PCEUT 527
Enzyme Induction: Biochemical Mechanisms
2/13/17

1. General Principles
2. Increase in enzyme content through transcription
3. Protein degradation
4. Protein stabilization

Why Does Induction Occur?

- An adaptive response to exposure to xenobiotics from the environment or endogenous compounds (e.g. hormones)

- Slow regulatory process (compared to enzyme inhibition which is rapid)
  - Genes involved in inductive response appeared early in the evolutionary scale

Consequences of Induction

- Change in pharmacological effect because of increased drug metabolism
  - Decreased pharmacological effect when activity associated with parent (unchanged drug)
  - Increased pharmacological effect when activity associated with metabolite (increased conversion of prodrug to active metabolite)
- Balance between “toxication” and “detoxification”
  - Decrease in toxicity due to accelerated detoxification
  - Increase in toxicity due to formation of reactive metabolites

Consequences of Induction

- Clinical or toxicological significance depends on:
  - Magnitude of change in the concentration of the active species (parent, active or toxic metabolites)
    - at the site of pharmacological action, and
    - the therapeutic/safety index of the drug/xenobiotic

Oral Contraceptives + St. John's Wort = Miracle babies!

Induction – General Principles

Definition:

- An increase in steady-state concentration of enzyme following exposure to an appropriate stimulus.

Kinetic Considerations:

- For a first-order metabolic process that follows simple Michaelis-Menten kinetics, intrinsic clearance defined as

$$Cl_{\text{int}} = \frac{V_{\max}}{K_m} = \frac{E_t \bullet k_{cat}}{K_m}$$

- Induction accelerates metabolism through an increase in V_{max}

Induction – General Principles

- Enzyme induction can occur by a change in rate of enzyme synthesis or rate of enzyme degradation

$$E_{ss} = \frac{R_o}{k_{\text{degr}}}$$

- Synthesis – usually considered zero-order process (constant rate)
- Degradation – first-order process (rate is proportional to the amount of protein)

Special Considerations

- Inducers can often affect more than one enzyme
  - Interactions with multiple cell signaling receptors and/or receptor binding to multiple gene targets (e.g., phenobarbital and CAR/PXR and *CYP3A4/CYP2C9/ CYP2B6)*

- A drug can induce Phase I, Phase II and Phase III (transporters) simultaneously (e.g., rifampin and CYPs/UGT/ P-gp)
  - Both parent and metabolite clearance and excretory routes can be affected
  - Interpretation of parent and metabolite AUCs can be challenging.

Other Considerations

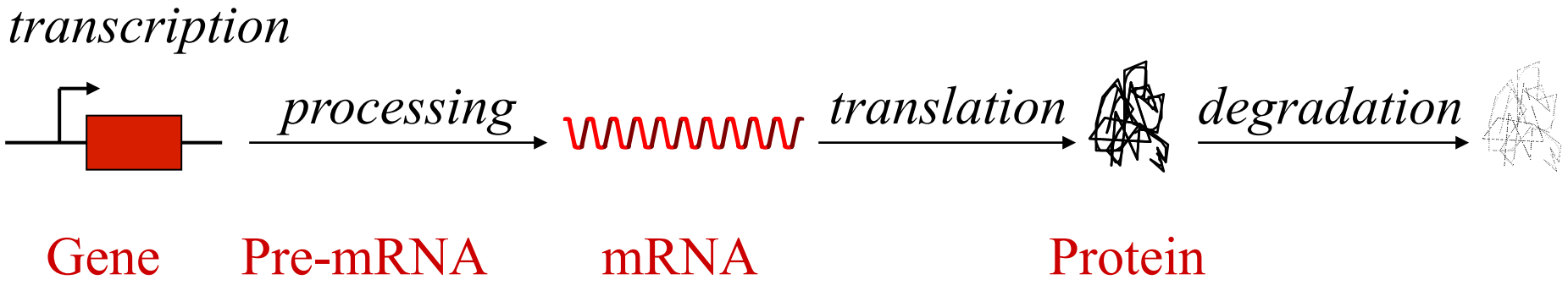
- Some drugs induce their own metabolism (“autoinduction” e.g., carbamazepine), but others act on non-self clearance enzymes

- Induction can occur in multiple tissues, but often associated with tissue-specific receptor or coactivator/repressor expression (ex. PXR-*CYP3A4*)
  - contrast clearance *vs.* toxicological importance

Possible steps in Induction

- Multiple steps which can be altered in the presence of an inducer

$$\text{Amt Enzyme}_{ss}(\text{mol}) = \frac{\text{Synthesis Rate}(\text{mol}/\text{hr})}{k_{deg}(\text{hr}^{-1})}$$



Receptor-Mediated DME Induction

- Constitutive, induced and repressed expression of drug metabolizing enzymes and transporters is largely under transcriptional control
- Most common and important mechanism of P450 induction involves nuclear receptor activation; other examples include
  - UDP glycuronosyltransferases (UGT)
  - Sulfotransferases (SULT)
  - Glutathione S-transferases (GST)
  - Multidrug resistance protein 1 (MDR1)
  - Multidrug resistance-associated proteins (MRP)
  - Organic anion-transporting polypeptides (OATP)

General: Nuclear Receptor (NR) Family

- Superfamily of intracellular transcription factors – 47 known human forms
- Structural similarity
  - Zinc finger DNA binding domain
  - Hinge region
  - Ligand binding domain
  - C-terminal activation function (AF-2)
- Known function and "orphan receptors"
- Affinity for endogenous ligand(s) varies widely: dissociation constants in nM (VDR) and μM (PPAR, LXR) range
- Often function as heterodimers (e.g., PXR/RXR)

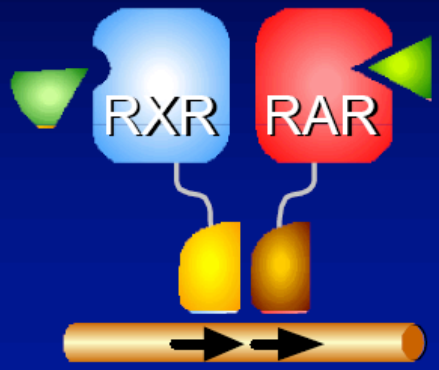
NRs Relevant to DMEs

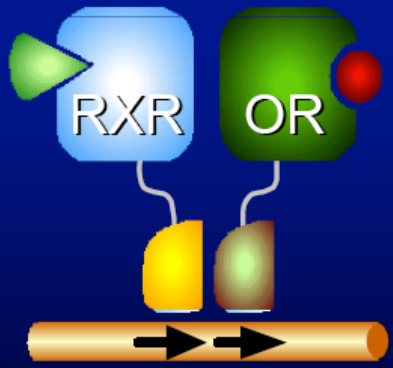
PXR (SXR), pregnane X receptor
CAR, constitutive androstane receptor
GR, glucocorticoid receptor
VDR, vitamin D receptor
RXR, retinoid X receptor (9-cis retinoic acid)
PPAR, peroxisome proliferator-activated receptor (fatty acids, eicosanoids)
RAR, retinoic acid receptor
ER, estrogen receptor
AR, androgen receptor
FXR, farnesoid X receptor (bile acids)
LXR, liver X receptor (cholesterol metabolites)
BAR, bile acid receptor
TR, thyroid hormone receptor

[some receptors have subtypes (α,β,γ,δ)]

Ref: Nagy and Schwabe Trends in Biochem Sci, 2005

RXR is a key partner for many pathways





Known ligands

RAR α,β,γ	all-*trans* RA
TR α,β	thyroid hormone
VDR	1,25-$(OH)_2$-VD3
PPAR α,β/δ,γ	fatty acids, eicosanoids
LXR α,β	oxysterols
FXR α,β	bile acids
BXR α,β	benzoates
EcR	ecdysteroids

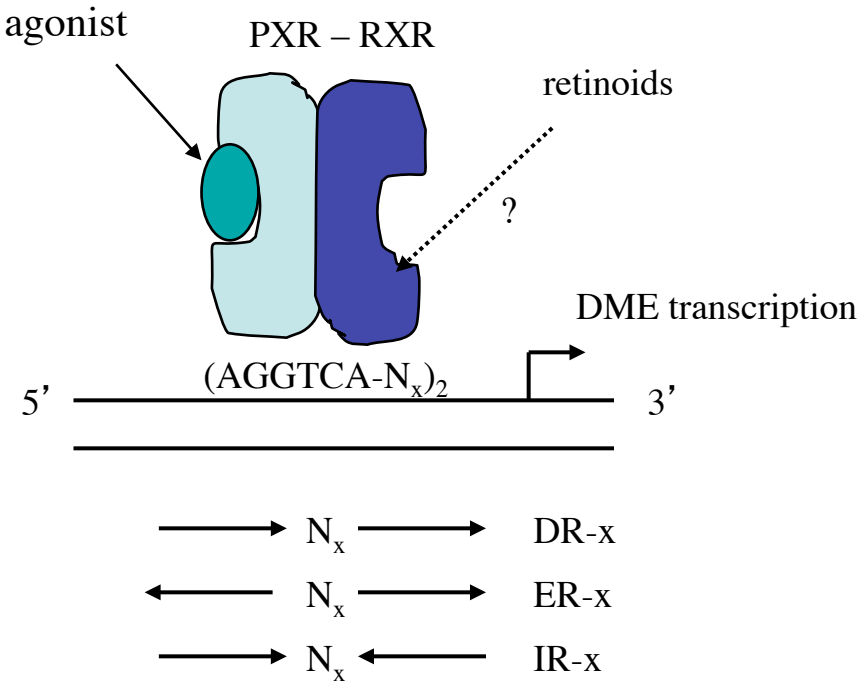
Activatable Orphans

SXR/PXR	steroids, xenobiotics
CAR	xenobiotics, androstanes

Inappropriate RXR activation may be expected to cause
wide ranging disturbances in the body's homeostatic hormonal controls

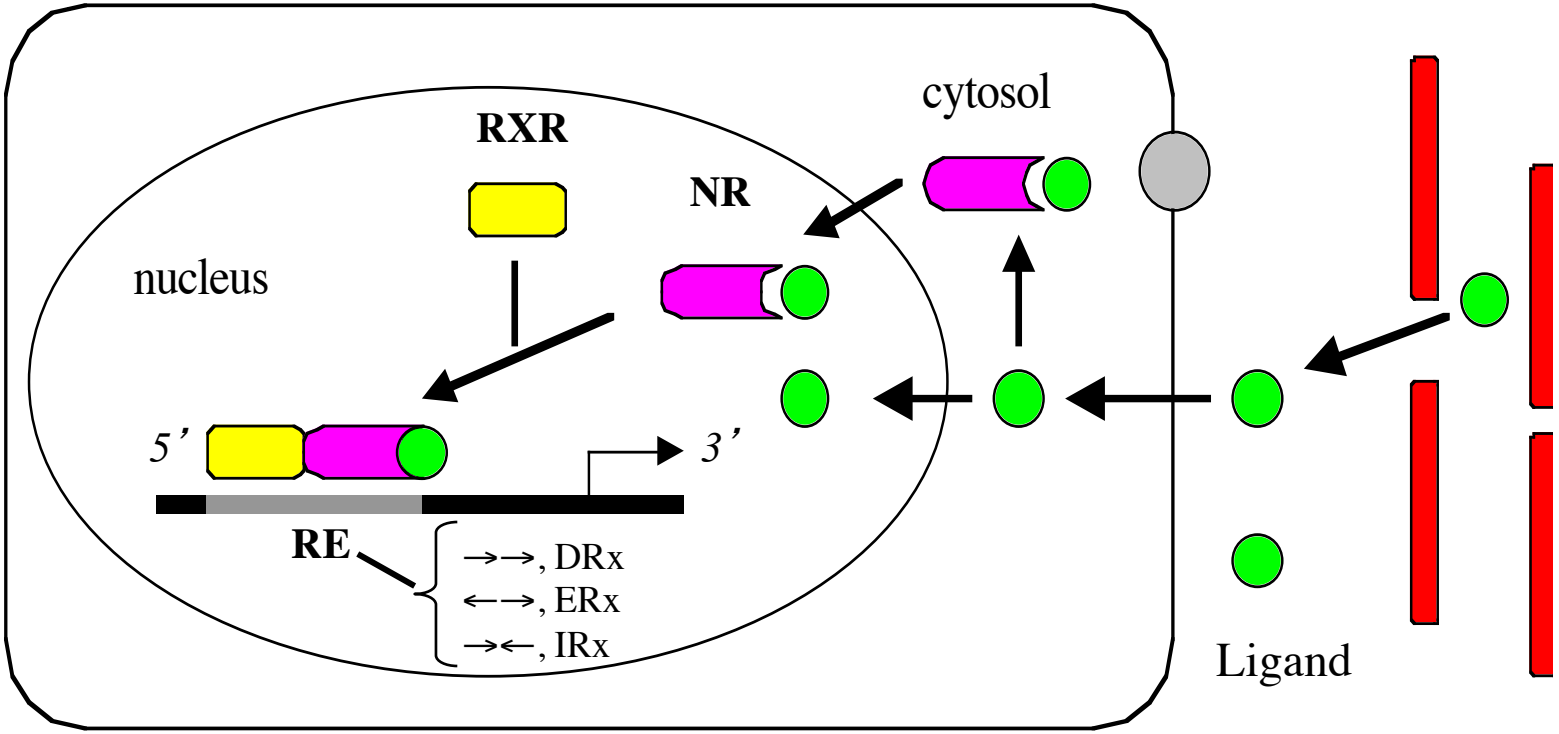
Transcriptional Activation – General Sequence of Events

- Nuclear receptor associated with corepressors
- Inducer binds and NR dissociates
- Translocation to nucleus (not always)
- Association of with dimerization partner
- Binding of heterodimer to response elements (hexameric repeats with spacer) of the target genes
- Release of corepressor proteins
- Recruitment of coactivators and general transcription machinery



