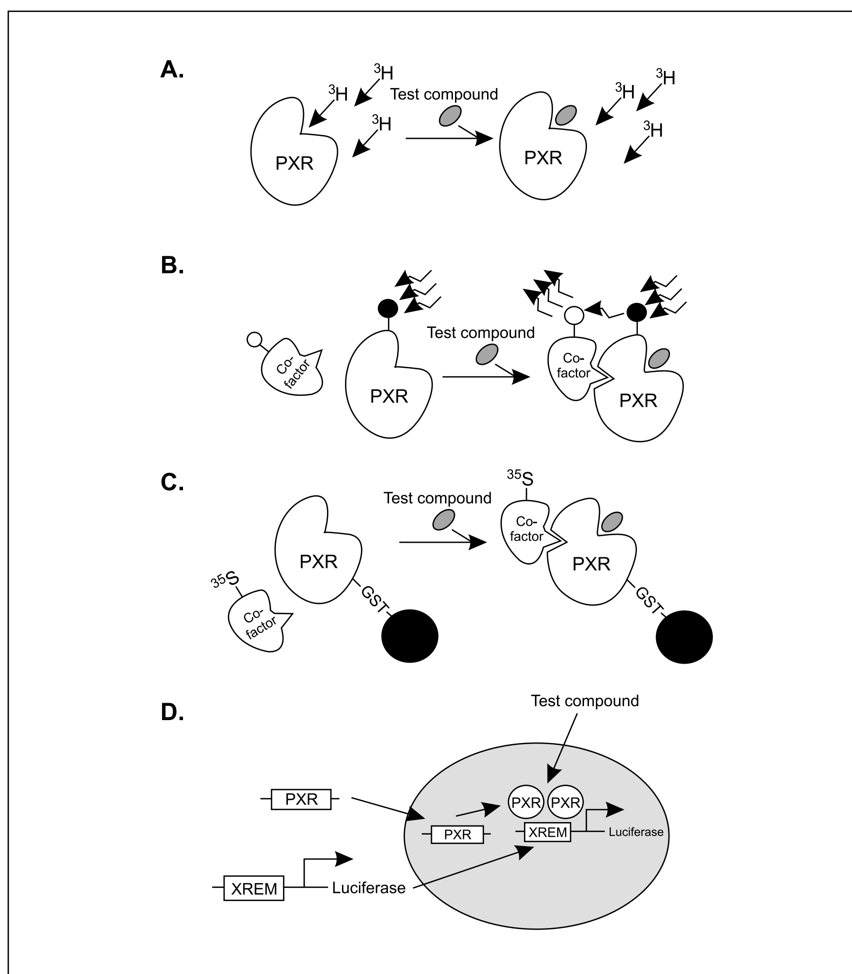

Fig. 2. *In vitro* assays to predict induction potential of different compounds. **A.** Radioligand displacement assay. An *in vitro*-expressed receptor protein is incubated with a radiolabeled ligand, and this ligand–receptor interaction is subsequently challenged by test compounds. After separation of the free and receptor-bound radiolabeled ligand, the amount of radioactivity is a measure of the binding affinity of the test compound to the receptor in comparison with the radiolabeled ligand. **B.** Fluorescent-resonance energy transfer. An *in vitro*-expressed receptor protein in combination with a known co-factor of this receptor is coupled to fluorescent dyes. After the addition of the ligand, interaction of the receptor with the co-factor allows energy transfer from one fluorescent dye to the other, and the strength of the emission of the second dye is correlated with the ability of the ligand to induce receptor–co-factor interactions. **C.** Co-activator–dependent receptor-ligand assays. *In vitro*-expressed receptor-glutathione S-transferase (GST) fusion protein is coupled to a sepharose column via glutathione. Addition of the ligand increases interaction of the receptor with a co-factor that has been radiolabeled by incorporation of ^{35}S-methionine. After washing the column, proteins are eluted, and the radioactivity recovered is dependent on the affinity of the ligand to the receptor. **D.** Transactivation assays. Suitable cells are transfected with expression plasmids for a receptor and a reporter gene plasmid under the control of a receptor-dependent enhancer element. When challenged with activators, increased reporter gene levels in the cells can be measured.

