Human CYP3A4 and Murine Cyp3A11 Are Regulated by Equol and Genistein via the Pregnane X Receptor in a Species-Specific Manner1–3

Yilan Li,4,7 Jennifer S. Ross-Viola,4 Neil F. Shay,5 David D. Moore,6 and Marie-Louise Ricketts5*

1Department of Biological Sciences, University of Notre Dame, South Bend, IN 46556; 2Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611; and 3Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

Abstract
Pregnane X receptor (PXR) is an important component of the body’s adaptive defense system responsible for the elimination of various toxic xenobiotics. PXR activation by endogenous and exogenous chemicals, including steroids, antibiotics, bile acids, and herbal compounds, results in induction of drug metabolism. We investigated the ability of the isoflavones genistein, daidzein, and the daidzein metabolite equol to activate human and mouse PXR in vitro using cell-based transient transfection studies and primary hepatocytes and in vivo in a mouse model. In transient transfection assays, the isoflavones genistein and daidzein activate full-length, wild-type mouse PXR, but not a mutant form, with genistein being the most potent. In contrast, equol was a more potent activator of human PXR than genistein or daidzein. In a mammalian 2-hybrid assay, isoflavones induced recruitment of the coactivator steroid receptor coactivator 1 to PXR. When tested against the native human Cytochrome P450 3A4 (CYP3A4) promoter, equol was the more potent activator and treatment of human hepatocytes with equol increased CYP3A4 mRNA and immunoreactive protein expression. Treatment of wild-type, but not PXR2/2, mouse hepatocytes showed that genistein and daidzein induced the expression of Cytochrome P450 3A11 (Cyp3A11) mRNA, whereas equol had no effect. Cyp3A11 mRNA was also induced in vivo in mice fed a soy protein-containing diet. The results presented herein demonstrate that there is a species-specific difference in the activation of PXR by isoflavones and equol. J. Nutr. 139: 898–904, 2009.

Introduction
Isoflavones present in soy have been shown to be bioactive compounds. When significant quantities of isoflavones are consumed as a component of soy or as a dietary supplement, their bioactivity has been proposed to favorably affect blood lipid levels, symptoms of menopause, osteoporosis, and even the development or progression of cancer (1–6). The widespread availability of isoflavone-containing supplements suggests that some individuals are exposed to these compounds at levels that well exceed those in individuals consuming soy products as part of their diet. Isoflavones may exert a hypocholesterolemic effect at least in part by modifying the activity of certain lipid-regulating transcription factors. For example, isoflavones have been shown to affect lipid metabolism via the activation of PPARα and -γ (7–9) and affect the expression of HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, through sterol regulatory element binding protein regulation in HepG2 cells (10).

Equol [7-hydroxy-3(4′-hydroxyphenyl)-chroman] is the major metabolite of the phytoestrogen daidzein and recent studies have shown that equol itself may be an important biologically active molecule in humans (11). The protective effects of soy protein observed with respect to cardiovascular disease and bone and menopausal health may in part be due to the biotransformation of soy isoflavones into equol (11).

The pregnane X receptor (PXR, NRI1)8 is a member of the superfamily of nuclear hormone receptors (NHR) that regulates transcription...
transcription by binding as a heterodimer with retinoid X receptors to DNA response elements in the regulatory regions of target genes. Metabolism and detoxification of foreign compounds, termed xenobiots, is a major function of the liver and PXR is a key regulator of xenobiotic metabolism. Disruption of PXR in mice results in severe defects in such responses, including an inability to upregulate Cytochrome P450 3A (Cyp3A) and Cyp2B expression in response to xenobiotic stimuli (12,13). In addition to the liver, PXR is also expressed in the intestine and in these 2 tissues, it is activated by a wide range of chemically and structurally diverse compounds, including various xenobiots [e.g. rifampicin (RIF), natural and synthetic steroids [e.g. progesterone 16α-carbonitrile (PCN) and dexamethasone], and bile acids [e.g. lithocholic acid]. These unrelated compounds compete for binding to PXR within the ligand-binding pocket, with the majority having dissociation constant values in the micromolar range (14). Consequently, in contrast to other NHR that bind to 1 or 2 ligands with a high affinity, PXR can bind a wide range of ligands with lower affinity. PXR shows the highest degree of cross-species sequence diversity of any of the vertebrate NHR, with differences in amino acid sequence identity leading to differences in response to xenobiots (12,13), accounting for the ability of different compounds to activate drug metabolism in different species.