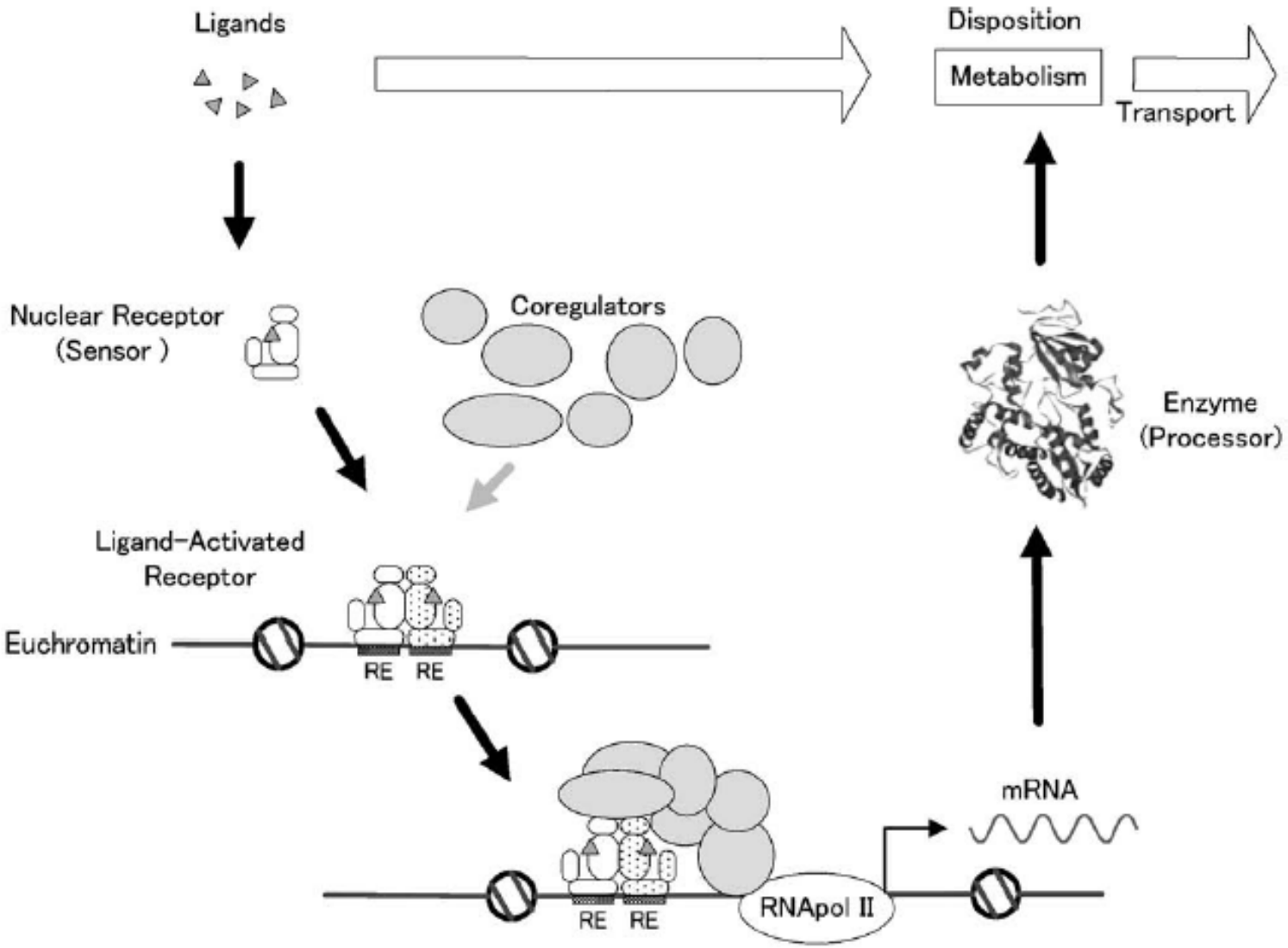
(Note, the hexameric sequences may be imperfect repeats of the canonical binding sequence)

Localization of Nuclear Receptor



- PXR localized primarily in the nucleus in the absence of ligand
- AhR translocates to the nucleus after ligand binding
- CAR translocates after a ligand initiated event (phosphatase activity?) that generally does not involve direct ligand binding to the receptor; consitutively active by endogenous signaling

Transcriptional Activation – Further Details

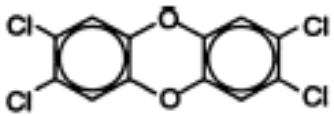


Summary of Nuclear Receptors Regulating P450s Following Exposure to Xenobiotics

CYP Gene Target	Receptor	Inducer
CYP1A1/1A2/1B1	AhR-ARNT	Antiestrogens, PAH
CYP2B6, CYP2C9, CYP2C8	CAR-RXRα	Androstanes, bile acids, phenobarbital
CYP3A4	PXR-RXRα	Pregnanes, bile acids, phenytoin, rifampin
CYP4A	PPARα-RXRα	Fibrates, glitazones

AhR

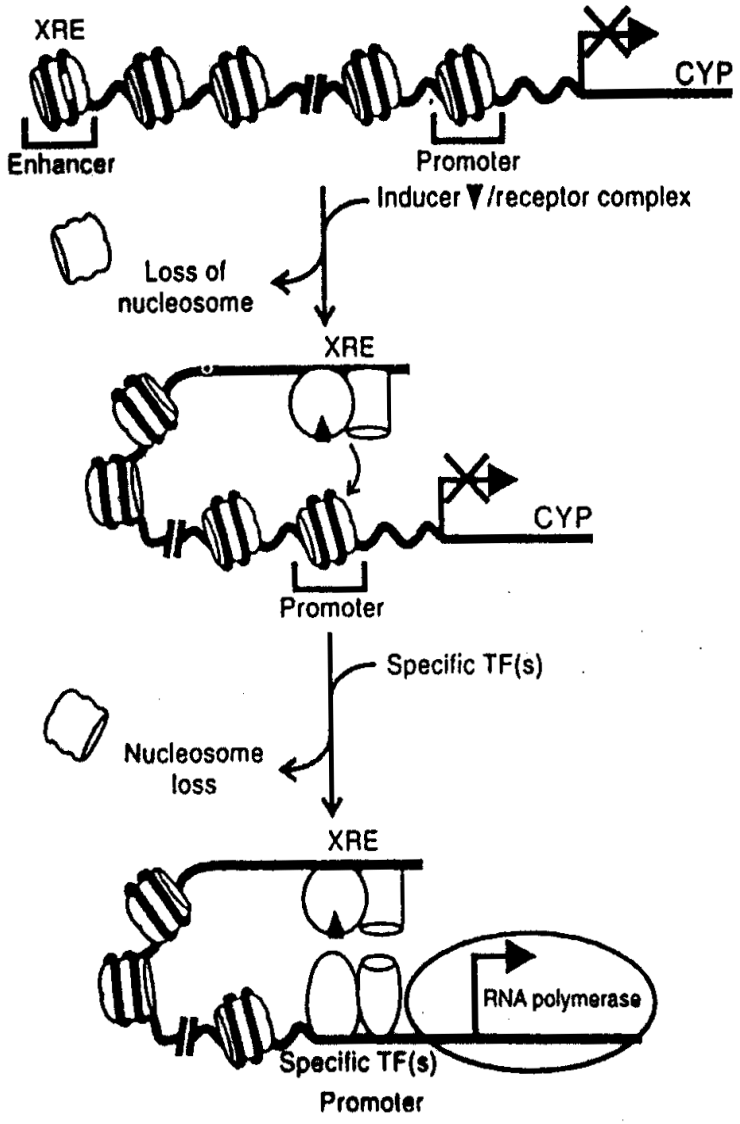
3MC



TCDD

- AhR: Aryl hydrocarbon receptor
  - Response element: XRE
  - CYP1A1, 1A2, 1B1
  - UGT1A1, 1A6
- Activators: planar lipophilic molecules, polycyclic aromatic or halogenated hydrocarbons (PAH, TCDD), β-naphthoflavone, antiestrogens
- Deactivators: 3,4-dimethoxyflavone
- Constitutively repressed at the nucleosome level and single gene level

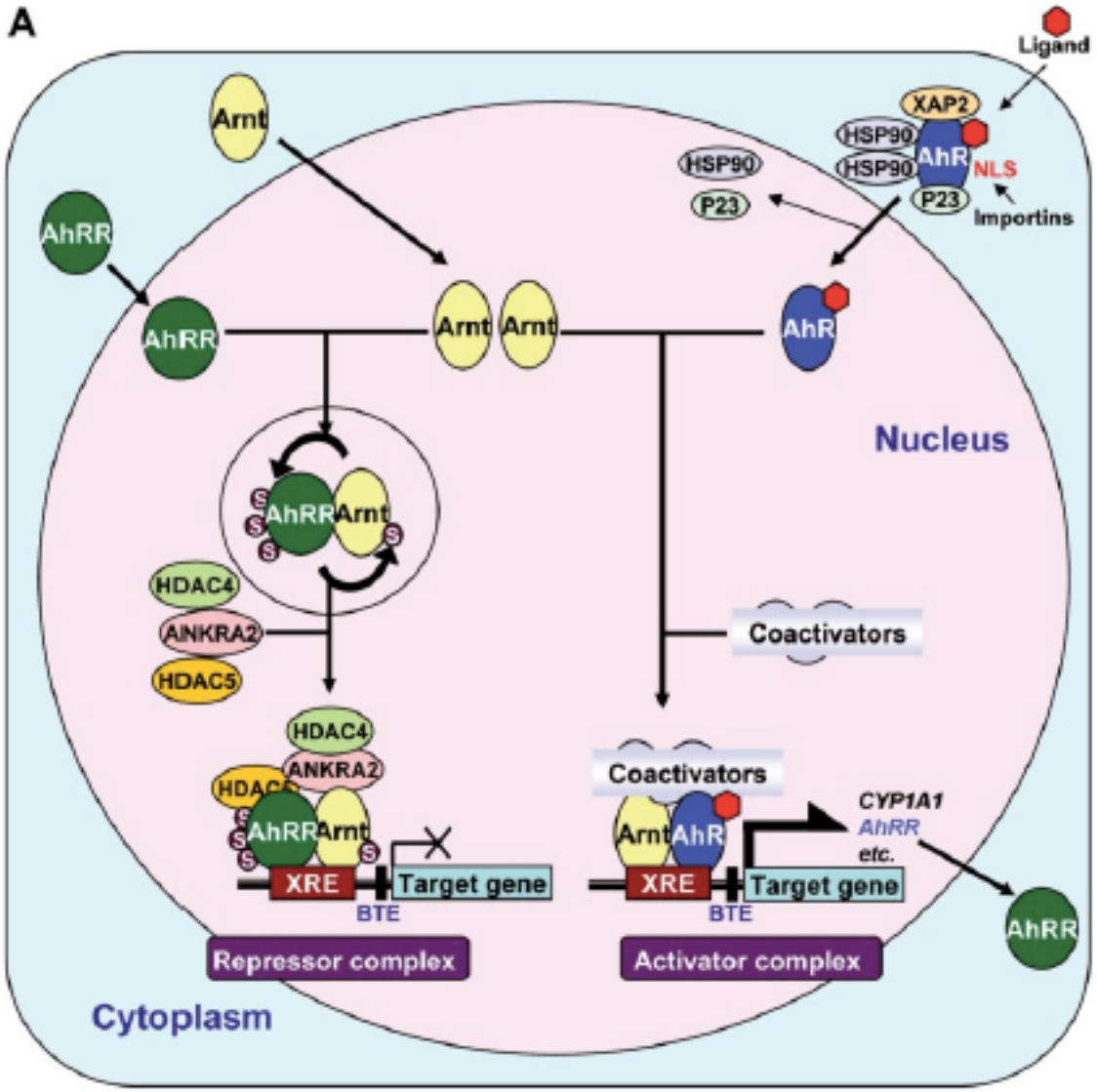
Transcriptional Activation: Promoter/Enhancer Effects



- Binding of receptor heterodimer disrupts chromatin structure, permitting binding interactions between promoter and enhancer regions (also requires binding of additional transcription factors, *e.g.,* Sp1)
- The new 3-D structure facilitates the binding of the polymerase II complex and initiation of transcription

Ref: Clin Exper Pharmacol Physiol, 1998

More Detailed Mechanism of Induction by AhR



Ligand binding to AhR triggers translocation to the nucleus and release of HSP90. Unmodified Arnt forms a heterodimer with AhR and recruits coactivators, such as CBP/p300, to form the transcriptional activator complex.

Arnt also forms a heterodimer with a repressor (AhRR), which significantly enhances the SUMOylation of both proteins. SUMOylated AhRR recruits corepressors ANKRA2, HDAC4, and HDAC5 to form the transcriptional repressor complex.

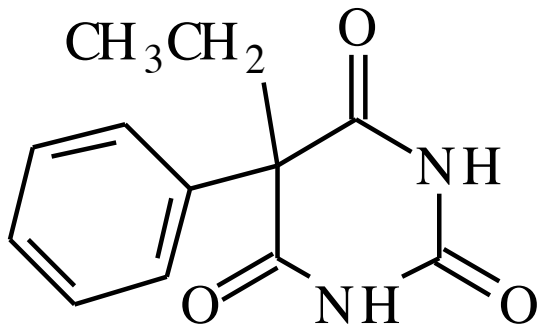
From: Fujii-Kuriyama and Kawajiri Proc Jpn Acad Ser B Phys Biol Sci. 2010 Jan; 86(1): 40–53..

CAR

- CAR: Constitutive androstane receptor
  - Response elements: DR-3, DR-4, ER-6
  - CYP2A6, 2B6, 2C8, 2C9, 2C19, 3A4
  - UGT1A1
- Constitutively active *in vitro*; can be quiescent in cytoplasm of hepatocytes *in vivo* unless activated by endogenous signaling
- Treatment with ligand, CAR translocates to nucleus but the mechanism is thought to be independent of ligand binding

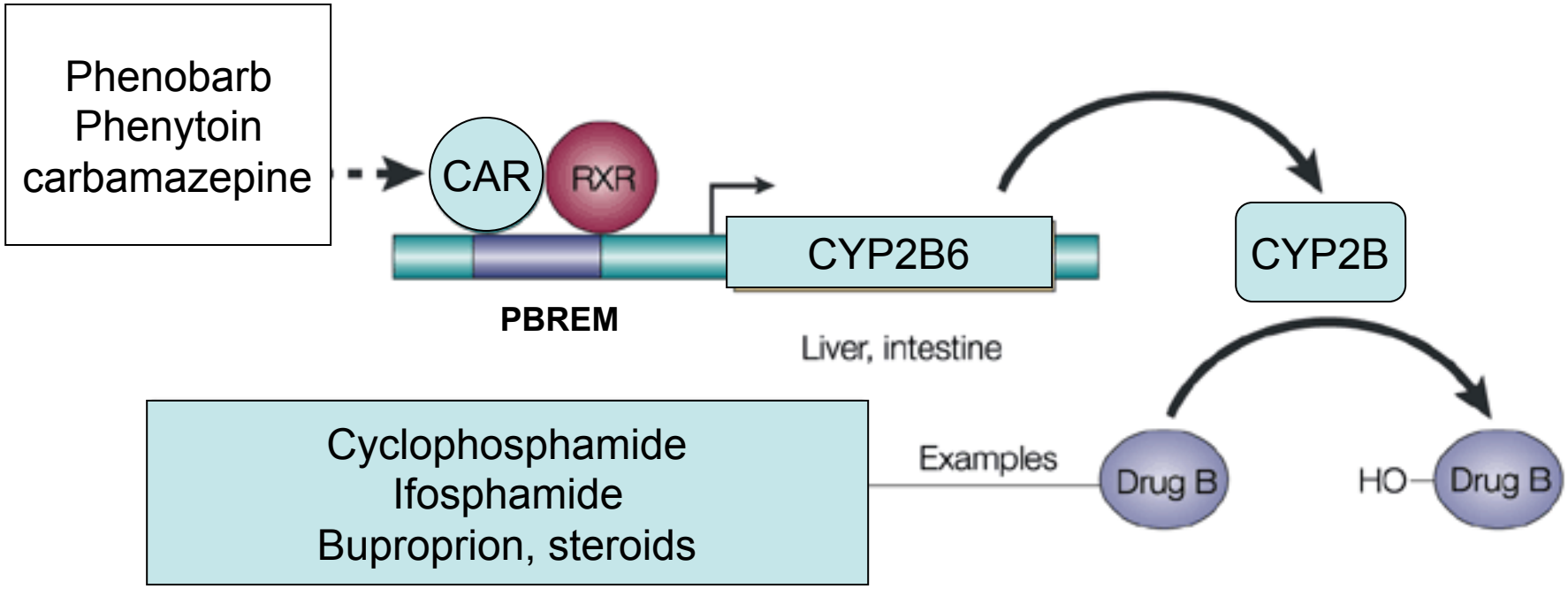
CAR

- Note: phenobarbital, prototypical inducer, is not a direct ligand – regulation of CAR function involves protein dephosphorylation, coactivators, cytoplasmic CAR retention protein, and p38 MAPK
- Activators: phenobarbital, TCPOBOP (mice), CITCO (human), clotrimazole, phenytoin
- Deactivators: Androstanes
- Physiology: bilirubin clearance, bile acid detoxification, glucose control



phenobarbital

Constitutive Androstane Receptor
CAR, NR1I3



CAR also regulates CYP3A4 by binding to the “PXRE”