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of a PXR-responsive element are cotransfected into a suitable cell line such as the human colon carcinoma cells LS174T or the monkey kidney epithelial CV-1 cells that endogenously express a considerable amount of RXR, the PXR heterodimerization partner. Then, as shown in Figure 2D, the level of reporter gene expression after treatment with different compounds is determined.[46] A variant of this assay is the expression of the PXR-ligand binding domain fused to the yeast GAL4-DNA binding domain and a reporter gene plasmid under the control of the GAL4 upstream activation sequence.[18] The latter is the method of choice if no or only a weak response element for a certain receptor is known. Nowadays, a combination of these approaches, ligand-binding assays as well as PXR activation assays, are widely used in early stages of drug development and elimination of unwanted compounds.[55–59]

The mechanism underlying activation of CAR upon drug treatment is less amenable to *in vitro* studies than PXR. This is due to the drug-induced cytoplasmic-nuclear translocation, the constitutive activity and the different modes of receptor modulation in the cytoplasm and the nucleus.[26,27] Furthermore, cell lines that are either transiently or stably transfected with CAR show an aberrant distribution of CAR in both the cytoplasm and the nucleus and are unlikely to reflect CAR-mediated induction *in vivo*.[60,61] One way to overcome these adversities and study the nuclear translocation of CAR triggered by several compounds including phenobarbital is to directly express CAR tagged with the green fluorescent protein in the liver of mice,[62] but obviously, this method is not adaptable to high-throughput screening of a large number of compounds. Other methods for determining the enrichment of CAR protein in the cell nucleus involve cellular fractionation with subsequent use of the nuclear fraction in Western blots, DNA-affinity columns or electromobility-shift assays.[43,61] Unfortunately, whereas potent inverse agonists for mouse CAR have been discovered,[29,] these androstanes inhibit only the mouse but not the human ortholog and therefore cannot be used for inhibition of CAR followed by subsequent derepression using candidate inducer compounds.[63] In summary, because of the peculiarities of the signaling mechanism of this xenosensor, no high-throughput *in vitro* assay for human CAR has been developed yet.

Although the current consensus recommendations state that reporter gene and ligand binding assays are only appropriate for initial screens because their predictive value still has to be established more clearly, these assays may serve well for early exclusions of potentially interacting compounds. They may be used to make predictions of the pharmacokinetics and pharmacodynamics in different species, and thus allow a more rational design of subsequent *in vivo* studies.[64,65]

Cell culture systems and liver slices

Hepatic induction of enzymes by drugs and other xenobiotics is a typical feature of differentiated hepatocytes. Therefore, immortalized cell lines do not exhibit CYP induction, or the pattern of expressed CYPs is aberrant.[66] The only cell line that has been found so far that is fully responsive to phenobarbital-type inducers comparable to primary cultures of hepatocytes is the LMH cell line.[16] These cells originate from a chicken hepatoma and thus respond in a chicken-specific way to xenobiotics, limiting their use for prediction of drug induction in humans.[15] Thus, the reliable usage of mammalian cell lines is restricted to assessing induction of CYP1As by polyaromatic hydrocarbon-type inducers, a mechanism that is well maintained even in immortalized, continuously dividing cell systems.[64]

The current cell culture system of choice is primary cultures of human hepatocytes that retain a human-specific hepatic drug induction response. In the future, the potential of primary hepatocytes for large-scale compound screening might be enhanced by improvement in cryopreservation, long-term cultures and reuse of cultures.[67] However, primary cultures have several major drawbacks including availability and logistic problems. Moreover, cultures are technically demanding and normally suffer from low cell homogeneity and stability. Furthermore, primary hepatocytes from different donors reflect the interindividual variability in drug response and might therefore lead to divergent results. Finally, primary cultures are not easily transfectable, although recent advances in the use of adenoviral vectors have considerably improved transfection efficiency.[68]

Some of the disadvantages of primary hepatocytes are overcome by using precision-cut liver slices. The hepatic tissue architecture is preserved in liver slices including cell–cell contacts. Moreover, preparation and culture of tissue slices are less demanding than with primary hepatocytes and do not require collagenase treatment of cells. Finally, liver slices can be more easily cryopreserved without altering the induction potential of different CYPs.[69–71] However, liver slices suffer from the same drawbacks as primary hepatocytes concerning availability and interindividual variability. In addition, the function of liver slices declines rapidly with culture time and allows only short exposures to drugs for a few hours. In summary, the optimal culture system for prediction of the human drug induction response has not been found so far, and right now we still depend on the availability of human liver tissue taken during surgery or from organ donors in order to prepare primary cultures or liver slices. In the future, genetically engineered cell culture systems or immortalized organ cultures might replace these cumbersome systems and eliminate the disadvantages associated with them.