In response to the wide variety of activating compounds, PXR coordinately regulates the expression of a spectrum of genes involved in the metabolism, transport, and, finally, elimination of those compounds from the body. CYP are regulated by a variety of mechanisms and the transcription of many of these genes is induced by xenobiots and endogenous compounds (15). CYP3A4 is a phase I enzyme that is responsible for the metabolism of endogenous steroids and many drugs and is the predominant Cytochrome P450 expressed in human liver. The CYP3A4 gene product catalyzes the hydroxylation of a broad range of substrates, rendering these compounds more hydrophilic and therefore subject to hepatic clearance (16). Human CYP3A4 and the murine homolog Cyp3A11 are the major CYP regulated by PXR. Soy isoflavones have been shown to affect the expression and activity of drug-metabolizing enzymes and multiple studies have demonstrated the regulation of several isoforms of Cyp1, 2, and 3 subfamily members by isoflavones in rodent models (17–20).

CYP3A4 is critically important in drug metabolism, being responsible for the metabolism of ~50% of all prescription drugs (21). Consequently, the present study is especially important for individuals concurrently using prescription medication and isoflavone-containing supplements. Because our previous observations using the Gal4-based assay demonstrated that the ligand-binding domain (LBD) of mouse PXR is activated by soy isoflavones (22), we investigated the ability of soy isoflavones to activate full-length mouse and human PXR and the subsequent effect on the expression of murine Cyp3A11 and human CYP3A4 in primary hepatocytes, as well as the expression of hepatic Cyp3A11 expression in vivo in mice consuming a soy protein-containing diet.

Materials and Methods

Materials. Purified genistein, daidzein, and equol were purchased from Indofine Chemical Company. PCN and RIF were purchased from Sigma Chemical. Mouse diets consisted of an isoflavone-stripped, ethanol-washed soy protein (LIS: catalog no. FSX-H0140; soy isoflavone concentration, ~4 mg/kg diet), or a soy protein diet containing isoflavones (HIS: catalog no. IB1.2UN30CA; soy isoflavone content, ~800 mg/kg diet) and were a gift from the Solae Co. (Dupont Protein Technologies International).

Plasmids. Full-length murine (23) and human PXR (24) were kindly provided by Dr. Steven A. Kliewer (University of Texas Southwestern Medical Center, Dallas, TX) and the PXR activation function 2 (AF2) mutants for mouse (E424K) and human (E427K) were described previously (25). The human CYP3A4 promoter-luciferase reporter plasmid was a gift from Dr. Christopher Liddle (University of Sydney at Westmead Hospital, Australia) (26). The pSV-β-galactosidase expression vector (Promega) was used as an internal control.

Cell culture. The human hepatocellular carcinoma cell line HepG2 and the African green monkey kidney cell line, CV-1, were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in antibiotic-free DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine and incubated at 37°C and 5% CO₂.

Transient transfections. For transfection assays assessing the effect of the isoflavones on wild-type and mutant PXR, cells were plated in 24-well plates in DMEM supplemented with 10% charcoal-stripped serum at a density of either 100,000 (CV-1) or 130,000 (HepG2) cells per well and were transfected using the calcium phosphate precipitation method, as previously described (22,25). Ligands were added for 24 h and luciferase and β-galactosidase assays were performed. Similar results were obtained from at least 3 independent experiments performed in triplicate.

To assess the effect on the full-length human CYP3A4 promoter, HepG2 cells were transfected using Lipofectamine 2000 (Invitrogen) as previously described (27). Typically, transfections included 300 ng of wild-type hPXR, 300 ng of the CYP3A4-luciferase reporter, and an equal amount of pSV-β-galactosidase plasmid as the internal control. Similar results were obtained from at least 2 independent experiments performed in triplicate.