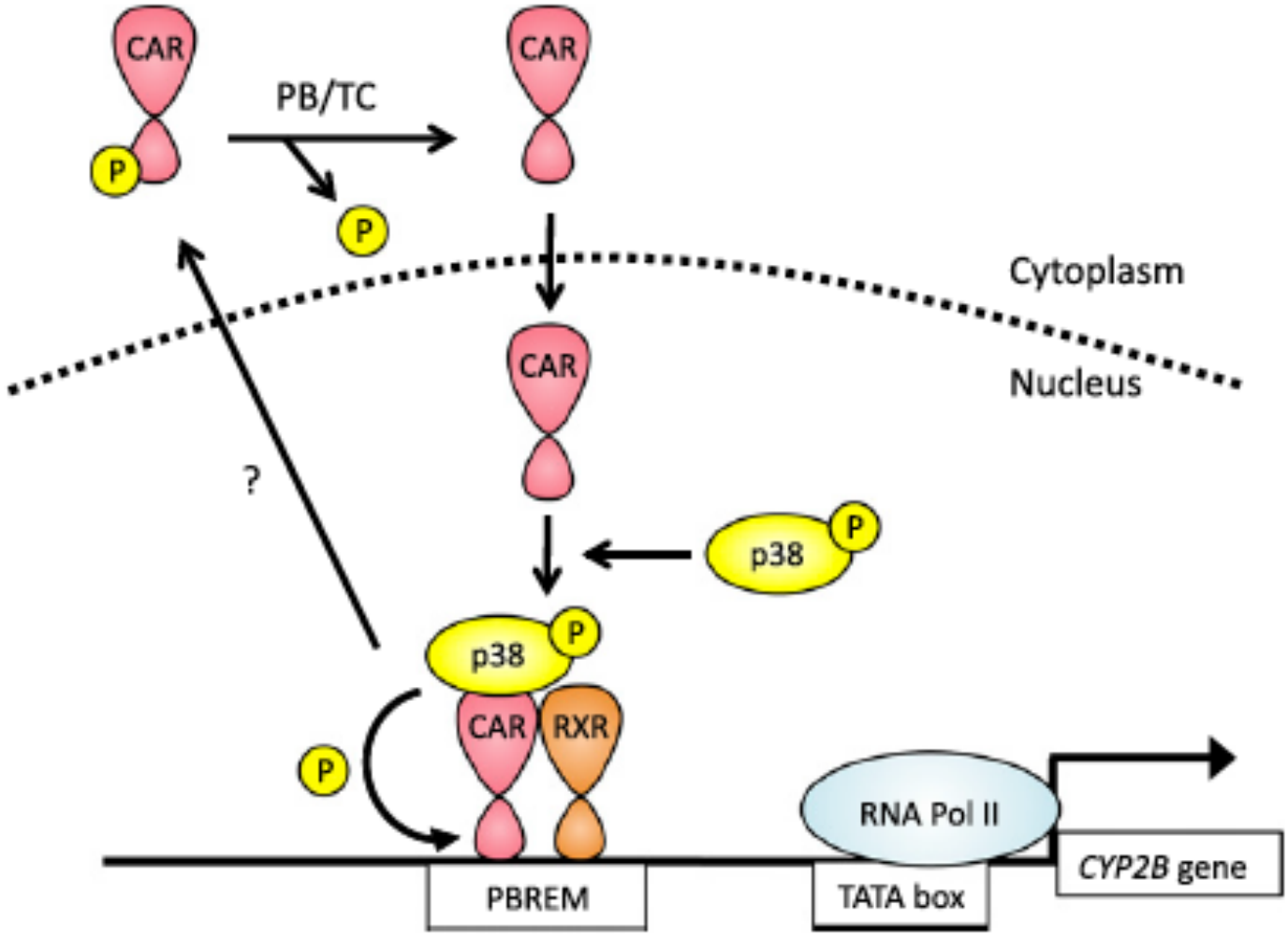
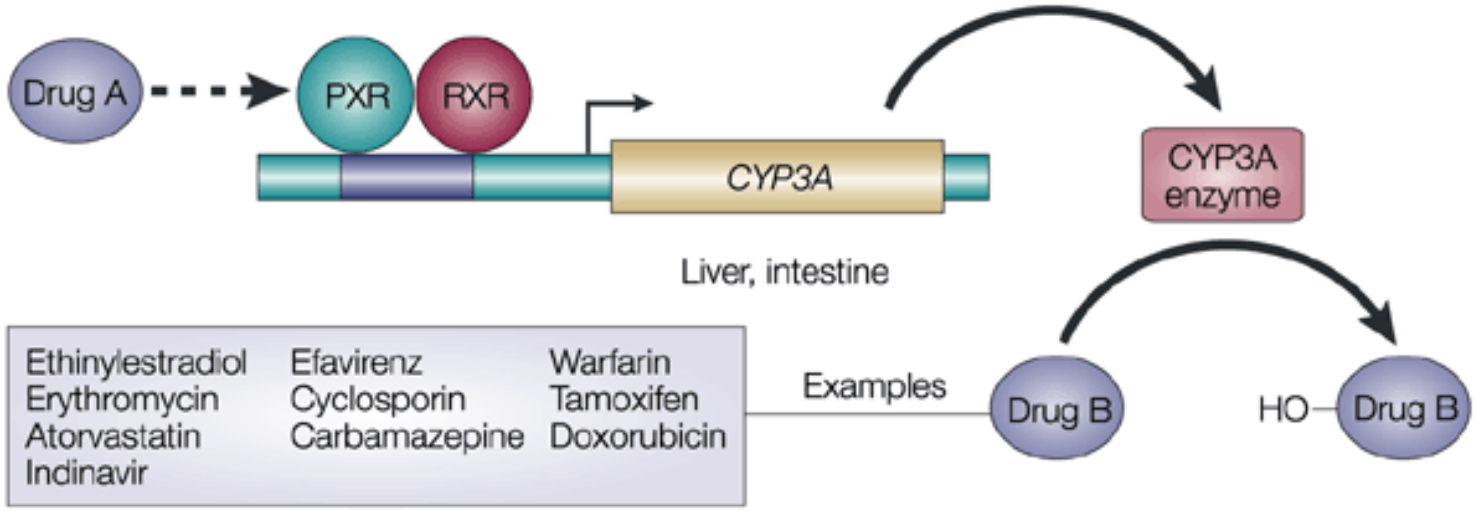


Fig. 5. Activation and inactivation of CAR-mediated transcription by p-p38 MAPK. CAR is phosphorylated at threonine 38 under normal conditions in livers. Phenobarbital (PB) or TCPOBOP (TC) causes dephosphorylation of CAR and translocates it to the nucleus. CAR interacts with p-p38 MAPK in the nucleus, and the CAR/p-p38 MAPK complexes activate transcription of the *CYP2B* gene. P-p38 MAPK phosphorylates threonine 38 of CAR and translocates it to the cytoplasm as an inactive form.

PXR

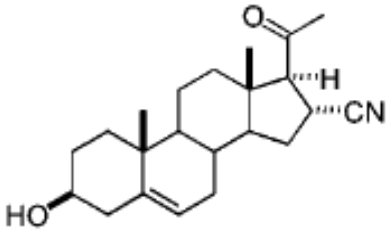
- PXR: Pregnane X receptor
  - Response element: DR-3, DR-4, ER-6, ER-8
  - CYP1A2, 2B6, 2C9, 2C19, 3A4, 3A7
  - SULT2A1, UGT1A1, 1A3, 1A4, MDR1, AHR
  - More than 36 different gene targets
- Treatment with ligand, PXR translocates to nucleus (or resides in nucleus)



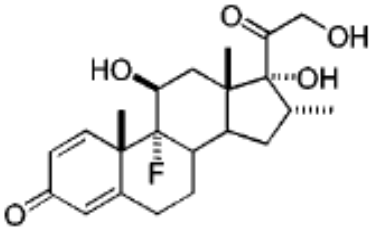
Nature Reviews | Drug Discovery

PXR

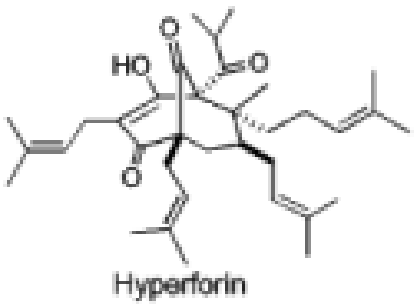
- Crystal structure solved – large binding pocket, promiscuity of PXR towards xenobiotics
- Structurally diverse molecules can induce CYP3A *via* the same biochemical pathway



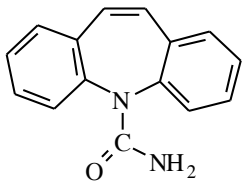
Pregnenolone 16α-carbonitrile



Dexamethasone



Hyperforin



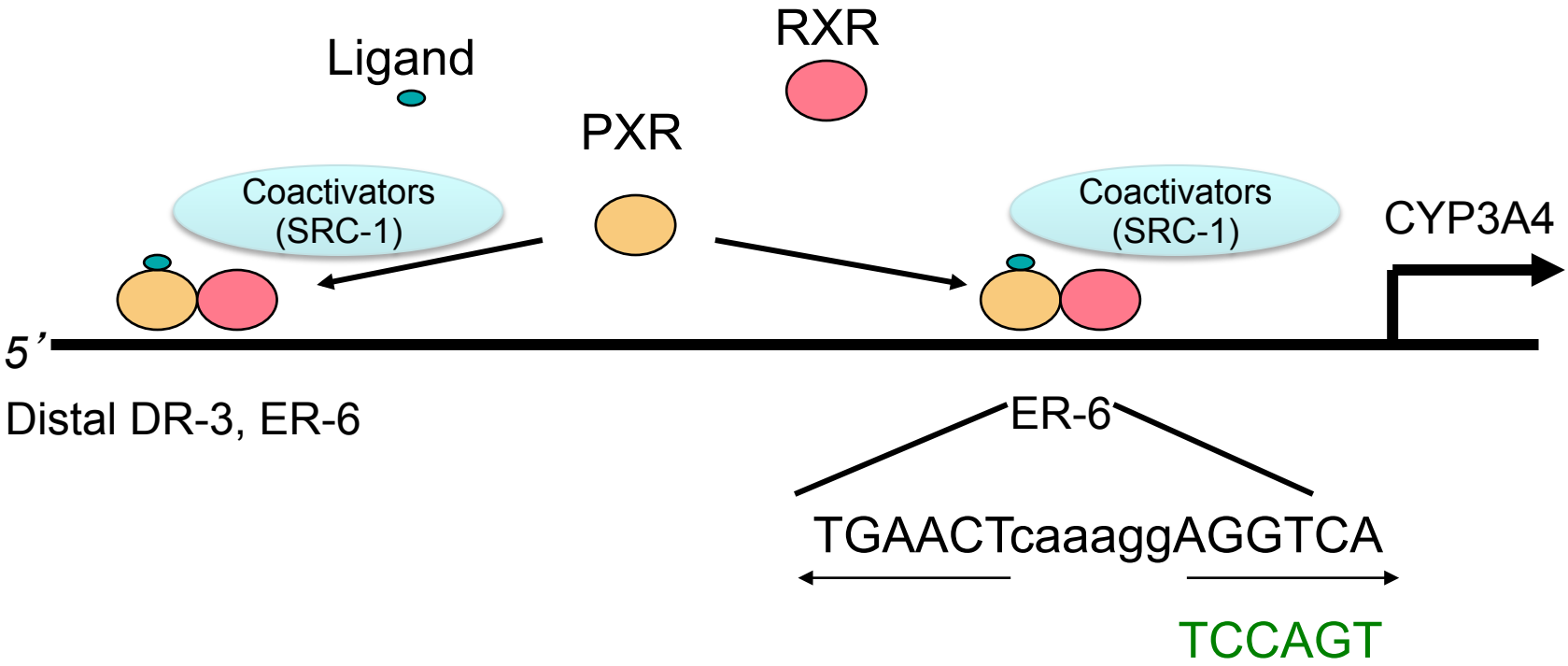
carbamazepine

- Activators: bile acids, rifampin, paclitaxel, nifedapine, clotrimazole*, ritonavir*, glucocorticoids, efavirenz, statins
- Deactivators: ET-743, sulfurafane

* also act as catalytic inhibitors

Induction of *CYP3A4 via* PXR

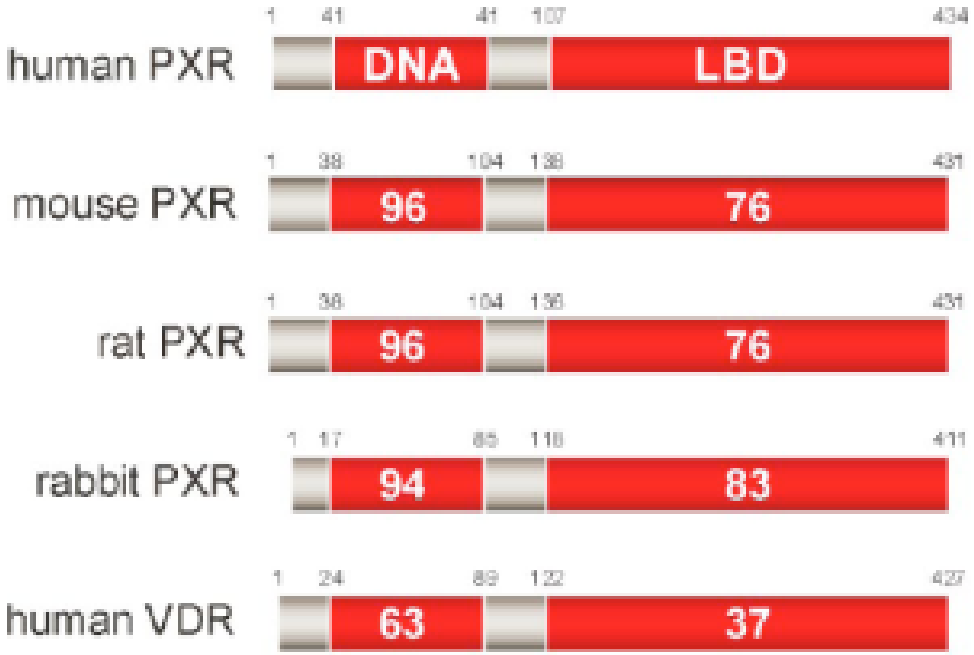
- Maximum induction of CYP3A4: binding of PXR/RXR to distal (DR-3, ER-6) and proximal (ER-6) response elements
- This feature distinguishes *CYP3A4* from the weakly-inducible *CYP3A5* (lacks distal elements)



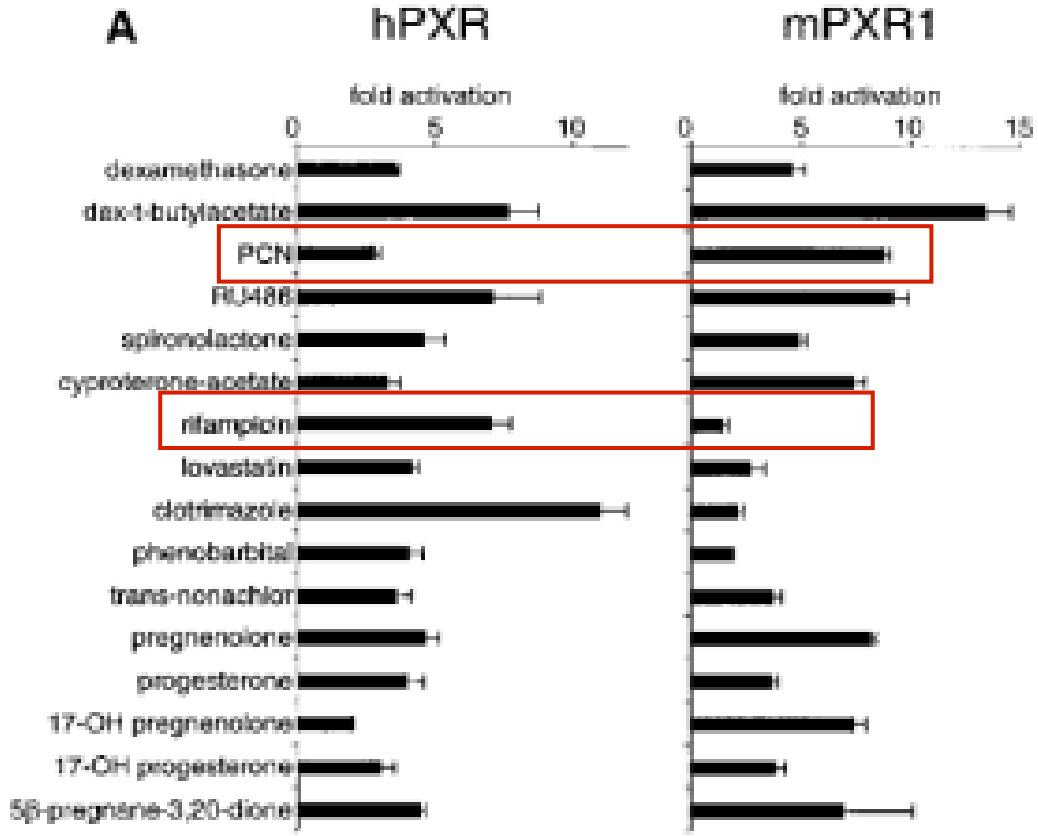
Ref: Goodwin, Mol Pharmacol, 1999

Species Differences in PXR

- Species dependency in CYP3A induction by different inducers (rifampin and PCN) – amino acid sequence difference in ligand binding domains of PXR
- Humanized mice (PXR knockout + human SXR) respond to “human” inducers



Species Differences in CYP3A Induction



- Interspecies differences in the inducibility of CYP3A by xenobiotics can be explained by the difference in binding affinity of the ligand to PXR (ligand binding domain variation).