Animal models and other *in vivo* techniques

In drug development, testing of compounds in different animal models

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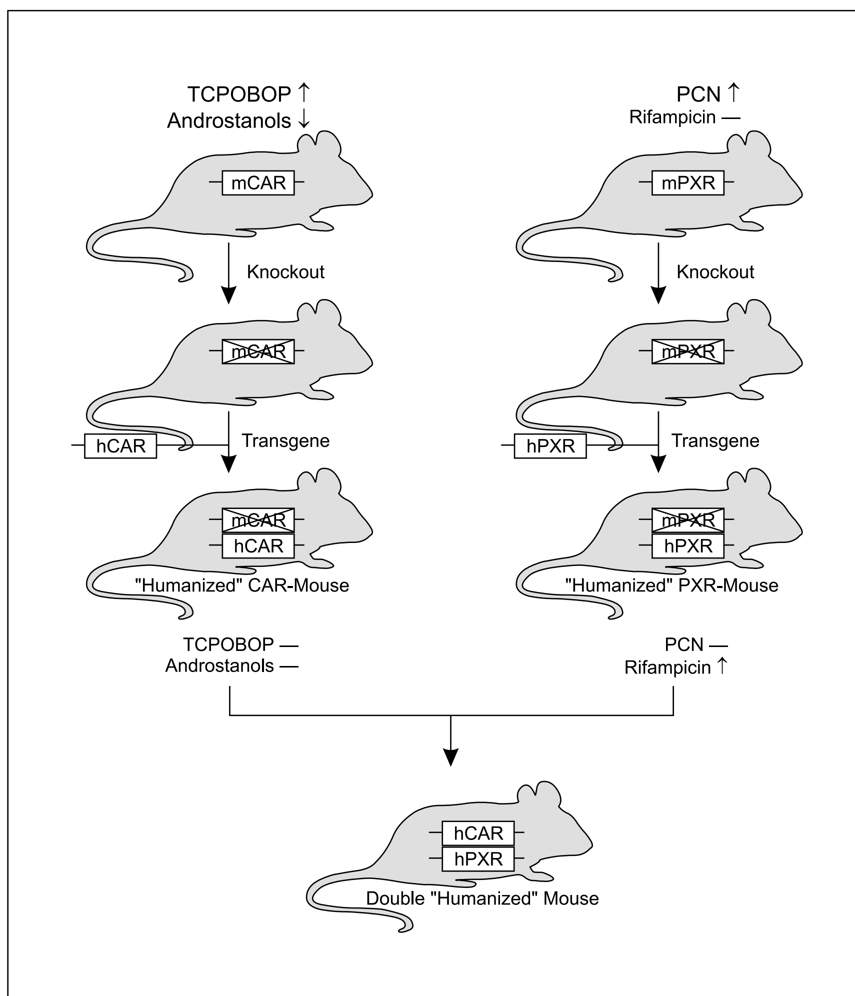

Fig. 3. Humanized mouse models. In order to generate mouse models that exhibit a human-specific response to drugs, the endogenous gene for pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are knocked out, and the mice are subsequently "rescued" by transgenic expression of the human orthologs under the control of a liver-specific promoter. These mice no longer react to rodent-specific inducers such as TCPOBOP, androstanols or pregnenolone 16 α-carbonitrile (PCN) but increase transcription of cytochromes P450 after rifampicin treatment, as observed in humans. The two humanized mouse lines can then be crossed and will result in a double humanized animal with both CAR and PXR replaced by the human orthologs.