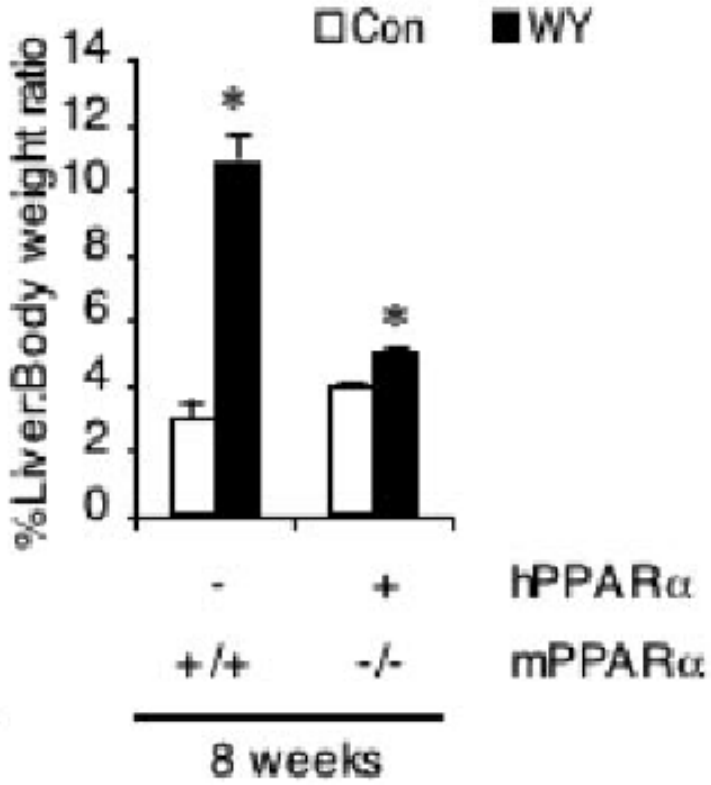
Ref: Goodwin, Ann Rev Pharmacol Toxicol, 2002
Lehmann, J Clin Invest, 1998

PPARα

- PPARα: Peroxisome proliferator-activated receptor
  - Response element: DR-1
  - CYP4A, UGT1A9, 2B4
- PPARα involved in lipid and glucose metabolism
- CYP4A induced – catalyzes ω-oxidation of fatty acids (e.g., lauric acid, arachidonic acid)
- Activators: phthlate ester plasticisers, fibrates, glitazones, certain herbicides, WY-14643

PPARα

- Exposure of rodents to peroxisome proliferators leads to increased size and number of hepatic peroxisomes, hepatomegaly and carcinogenesis
- This does not seem to occur to the same extent in humans

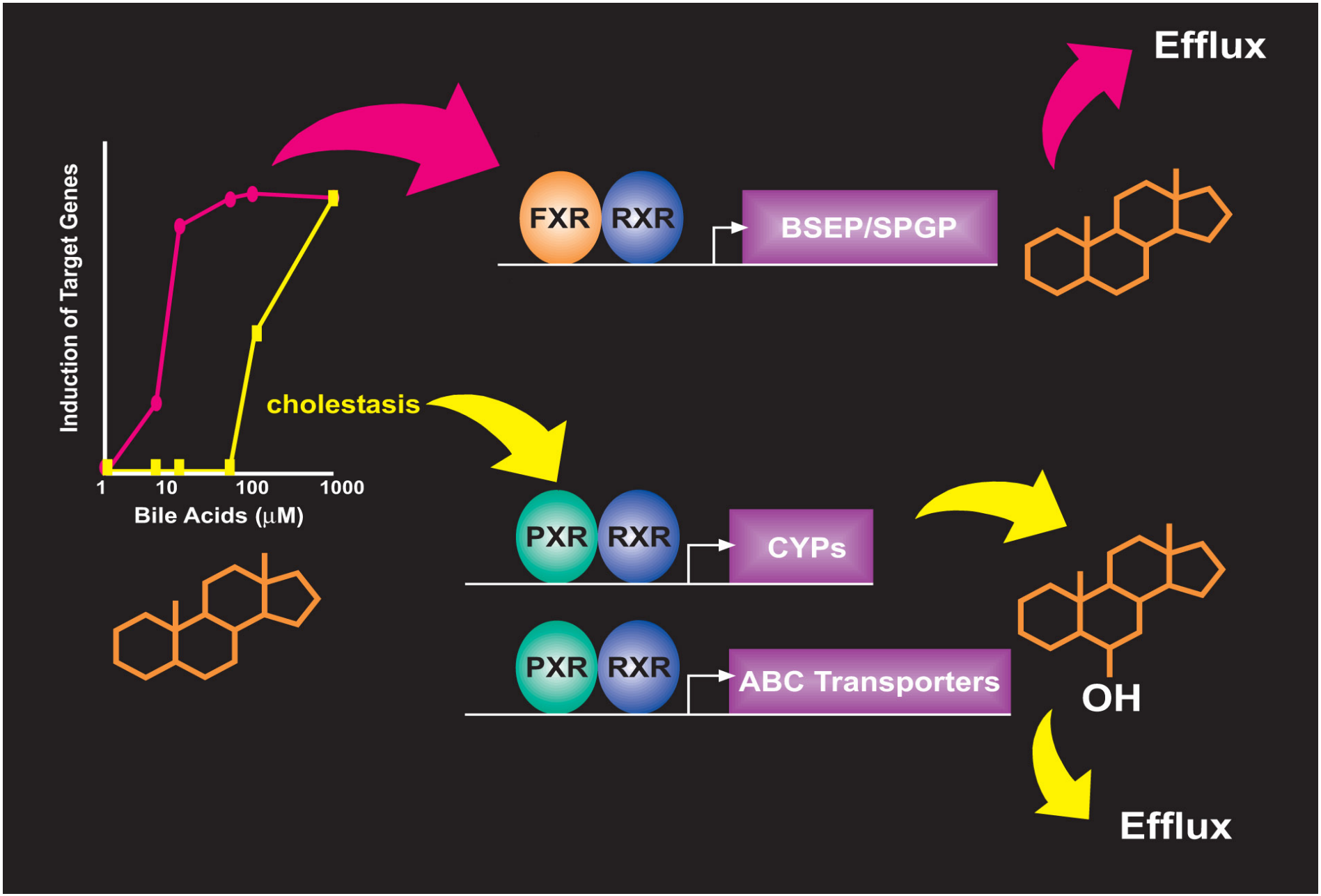


Ref: Cheung, Cancer Res, 2004

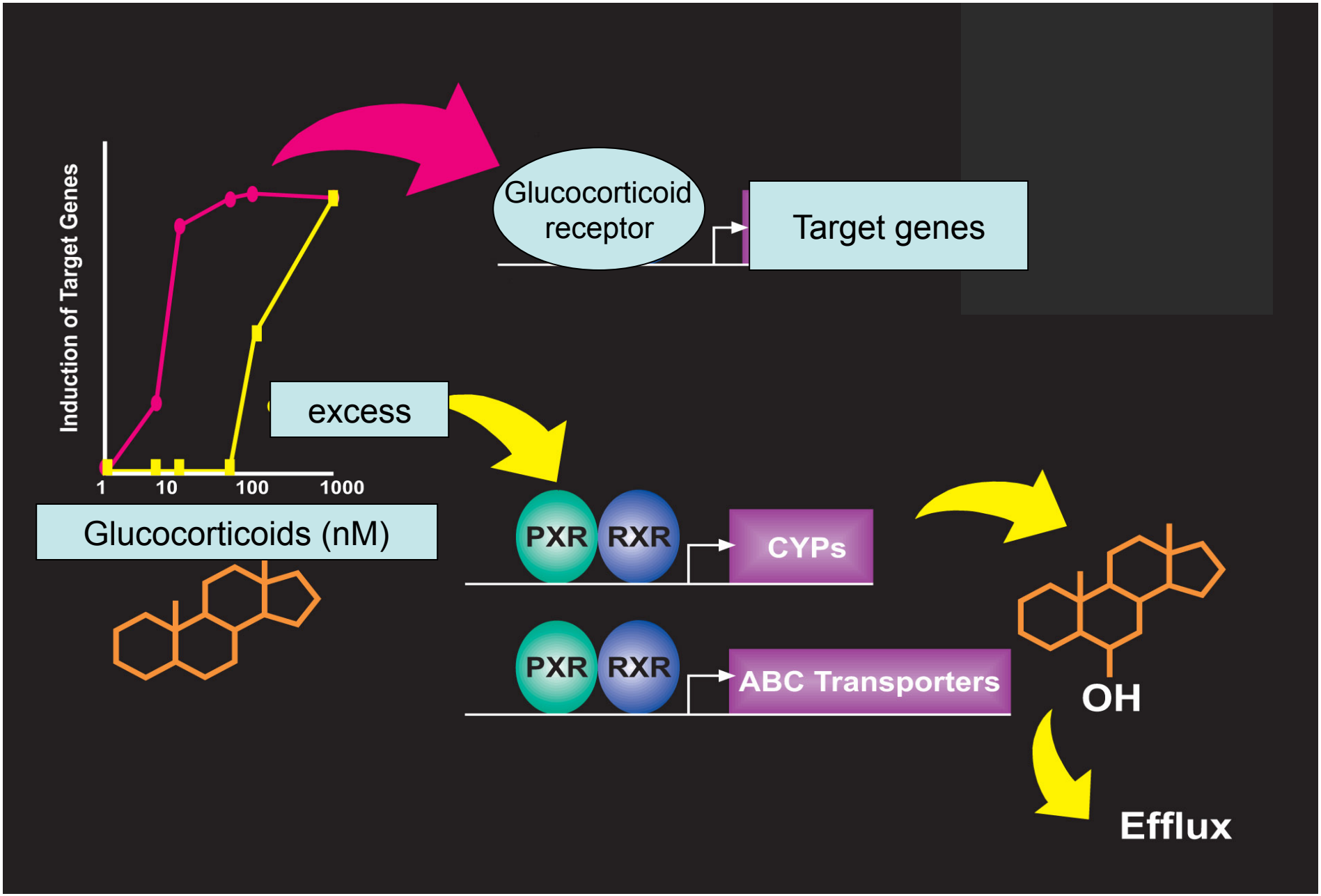
Additional Complexities

- NR Cross-talk; ligand concencetration-dependent effects
- Tissue expression of nuclear receptor (PXR)
- Nuclear receptor splice variants
- Response element of target gene inducer (PXR activation of CYP3A4 and P-gp)
- Inducer (PXR activation of CYP3A4 and P-gp)
- Tissue specific corepressors, coactivators, transcription factors

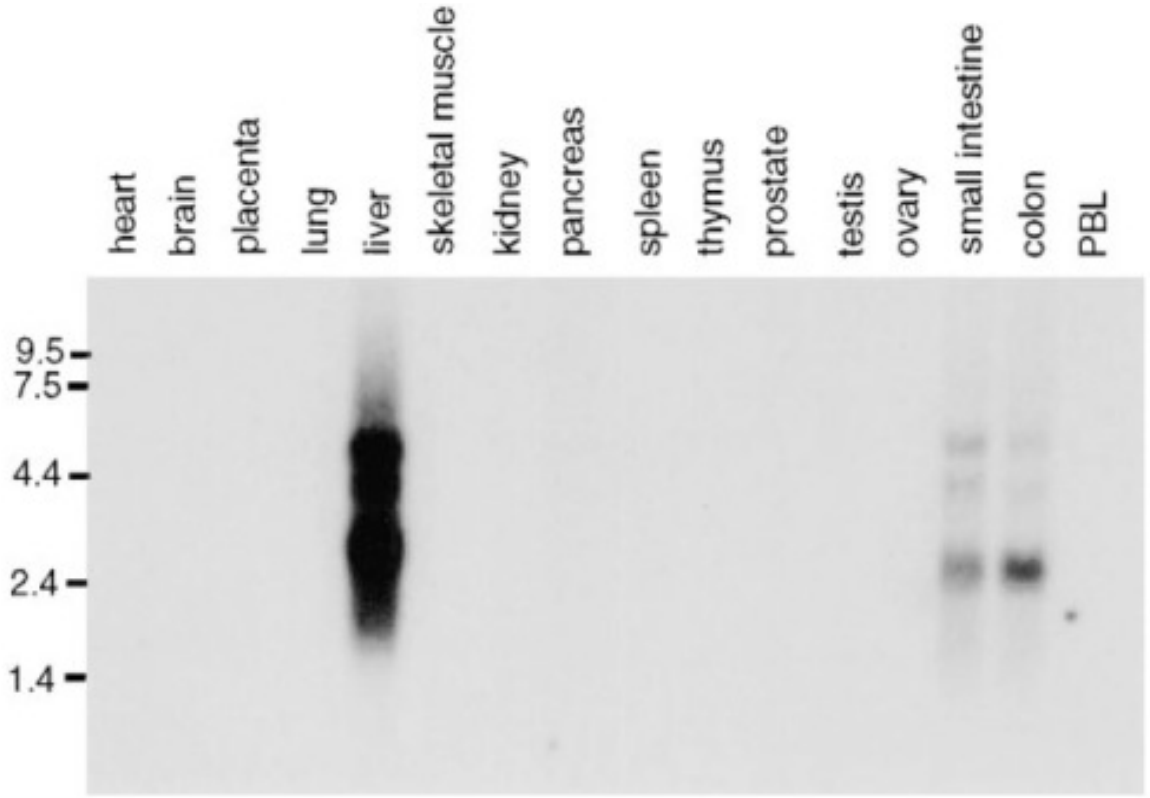
Cross-Talk Between Nuclear Receptors



Pharmacological concentrations of endogenous molecules activate PXR to induce metabolizing and transport genes to enhance steroid elimination



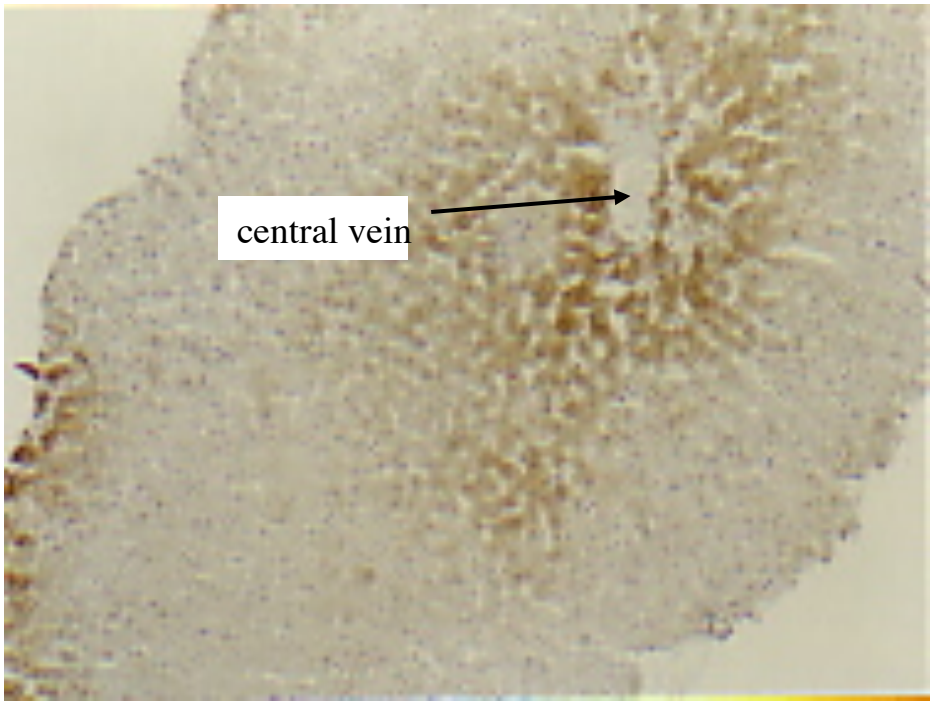
Tissue Expression of hPXR

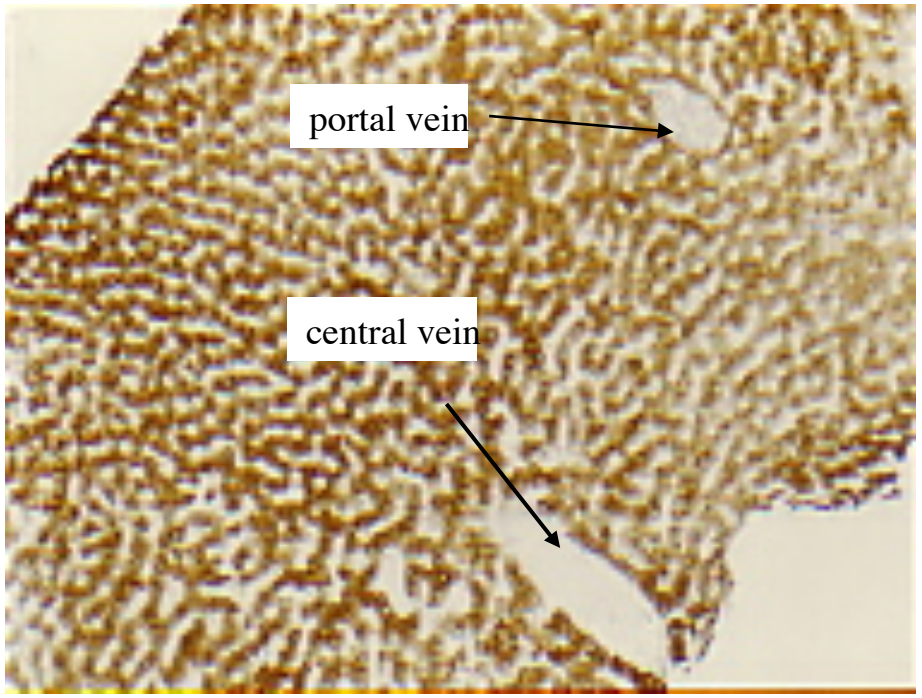


- Northern blot of PXR hRNA in human tissues
- Major inducible organs express hPXR

Ref: Lehmann, J Clin Invest, 1998

Induction of Hepatic CYP3A by Phenytoin





- Biopsies collected from a liver transplant patient placed on phenytoin for seizure control (presumed CsA-induced). Long-term treatment with phenytoin induces enzyme expression in every hepatocyte.

Ligand-Specific and Promoter-Specific Induction

- Although multiple genes can be activated by PXR, the magnitude of response for each gene depends on the ligand; this is the result of co-activator specific interactions.

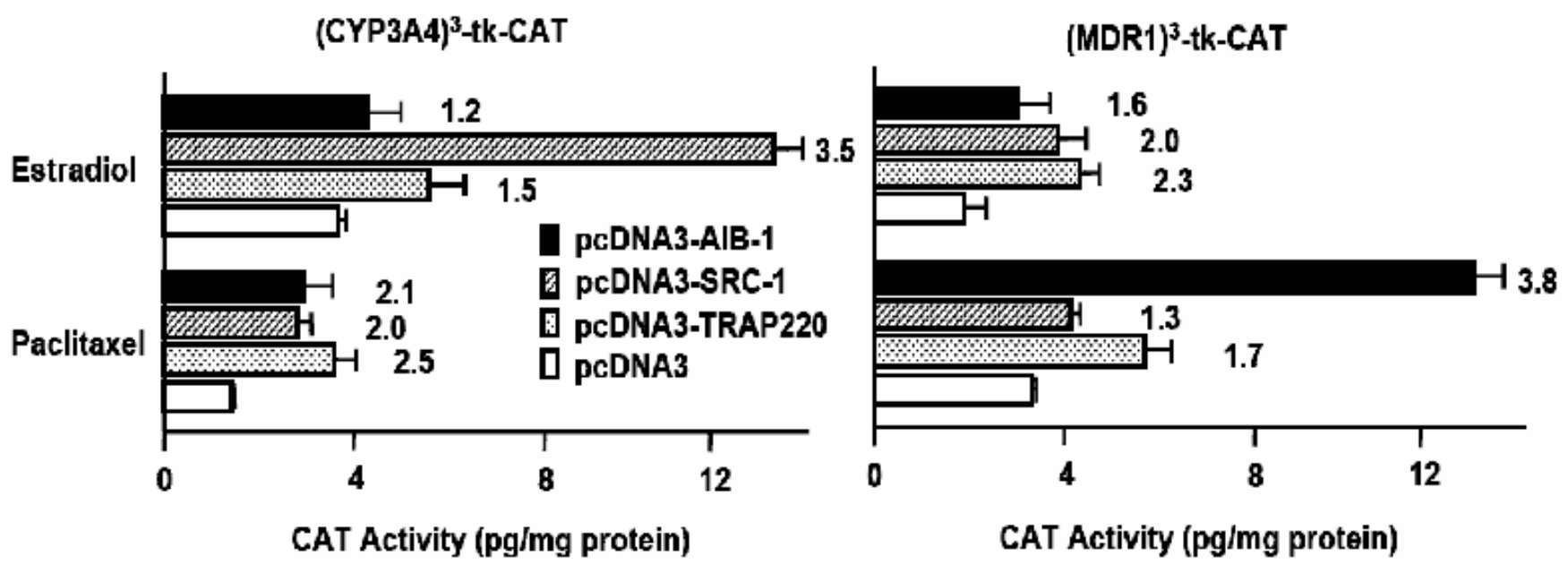
- Note (next slide) differential effects of PXR ligands on the DR3 and DR4 elements of MDR1 (ABCB1) and CYP3A4 when certain co-activators (SRC-1 and AIB-1) are present

5'-gggtca gca agttca-3' (DR-3 motif – CYP3A4)

5'-aggtca agtt agttca-3' (DR-4 motif – MDR1)

Ref: Masutyama, Mol Endocrinol, 2005.

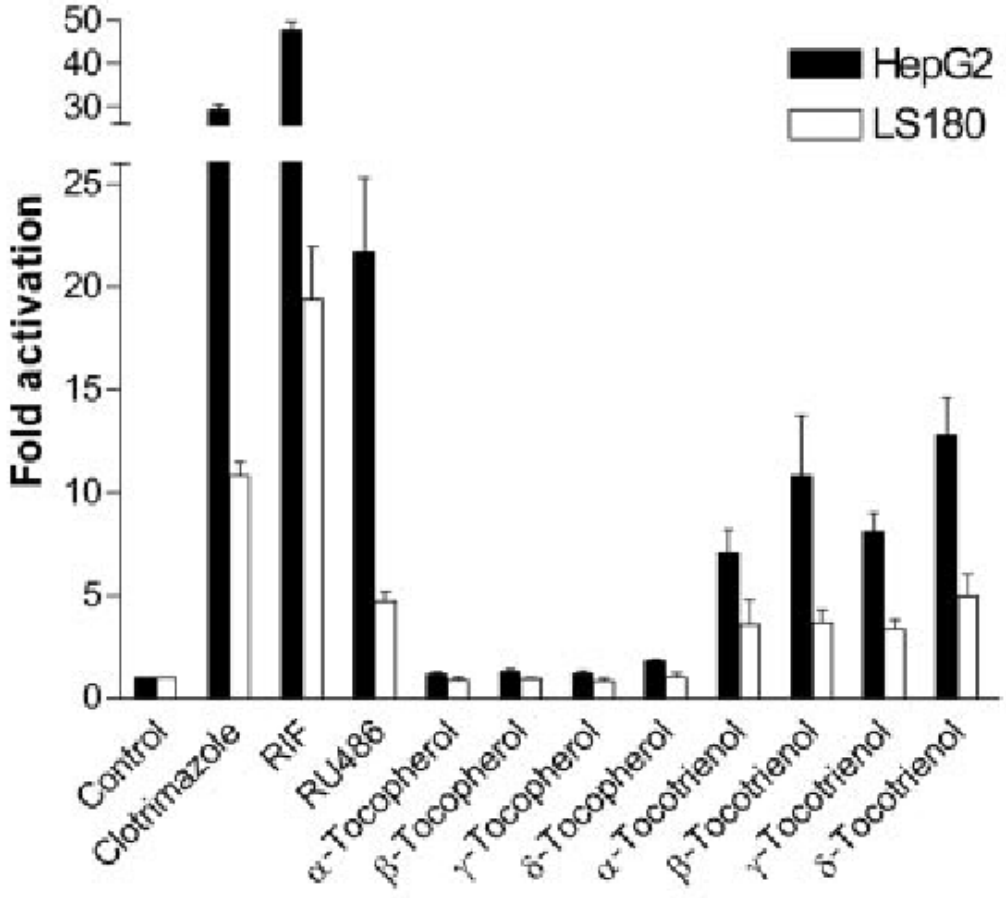
Coactivator-Selective Effects



- Transient transfection of coactivator with PXRE–CAT reporter construct
- Note coactivator-selective effects of estradiol on DR3 (CYP3A4) activation *vs* paclitaxel on DR4 (MDR1) activation

Ref: Masutyama, Mol Endocrinol, 2005.

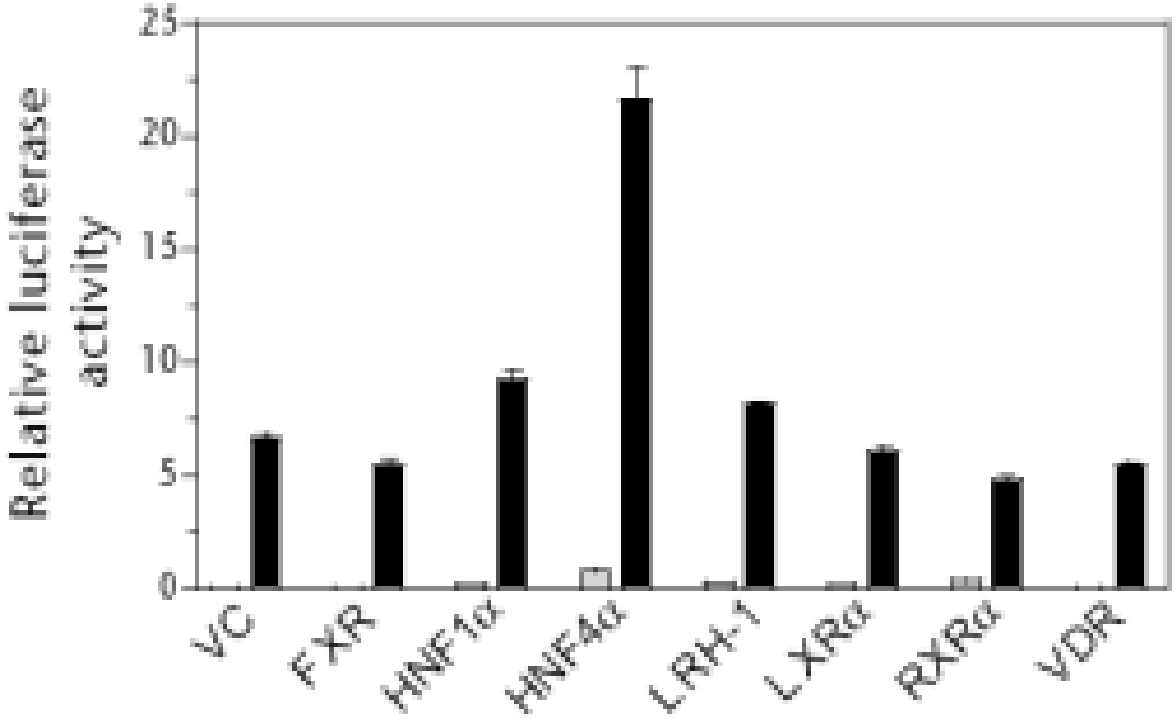
Tissue-Selective Expression: Effect of Co-repressors



- Cells transfected with PXR and PXRE-reporter
- NCoR, nuclear receptor corepressor highly expressed in LS180 cells (intestine), low in hepatocytes

Ref: Zhou, DMD, 2004.

Maximum PXR Activation also Requires HNF4α



- HNF4α stimulates transcription 4- to 10-fold above that achieved with PXR alone; (shown basal expression in the absence of exogenous inducer)
- Effect appears to be mediated presumably binding of HNF4α to a DR1 motif in the distal (-7783 and -7771) region of the CYP3A4 gene that contains PXREs (DR3 and ER6).