is one of the key steps. However, only limited information regarding pharmacokinetics and pharmacodynamics of drugs can be concluded from these tests because of the species-specific drug response. Recently, the knowledge of PXR and CAR playing crucial roles in drug induction and the discovery that the divergent ligand-binding domains of the receptor orthologs account for a large part of the species differences [37,72] led to the generation of so-called "humanized" mice (Fig. 3). In these experiments, the endogenous gene encoding for mouse PXR or mouse CAR were knocked out, and the resulting mice were then "rescued" by a transgenic expression of human PXR and CAR, respectively, under the control of a liver-specific promoter. [24,42] When treated with species-specific inducer compounds, these mice exhibit a regulation of Cyp3a11 and Cyp2b10 that closely resembles the regulation of CYP3As and CYP2Bs in humans. As examples, the rodent-specific CYP3A inducer PCN is no longer active in the humanized PXR mouse, whereas these animals now strongly react to rifampicin treatment. Thus, humanized animal models might prove to be invaluable tools for testing candidate drugs and prediction of their induction potential in humans. So far, only the single humanized mice with either human PXR or human CAR have been generated, but crossing of these two lines might result in an even better model concerning drug induction typical for humans. [73] Recently improved methods of *in vivo* DNA-delivery systems combined with powerful noninvasive, extracorporal monitoring of bioluminescent reporter gene assays can further improve the cost, time and number of animals used in drug-induction studies. [74] However, it should be cautioned that these animal models only cover our present knowledge of the drug-induction signaling pathways, and further discoveries of receptors and transcription factors will have to be taken into account to improve the model.

Probe drugs are the current method of choice to assess the potential of a compound to modify CYP activity, particularly in human subjects. The following enzymatic reactions are widely used to study single CYP activities either *in vitro* or *in vivo*: phenacetin *O*-deethylation and caffeine N3-demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), 7-ethoxy-4-trifluoromethyl coumarin *O*-dealkylation and *S*-mephenytoin *N*-demethylation and bupropion hydroxylation (CYP2B6), tolbutamide 4′-hydroxylation and *S*-warfarin 7′-hydroxylation (CYP2C9), *S*-mephenytoin 4-hydroxylation (CYP2C19), bufuralol 1′-hydroxylation and dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation and midazolam 1′-hydroxylation (CYP3A4). Whereas some of these reactions are quite specific for a single CYP, others might target a battery of different enzymes and thus only vaguely reflect the respective CYP activity. [75] In the case of drug-mediated CYP induction, it would furthermore be useful to be able to measure an endoge-

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nous marker compound that closely correlates with inducible CYP levels. Since steroid hormone levels are tightly regulated, increase of hydroxylated steroid metabolites is only observed in patients after prolonged treatment with strong inducers such as rifampicin and are therefore poor sentinel compounds for induction.[76] However, the recent discovery of the CYP3A4 metabolite 4β-hydroxycholesterol that is increased in plasma after drug treatment of patients with CYP3A4 inducers is promising because of its presumed specificity for CYP3A4, its high elevation following transcriptional increase of CYP3A4 and its long apparent half-life of 52 hours in patients.[77,78] Thus, determination of 4β-hydroxycholesterol in the blood might be an easily accessible marker for drug induction.[79] Other endogenous metabolites of CYP3A4 that have recently been described include 25- or 6α-hydroxylated bile acids that are excreted via urine[80–83] and therefore also could be useful as markers, although plasma levels of these hydroxylated bile acids are relatively low in noncholestatic patients.

In silico approaches

In today's laboratory, experiments carried out without assistance by computers are nearly extinct. The planning of experiments, evaluation of experimental data and application of statistical tools to mine relevant information from large sets of data are commonplace applications of computer technology that, while not exactly being computational biology, fit under the general term of bioinformatics. Several developments in this regard are noteworthy: Especially apparent and welcome are specific integrated databases or knowledge bases that store large amounts of diverse toxicological, pharmacogenomic or biological data on systems relevant to drug induction. Some selected examples are TOXNET as a meta-database for toxicology, PharmGKB, the pharmacogenomics knowledge base, or NUREBASE, a database that collects many types of information about nuclear receptors. These databases can be found at http://toxnet.nlm.nih.gov/,[84] http://www.pharmGKB.org[85] and http://www.ens-lyon.fr/LBMC/laudet/nurebase.html,[86] respectively. They share a trend toward the integration of disparate data relevant to a common theme, collecting data such as nucleotide and protein sequence, phylogenetic data, known allelic variants and experimental results within a common framework. Moreover, there are a number of Internet resources that provide manually compiled clinical advice in regard to drug–drug interaction (see http://medicine.iupui.edu/flockhart/) or in regard to general information about cytochromes P450 (see http://drnelson.utmem.edu/matrix.html). The holy grail of *in silico* biology, however, is the creation of new knowledge and the prediction of behavior, based on an intellectual abstraction of previous experimental work. Set in the context of drug induction, specific *in silico* goals could be the prediction of a novel compound's propensity to induce drug metabolism or to estimate the subset of genes that will be part of the pleiotropic response to a given drug.