Ref: Tirona, Nature Medicine, 2003

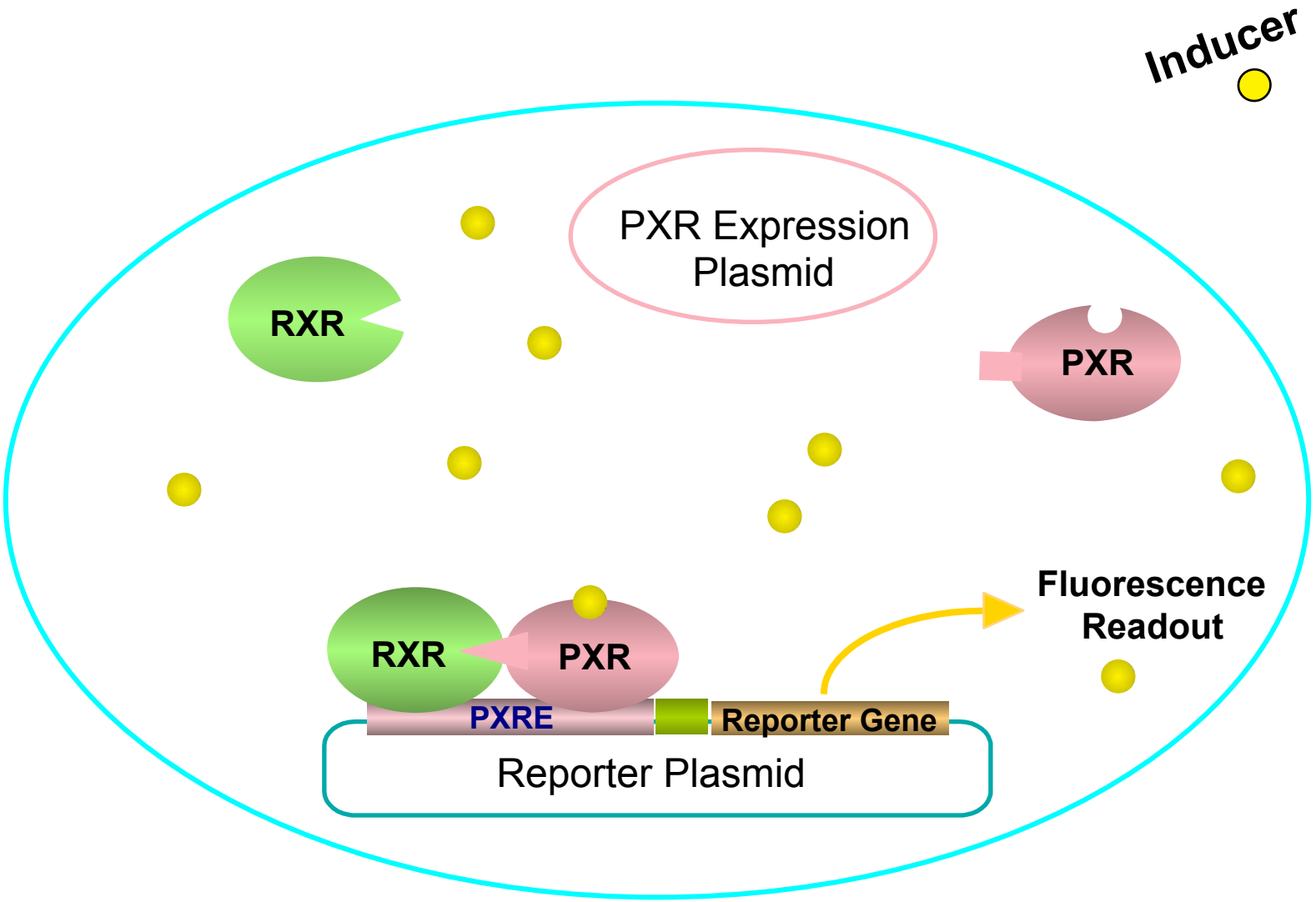
Experimental Techniques: Transcription

Method	Advantages	Limitations
Ligand-PXR Displacement	reproducible, high throughput low cost	false positives, access to technology
Co-transfection (NR & reporter gene)	reproducible, adaptable to enzyme - receptor of interest	single enzyme screen, lower throughput higher costs
Human Hepatocyte	functional kinetic data quantitative RT-PCR multi-enzyme	high variability livers, access to cells, slower turnaround
In vivo animals	accessibility, experience,	species differences* low throughput
In vivo humans	clinical applicability	staging in development, high cost

* May be circumvented with the availability of hPXR transgenic animals

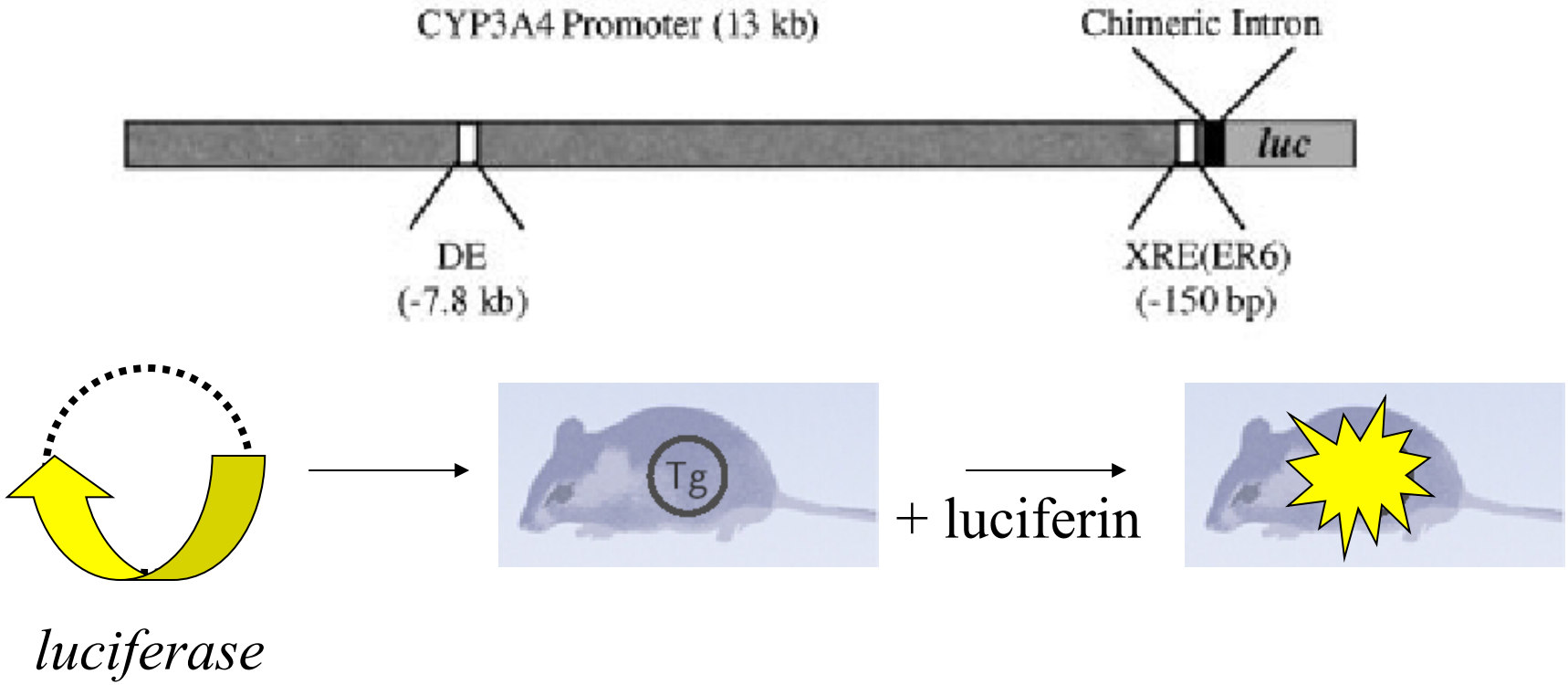
hPXR Transient Transfection in Cells

- Assay of gene activation



CYP3A4 Induction in Genetically Modified Mice

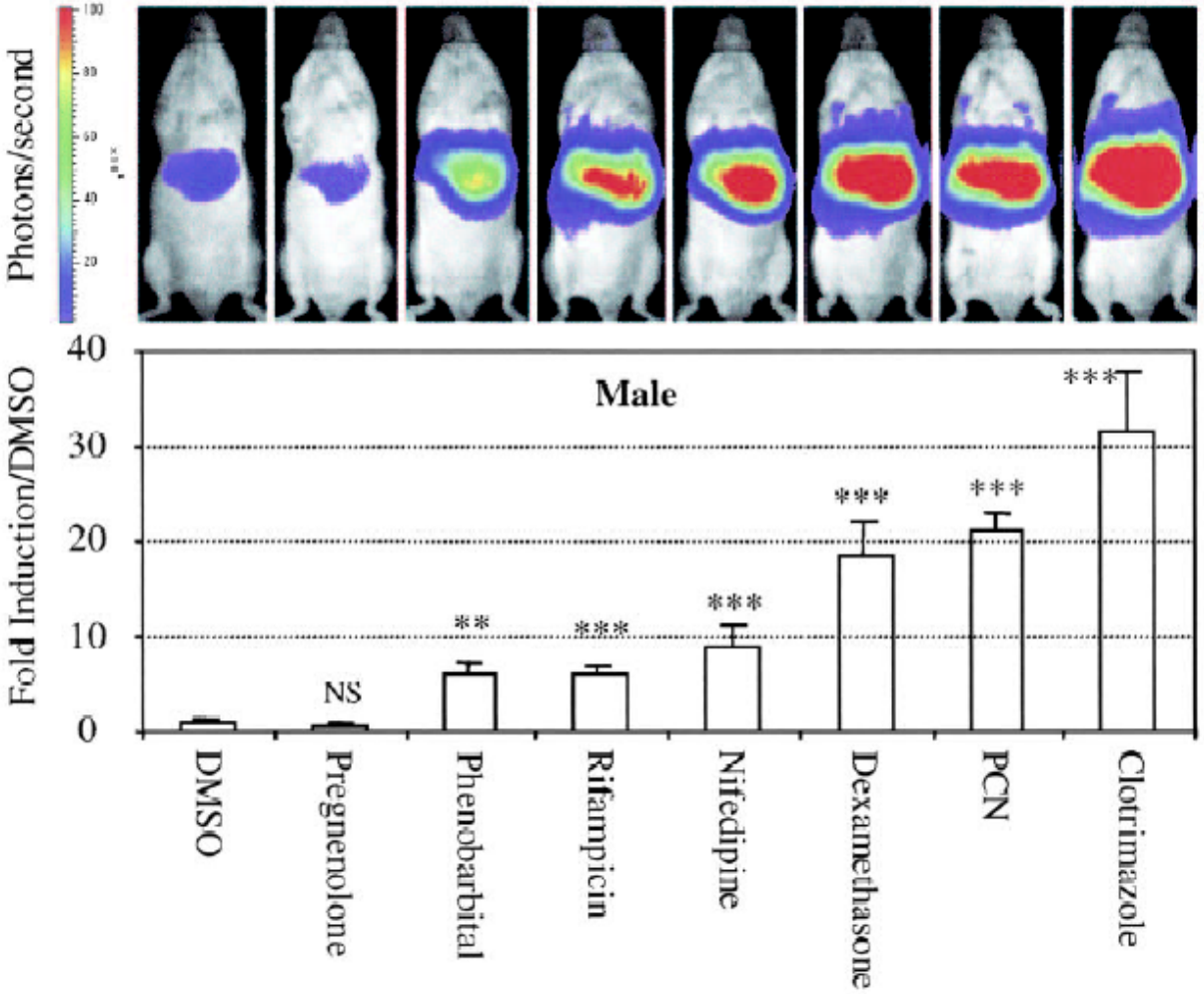
- Generation of transgenic animal expressing human CYP3A4 promoter + luciferase reporter



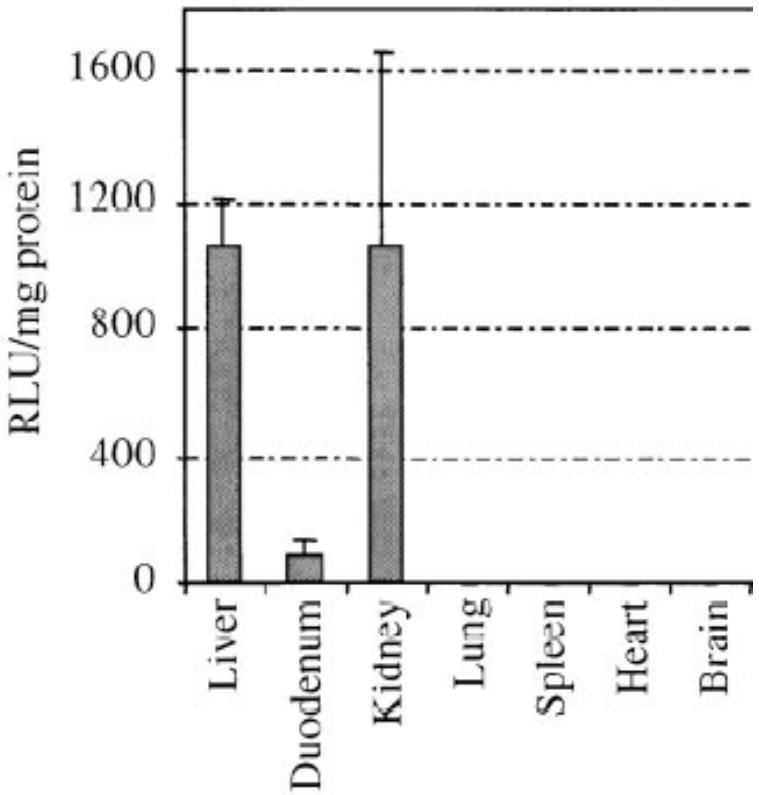
Ref: Zhang, DMD, 2003

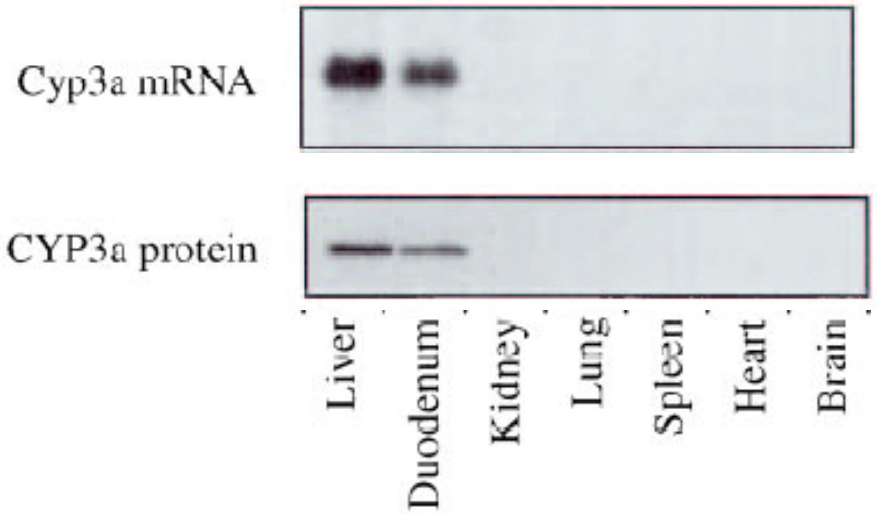
CYP3A4 Induction in Genetically Modified Mice

- Permits in vivo inductive response
  (mouse PXR with human CYP3A4 luciferase reporter)



CYP3A4 Induction in Genetically Modified Mice

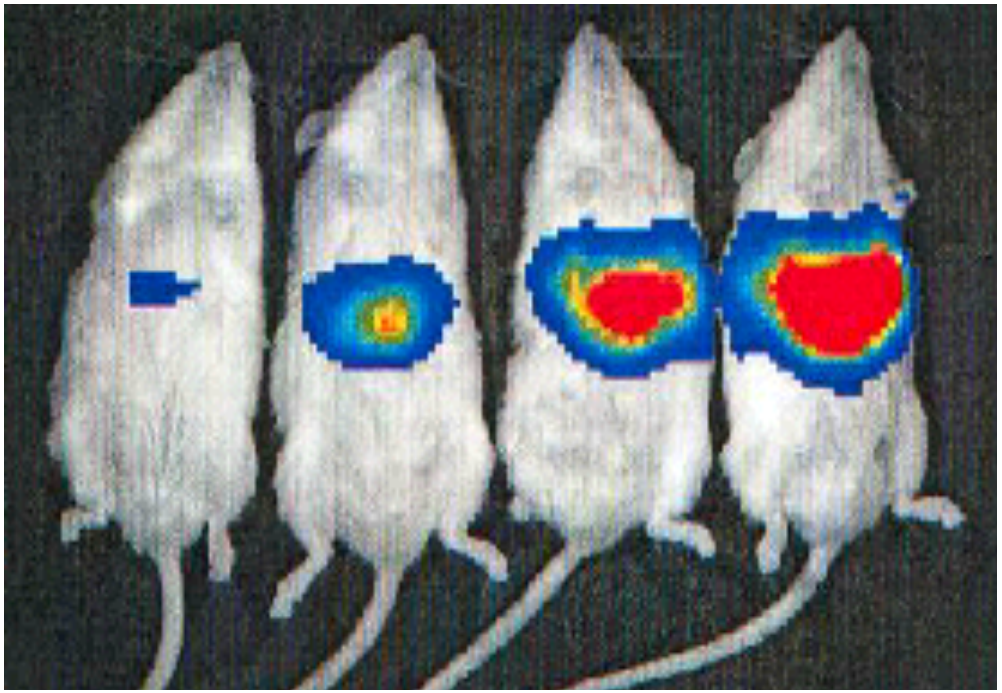




Ref: Zhang, DMD, 2003

Hydrodynamic DNA Infusion: CYP3A4 & hPXR

rifampin (mg/kg): 0 5 10 50

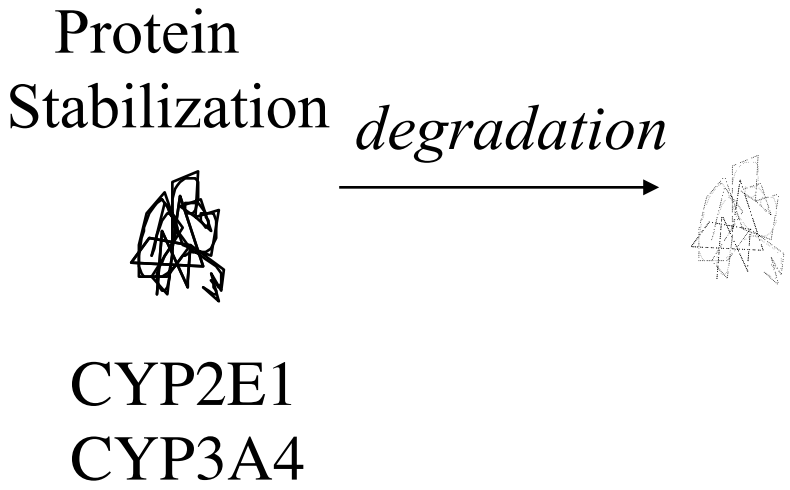


- Transient transduction of hCYP3A4-LUC and hPXR in mice
- Permit rapid quantitation of inductive response in context of in vivo PK (hPXR or other nuclear receptor, hCYP3A4-LUC or other reporter)

Ref: Schuetz, Mol Pharmacol, 2002

Protein Stabilization: Changes in k_{degr}

- Protein stabilization – decrease in degradation
- Degradation pathways:
  - ubiquitination
  - lysosomal degradation



Induction by Changes in k_{degr}

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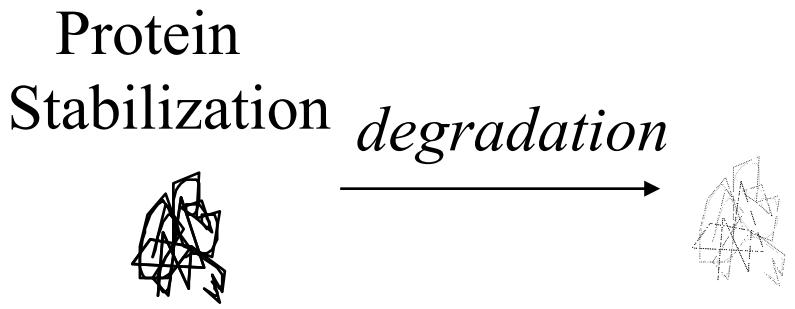


CYP2E1 (in vitro and in vivo)
CYP3A4 (in vitro)

Protein Degradation

- Quality control system to degrade proteins
  - Unassembled and/or misfolded proteins
  - Metabolic regulation
  - Oxidative damage
- Cytosolic ubiquitin (Ub)-dependent 26S proteasome system
  - (enzymes with short half-lives)
- Lysosomal pathway
  - Recycles membrane proteins, extracellular proteins and proteins with long half-lives

Degradation Pathways

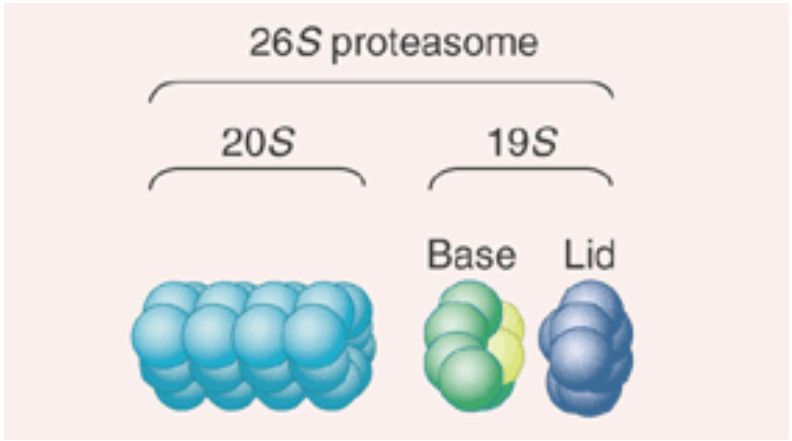
Protein

Ubiquitination

Polyubiquitinylated protein

26S Proteasomal Degradation

Lysosomal Degradation



vacuole

Ref: Murray, Mol Pharmacol, 2002

Ubiquitination & Degradation

- Ubiquitin
  - 76 amino acids (8.5 kD)
  - Highly conserved (present throughout eukaryotic kingdoms)
  - 3 enzymes participate in conjugation of ubiquitin to proteins (ATP-driven)
  - Results in a polyubiquitinylated protein
- Digestion by 26S protease complex
  - ATP-driven multisubunit protease
  - Multiple rounds of ATP hydrolysis enable protease to unfold and processively digest the protein
  - Ubiquitin recycled

Ubiquitination & Degradation

- Proteolysis of ubiquitinylated proteins is a feature of many cellular processes including:
  - Chromosomal stabilization
  - Cell division
  - Apoptosis
  - Cell differentiation
  - Stress response
- Ubiquitin-tagged proteins (that do not undergo proteolysis)
  - Endocytosis
  - Localization of certain proteins in the nucleus

Degradation

- Exhibit asynchronous turnover
- "short $t_{1/2}$" (e.g. CYP3A4) ubiquitin-dependent 26S proteasome pathway
- "long $t_{1/2}$" (e.g. CYP2B1 and POR: $t_{1/2}$ ~ 30 hours) lysosomal degradation
  - Electron micrographs of livers cells of rats treated with leupeptin (serine protease inhibitor) show "lysosomal constipation" and consequent accumulation of CYP2B1 and POR

CYP2E1, biphasic turnover

- t1/2 ~ 7 hours: degradation by proteasomal pathway
- t1/2 ~ 37 hours: lysosomal degradation

Hepatic CYP Half-life

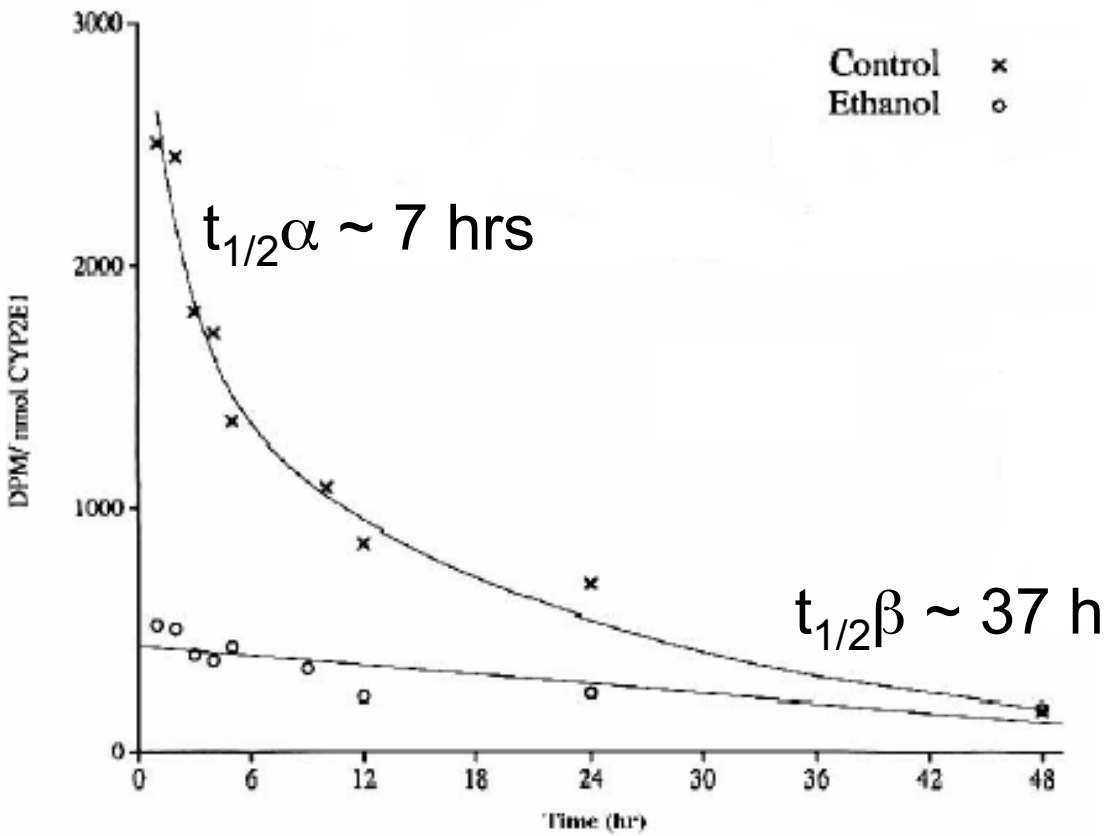
- Although there is no direct data for human CYP half-life in vivo, animal and hepatocyte data suggest values between 6-25 hrs; proteasomal mechanisms associated with a short $t_{1/2}$.
- Time-course of induction effects in vivo in humans suggests even longer $t_{1/2}$ – 36-48 hr for some enzymes (e.g., CYP3A4).

Approximate CYP Half-lives

Enzyme	t1/2 (hours)	Degradation by Ubiquitination
CYP1A1	15-16	No
CYP1A2	10*	
CYP2B1	19-25	No
CYP2B2	19-25	No
CYP2E1	6-7* 37	Yes No
CYP3A	9-14*	Yes
CYP4A		Yes
NADPH reductase	29-35	No

Adapted from Roberts, JBC 272: 9771-8, 1997

Biphasic Kinetics for CYP2E1 Elimination



- Rats injected with $NaH^{14}CO_3$
- ^{14}C-labeled CYP2E1 (Western blot – scintillation counting of band)

Ref: Roberts, JBC, 1995

Experimental Techniques: Degradation

- Degradation studies of long-lived hepatic P450s difficult to perform because of technical issues
- Hepatocytes or cultured hepatocytes
  - limited viability 6h
  - P450 instability due to accelerated loss of heme

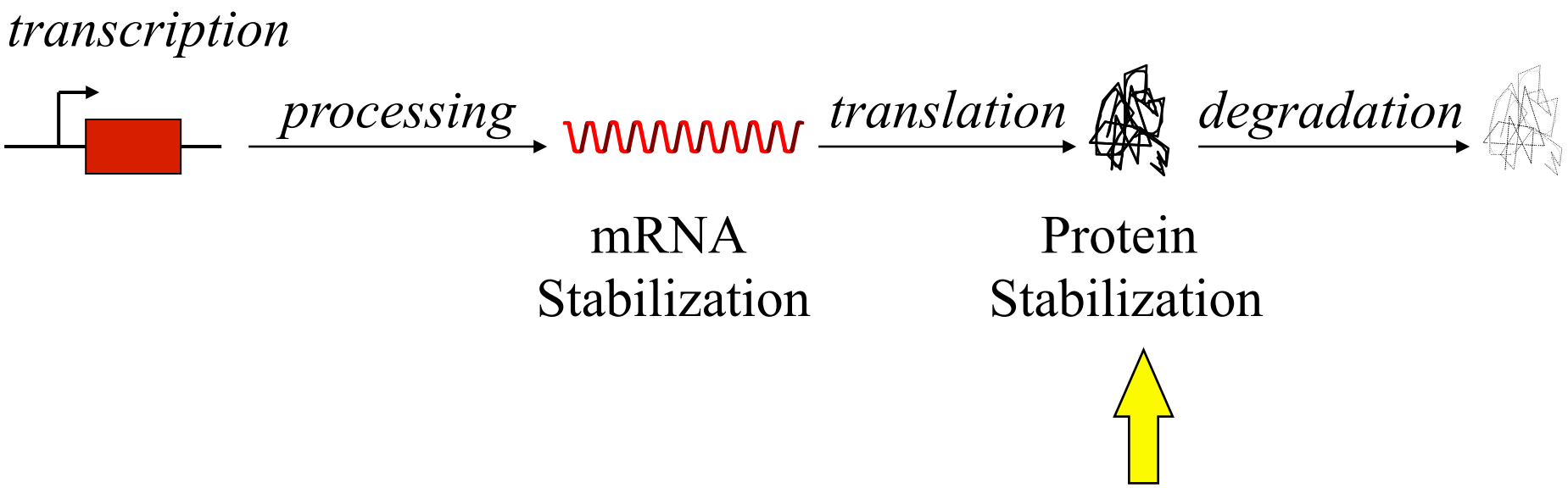
- In vivo – need to “label” enzyme and sacrifice animal at specific timepoints
- Yeast models of expression with various deletions of key enzymes in degradation pathways

Increased Degradation

- Structural changes to P450s can involve heme oxidation or adduct formation, or protein modification:
  - Oxidation of labile amino acids: Met, Pro, Arg, Lys, His
  - Uncoupled oxidation - generating reactive oxygen species
  - Phosphorylation of Ser129 (CYP2E1)
  - Ubiquitination (CYP3A4)