Optimally, an integrated approach to predicting drug induction would utilize a combination of high-throughput *in vitro* techniques and *in vivo* studies, but would be accompanied from beginning to end by *in silico* methods. These methods are employed to design the corresponding tests and experiments, but also to assist in understanding and explaining the obtained experimental results. Since the discovery of CAR and PXR as key molecules in drug induction, understanding the way that the multitude of structurally diverse inducer compounds activate drug metabolism has now largely become a question of understanding the structure of these receptors and their interactions with their ligands on one hand and with their DNA response elements on the other.

The interaction between xenobiotic receptors and their ligands is clearly a field that lends itself to molecular modeling and ligand docking approaches. The ligand-binding domain of human PXR has recently been crystallized in the presence or absence of a high-affinity ligand, and its structure has been published.[72] PXR was found to contain a ligand-binding domain that is about three times as large as those of other nuclear receptors, and the change of two helices to a turn and a flexible loop provides the structural prerequisites to accommodate both small and large ligands. Furthermore, the structure suggests that excessively large ligands, such as rifampicin, could displace the flexible loop and thus further increase the binding cavity volume. The oval binding cavity is hydrophobic, with a small number of polar residues spaced throughout it. This reflects well the overall characteristic of PXR ligands, which are often hydrophobic with a few polar groups that would permit hydrogen bonding to critical residues in the cavity. Indeed, a number of amino acid residues were found that are critical for the recognition of ligands and moreover are responsible for the species specificity of PXR activation profiles. Interestingly, the high-affinity ligand SR-12813 co-crystallized with PXR was found to bind in three distinct configurations to the ligand-binding pocket. Such multimodal binding may be the case for many of the smaller compounds.

At present, the crystal structure of CAR has not been solved. However, based on the crystal structure of the closely related human PXR, models for the ligand-binding domain of human CAR have been proposed.[87,88] The predicted structure for the CAR ligand-binding domain is structurally more similar to those of classical nuclear receptors than the slightly unusual structure of PXR.[89] Nevertheless, the volume of the binding pocket is expected to be only marginally smaller than that of PXR. Modeling CAR also provides us with a credible explanation of the constitutive activity of this receptor in the absence of any ligand. The C-terminal helix 12 of nuclear receptors is associated with the ligand-dependent activation function (AF2) of nuclear receptors. Its spatial position

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serves as ligand-dependent switch between basal, activated and repressed states. In the CAR ligand-binding domain, the loop connecting helix 12 to helix 11 is particularly short, limiting the ability of helix 12 to assume the inactive position and promoting docking of this helix in the AF2 groove, a position found in ligand-activated receptors. This active position is further stabilized by a charge clamp between the carboxy-terminal end of the helix and the K205 residue in the groove. Moreover, the interaction between helix and groove is deeper and more stable than with PXR, because of the presence of smaller hydrophobic residues in helix 12. Docking studies with various agonist or inverse agonist compounds of CAR isoforms predict that many of these ligands have multiple, spatially distinct ways of interacting with the ligand-binding pocket. This could indicate the possibility of a noncompetitive binding of two ligands simultaneously to the ligand-binding pocket. Interestingly, one of the models further suggests an alternative binding site for the inverse agonist androstanol, distinct from the ligand-binding pocket. Androstanol might directly interact with the AF2 groove and displace helix 12 from its location in the active conformation, thus directly inhibiting constitutive activity.