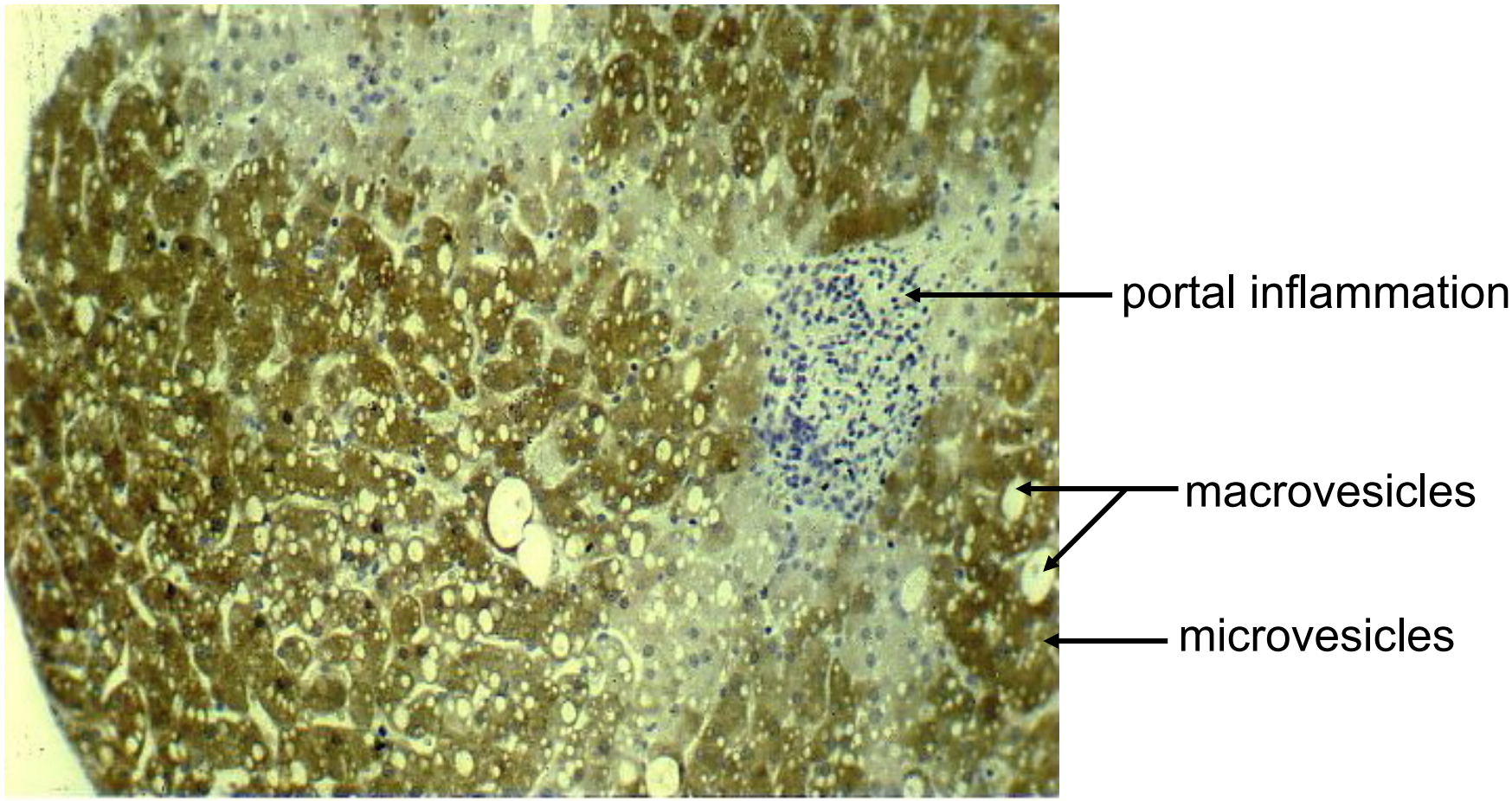
- Once modified, protein destruction occurs rapidly

Induction by Protein Stabilization



$$E_{ss} = \frac{R_o}{k_{\text{degr}}}$$

Induction of CYP2E1 in Steatotic Liver



- Immunohistochemistry of CYP2E1 (brown stain)
- Hepatic steatosis occurs in ~ 5-10% of the population; most commonly seen with obesity (90% with morbid obesity)

Conditions Inducing CYP2E1

- Xenobiotics
  - Ethanol, acetone
  - Pyrazoles, pyridines, primary alcohols

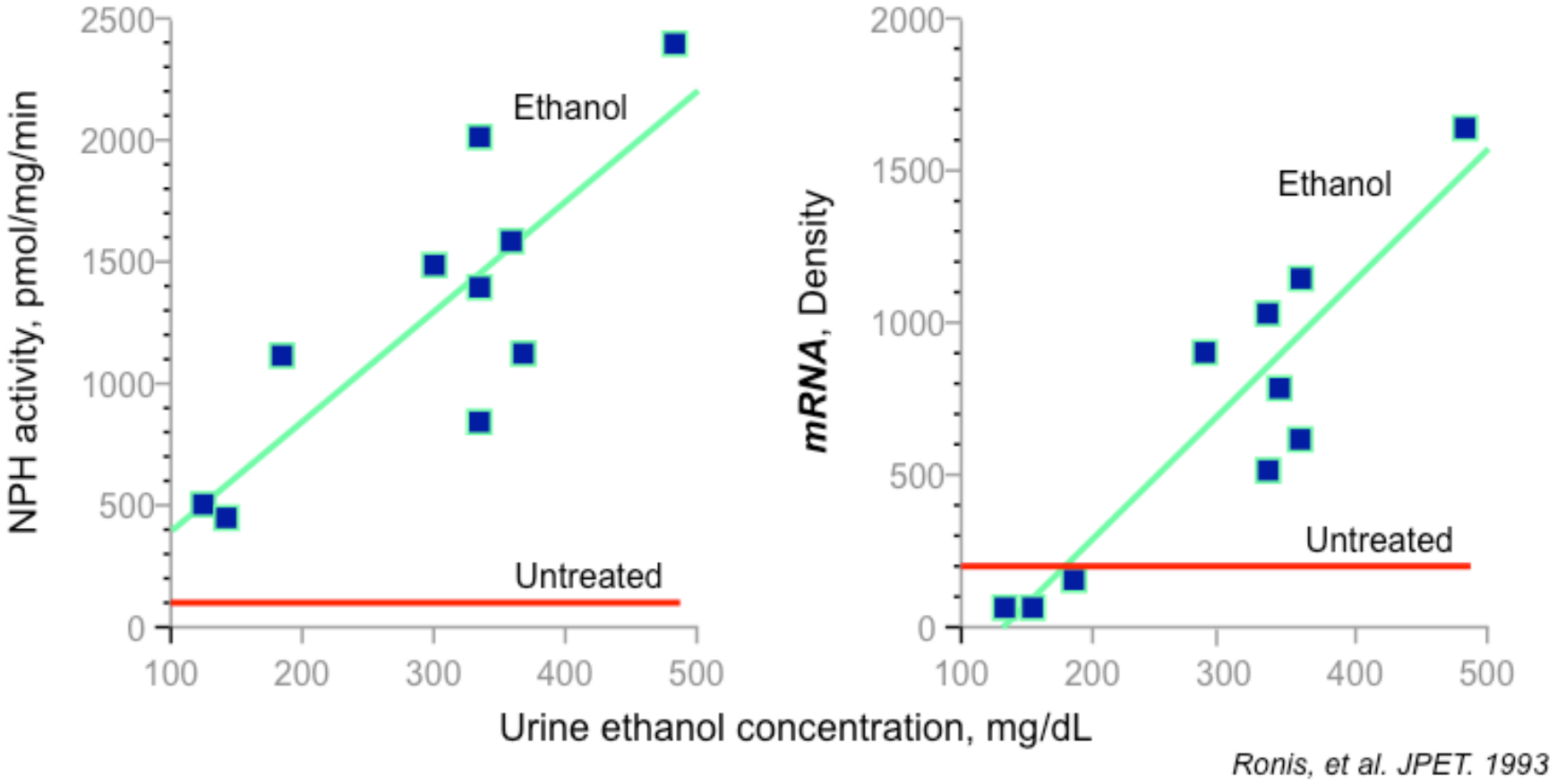
- Pathophysiological Conditions
  - Chronic fasting (ketones)
  - Steatosis
  - Birth

Transcriptional Activation of CYP2E1

- Most studies conducted in adults have failed to find evidence of increased mRNA synthesis following treatment with CYP2E1 inducers (ethanol, pyridine, acetone, pyrazole)
- Only birth triggers gene activation
- CYP2E1 mRNA in hamsters may be increased by ethanol and pyrazole. 2-stage induction process:
  - high BAC - increased mRNA (stabilization)
  - low BAC - protein stabilization
- There is also evidence that mRNA translation efficiency may be enhanced by inducers (blocked by translation inhibitors - NaF)

Ref: BBRC 150:304-10, 1988
Eur J Pharmacol 248:7-14, 1993

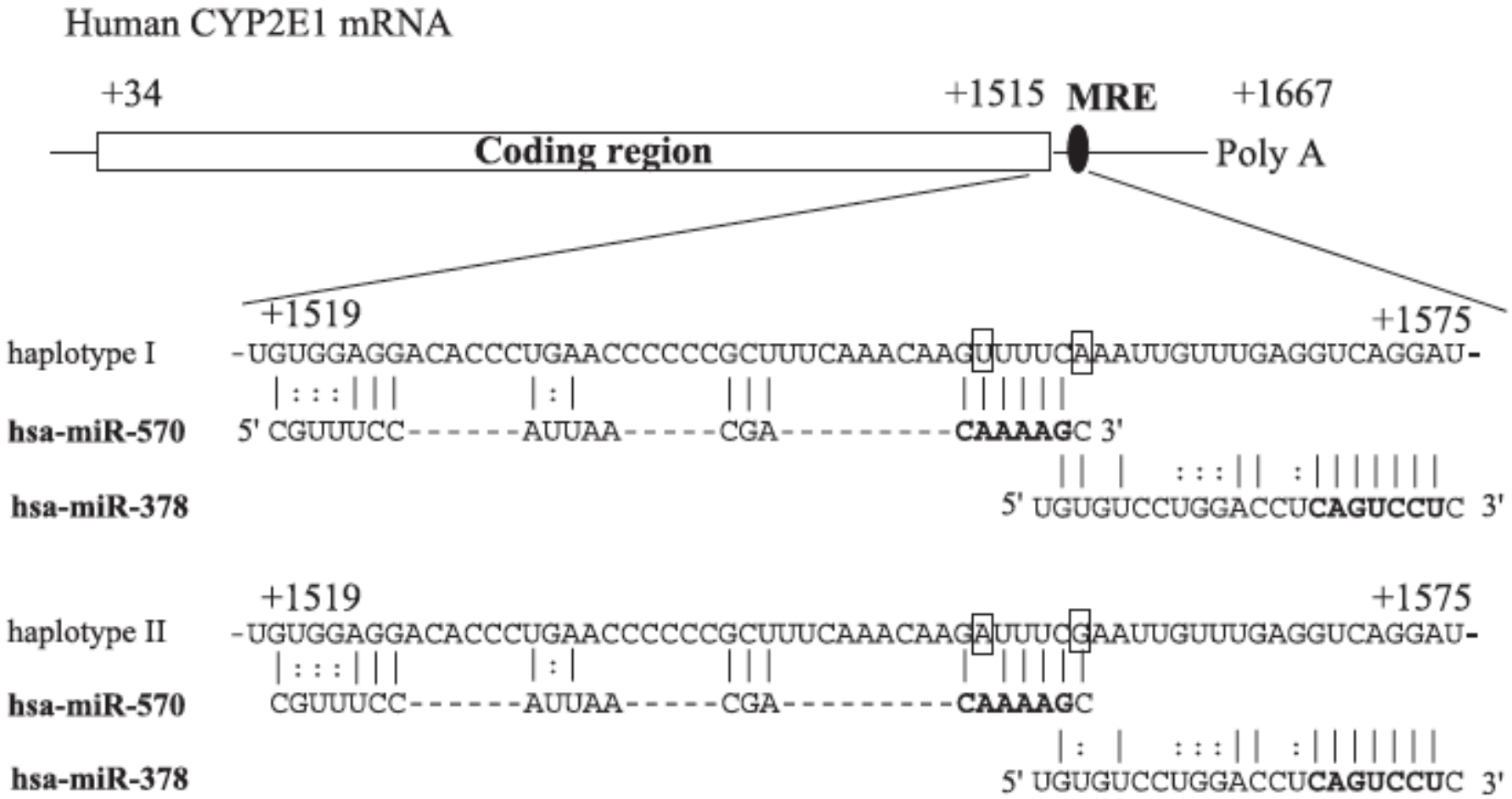
Effect of Ethanol on *CYP*2E1 Synthesis and Ex Vivo Activity



Ronis, et al. JPET. 1993

- induction of ex vivo hepatic CYP2E1 activity correlates with ethanol exposure; interestingly, there is with discontinuity in mRNA (300 mg/dL)

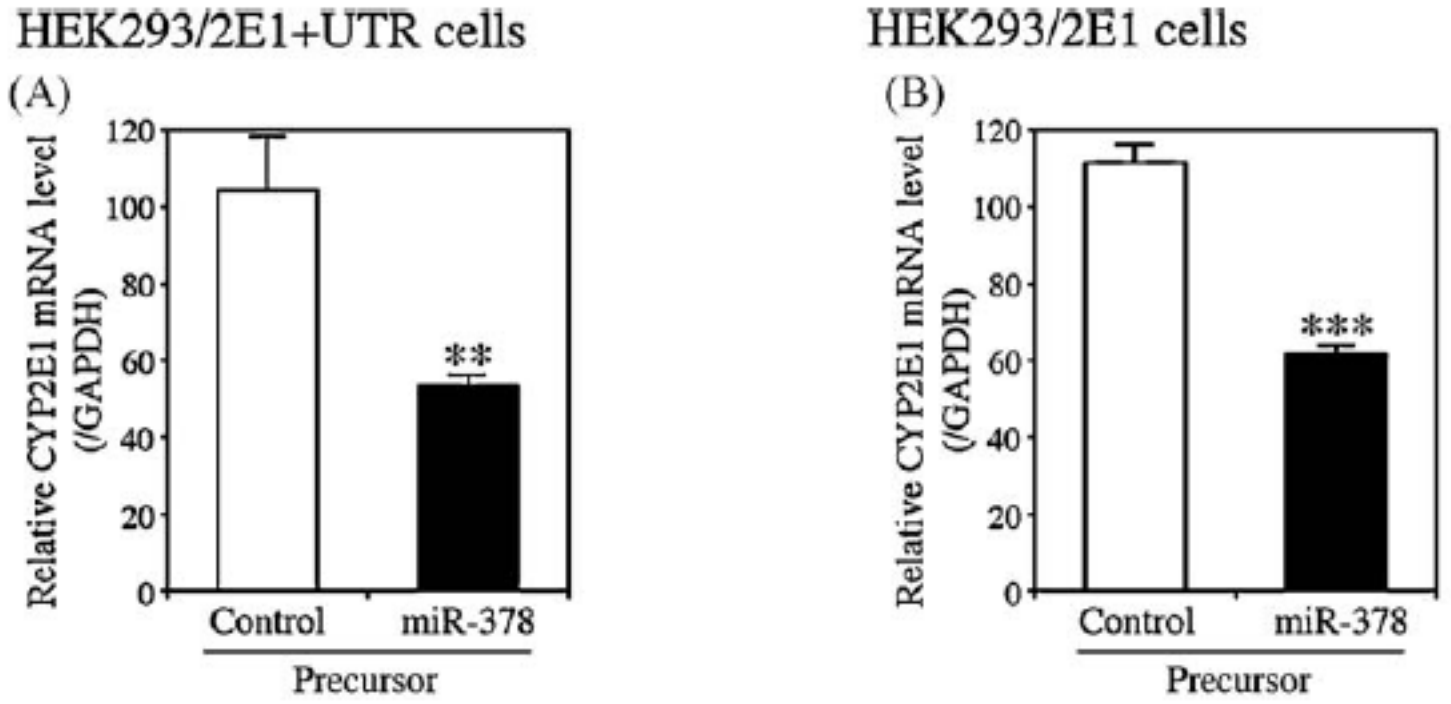
Regulation of CYP2E1 by miRNA



- A growing body of evidence indicates that CYP2E1 is regulated by multiple miRNA, including miRNA-378, and miRNA-570. Variation in the 3'-flanking region results in differential expression.

Nakano et al, DMD, 2015

Regulation of CYP2E1 by miRNA-378



- Mohri et al (Biochem Pharmacol, 2010) provided evidence that CYP2E1 is regulated by miRNA-378
- Speculated that the effects of xenobiotics and disease (diabetes, steatosis) on CYP2E1 levels may be mediated by repression of miRNA-378