In the future, in-depth structural knowledge about the ligand-binding domains of xenobiotic receptors will hopefully lead us toward rational, computer-aided design and test of compounds that either fit or do not fit into the pocket.[90,91] These approaches can be further complemented by the creation of a pharmacophore from known PXR (or CAR) ligands and might help to select promising lead compounds that show no interaction with PXR.[92,93] Moreover, understanding the interaction between ligand and receptor may prove useful in rescuing compounds rejected after testing positive for drug induction in an *in vitro* screening. Careful analysis of the compound's structure in relation to the ligand-binding pocket may suggest minor chemical modifications to the molecule that, while preserving therapeutic effect, likely abolish or diminish induction potential. A more integrated understanding of inducer compounds may also emerge from the recent report of the first crystallized mammalian CYP. Since inducers are often CYP substrates, and CYP substrates must be suspected to be inducers, the combination of *in silico* screening for receptor ligands and CYP substrates may prove to be an interesting insight in the co-evolution of these ligand–protein interactions.[94] However, one has to keep in mind that for most of the typical CAR activators no direct interaction with the CAR ligand binding domain was observed. Thus, in order to predict compounds that trigger cytoplasmic–nuclear translocation of CAR in the liver, alternative methods to crystallography may be required.

The combination of protein crystallography and *in silico* structural biology has enabled us to start apprehending the interactions between xenobiotic receptors and their ligands. Comprehensive studies, such as the correlation of structure and activity of a compound and several chemical derivatives thereof, or the interaction of one compound with engineered receptor mutants, may provide an even deeper understanding of the functional interactions between ligand and receptor in the future. Such information will, in turn, feed back into improved predictive capability of *in silico* methods. The interaction between receptors and their ligands is only half of the story, though. Equally important in determining the effect of inducers on gene transcription is the contact formed between the receptor and its cognate DNA response elements. This interaction, too, is amenable to *in silico* studies aiming to predict the target genes of a specific receptor. In the postgenomic era, where sequence information about genes and their regulatory regions is abundant, large-scale gene scanning for nuclear receptor binding sites in promoter or enhancer regions becomes an option. Moreover, expression array techniques contribute by providing a comprehensive view of all genes affected positively or negatively by an exogenous challenge, such as inducer drugs.[95] Indeed, the response to inducers is quite pleiotropic in nature, with up to 200 genes affected by the prototypical inducer phenobarbital.[96,97] This pleiotropy of response has to be taken into account when dealing with these substances. Besides CYPs, many other genes have been identified to be directly influenced by PXR and CAR.[46,49] Among others, PXR and CAR induce or repress genes encoding for phase II xenobiotic-metabolizing enzymes and drug transporters but also genes involved in heme, steroid, cholesterol and bile acid homeostasis as well as genes important in energy metabolism.[98,99]

Recently, an algorithm called NUBIScan (accessible at http://www.nubiscan.unibas.ch) was developed in this laboratory that provides assistance in the identification of likely nuclear receptor binding sites in large sequences, including those activated by the xenobiotic-sensing nuclear receptors PXR and CAR.[100] In general, nuclear receptor binding sites have a common structure, consisting of a repeat of two loosely conserved hexameric half-sites, separated by a variable number of spacer nucleotides. The orientation of the two half-sites toward each other is variable, and it is the combination of inter-half-site orientation and spacer length rather than the sequence of the half-sites themselves that to a large extent determines the specificity of a given binding site for a particular receptor dimer. Moreover, conspicuous promiscuity is found between receptors and response elements: A particular receptor dimer may recognize more than one repeat configuration, and a particular repeat configuration may serve as response element to several receptors. Both of these features are recognized as important factors in receptor crosstalk. At the same time, the diversity of valid nuclear receptor response elements presents problems when trying to create an *in silico* model of such an element in order to search genomic

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sequences. NUBIScan seeks to overcome these difficulties by focusing on the hexameric half-sites, constant features of nuclear receptor response elements. Half-sites that are in suitable arrangement toward each other are sought for, and each of the half-sites is recognized using weighted nucleotide distribution matrices. This flexible approach can be adapted to any possible type of repeat orientation and spacer length, without having to compile separate matrices for every binding configuration of a particular nuclear receptor. Moreover, focusing on the combination of two core components of these response elements enhances the sensitivity of this approach, bringing well-founded, useable analysis of large sequences into the realm of the possible. DNA sequence analysis with NUBIScan may provide insight into major questions in drug induction: 1) Highlighting the precise location of nuclear receptor–DNA interaction in a drug-responsive enhancer sequence. To understand the functionality of a complex drug-responsive enhancer element (which may be up to a few kilobases in length), it is vital to know where in the sequence xenobiotic-sensing receptors can bind, and *in silico* analysis followed by experimental verification is more efficient than classical methods; 2) Assessing the effect of interindividual variations in noncoding parts of the genome. Polymorphisms in the regulatory regions of target genes of xenobiotic receptors may cause differences in the ability of individuals to respond to drugs.[101–104] Differential NUBIScan analysis, using allelic variants of a particular target gene, may predict such regulatory polymorphisms; 3) Application of genome-wide NUBIScan analysis to predict target genes of particular receptors. Such an endeavor, however, must be combined with a previous selection of a subset of the genome, that is, focusing on stretches of DNA immediately around a gene's coding region. Otherwise, the vast amount of noncoding DNA swamps the algorithm with too much unspecific data, precluding the production of useful predictions; and 4) Lastly, judicious NUBIScan analysis combined with expression array data from control and drug-treated samples is a promising two-pronged approach. Analyzing the regulatory regions of genes induced by drugs can immediately reveal candidate nuclear receptor response elements and also stipulate a distinction between genes directly affected and those likely to be regulated by downstream mechanisms.

Conclusions

Although major breakthroughs in elucidating the molecular signaling pathways underlying hepatic drug induction of CYPs have been made in recent years, we are still far from understanding the details of its mechanism. Nevertheless, the discovery of the xenosensors PXR and CAR lifted the veil on many mysteries that haunted the field for years. Recent findings can explain the divergence in drug-responsive DNA-enhancer elements in diverse CYPs and species as well as the species-differences in the CYP-induction after drug treatment. Moreover, the structure of the PXR ligand-binding domain can account for the promiscuity of that receptor and for the structural variety of inducer compounds. Thus, several *in vitro*, *in vivo* and *in silico* assays have been developed on the basis of PXR and CAR that allow rapid screening of a large number of compounds. These assays allow early elimination of inducer compounds in the drug-discovery process and a rational choice of species in preclinical toxicology tests as well as better interpretation of the results. Nevertheless, the optimal, robust, easy-to-handle, high-throughput assay system that truly reflects drug induction in humans has not been found yet.

Moreover, few of the currently used assays also take into account interindividual variability of drug-response. So far, this variability has been largely associated with polymorphisms in the CYP genes,[105] but recent data suggest that polymorphisms might also occur in xenobiotic-sensing nuclear receptors and in drug-responsive enhancer elements.[101–104] In the future, it might be possible to genotype patients for polymorphisms in CYPs, nuclear receptors and enhancer elements and then provide a treatment that has less potential for drug–drug interactions or adverse drug reactions because of an individualized dosage and appropriate choice of medication deduced from the genotype of the particular patient. Nevertheless, genotyping should always be accompanied by "phenotyping" because induction, repression or inhibition of CYPs and other enzymes can alter the pharmacokinetics and pharmacodynamics of drugs dramatically.[106] Similarly, genome-wide analysis of target genes of the signaling pathways activated by different inducer drugs may support the effort of identifying more suitable markers of induction of different CYPs that can easily be measured in blood samples of patients.

In summary, our understanding of hepatic drug induction in humans today has an enormous impact on the drug-discovery process to an extent that safer drugs can be designed more rationally and unsafe compounds can be eliminated at a very early stage. Today, compounds can be developed that have a high potency on their target but a low impact on the drug-induction mechanism and that subsequently can be given in moderate daily doses.[107] These features contribute to the reduction of unwanted effects associated with certain drugs. Doubtless however, the next years will see a wealth of new information and findings that brings us closer to the goal of rapid and inexpensive development of safe drugs.

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ACCELERATED DEVELOPMENT OF VIRAL INHIBITOR THIOVIR FOR HIV

Adventrx Pharmaceuticals, Inc. announced September 23, 2003, that it has accelerated internal development efforts for *Thiovir*™, a nonnucleotide reverse transcriptase inhibitor (NNRTI) for the treatment of HIV/AIDS. *Thiovir* is an oral prodrug of foscarnet, which is an FDA-approved intravenous treatment for HIV and HIV-related opportunistic infections caused by cytomegalovirus (CMV) and herpes simplex virus (HSV). *Thiovir* has demonstrated *in vitro* efficacy against HIV, CMV and HSV-8, as well as topical efficacy in an animal model of HPV (human papillomavirus). It may also have broad efficacy against opportunistic infections associated with HIV/AIDS. *Thiovir* is protected by five issued patents, which Adventrx has licensed from the University of Southern California on an exclusive worldwide basis.

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