Primary human hepatocytes. Primary human hepatocytes were obtained through the Liver Tissue Procurement and Distribution System (NIH contract no. NO1-DK-9–2310). Hepatocytes were cultured in serum-free Williams’ E Medium supplemented with 100 nmol/L dexamethasone and 1% insulin-transferrin-selenium (ITS-G; Invitrogen).

Human hepatocytes were treated with control [media containing dimethyl sulfoxide (DMSO) or DMSO + ethanol], RIF, as the positive control, or individual isoflavones for 24 (mRNA analysis) or 48 h (protein expression). DMSO or DMSO + ethanol was always ~0.1% (w/v). After treatments, cells were harvested for protein or RNA isolation.

There were variations in the expression levels of CYP3A4 immunoreactive protein following treatment with various isoflavones among individual hepatocyte donors, which were not dependent on age or gender. Five different hepatocyte preparations were tested and the results presented herein are a representation of consistent results obtained from those 5 hepatocyte preparations.

Isolation of mouse hepatocytes. Primary mouse hepatocytes were prepared using a modified version of the 2-step perfusion technique developed by Seglen (28). After isolation, the cells were plated in supplemented William’s E medium on collagen-coated dishes at a density of 1 million cells/60-mm dish in 3 mL media. Cells were allowed to attach overnight at 37°C, after which media was replaced every 3 days and William’s E medium containing treatments and incubated at 37°C. After 24 h, total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions.

Immunoblotting. Primary human hepatocytes were plated in medium without serum for 48 h and then incubated with different treatments for a further 48 h. After the treatment period, cells were lysed, protein was isolated, and protein concentrations were determined using a DC protein assay (BioRad). Immunoblotting was performed as previously described (27) using anti-human CYP3A4 (57) and anti-β-actin antibodies (Chemicon). The enhanced chemiluminescence-plus chemiluminescent detection system (Amersham) was used to identify the proteins of interest and quantification of the relative amount of protein/ band was determined by densitometric scanning and analysis using the Quantity One System (BioRad).

Real-time PCR. Five micrograms of total RNA was used to make first-strand cDNA with Moloney murine leukemia virus RT (Invitrogen)
using random primers. Real-time PCR were performed using a Sybrgreen PCR master mix (Applied Biosystems) in an ABI Prism 7700 sequence detector system (Applied Biosystems). Primer sequences are listed in Supplemental Table 1. The results of cycle threshold were plotted into the standard curve separately and the final value of the target gene was normalized to 18s (mouse) or β-actin (human).

**Mouse diet studies.** Wild-type mice purchased from the Jackson Laboratory (strain 129S1/SvImJ, catalog no. 002448) were housed under standard conditions and consumed food (Purina rodent diet no. 5015) (27,29) and water ad libitum. Experimental procedures were approved by the local Committee for Care and Use of Laboratory Animals at the University of Notre Dame. Age-matched groups of 11- to 12-wk-old male mice were used in all experiments (n = 7–9 per experimental group). At 11 wk of age, the mice were acclimated to a powdered diet containing LIS (soy isoflavone content −4 mg/kg diet) for 1 wk. At 12 wk of age, the mice were randomly assigned to 2 groups: group 1, control diet of soy protein stripped of isoflavones (LIS), or group 2, a soy protein diet with intact, endogenous levels of isoflavones (HIS: soy isoflavone content 800 mg/kg diet), and were fed for 6 wk. Diet formulations were according to Mezei et al. (29) and contained 35% of the energy from fat, 20% protein, 45% carbohydrate, and 1.25% cholesterol. At the end of the experiment, diets were removed for 16 h prior to killing the mice. Liver tissue was collected, mRNA was isolated, and gene expression was measured by real-time PCR, as described above.

**Statistical analysis.** All transfection experiments were analyzed by 1-way or 2-way ANOVA, as indicated and Newman-Keuls method post hoc comparisons. One-way ANOVA and LSD post hoc comparisons were used to analyze the data obtained using primary hepatocytes. One-way ANOVA was used to analyze the experimental data obtained from the in vivo mouse studies. Data are presented as mean ± SEM. Differences were considered significant at P < 0.05.

**Results**

The effect of isoflavones on PXR directed-luciferase expression. The ability of the individual isoflavones, genistein, daidzein, and the daidzein metabolite, equol, to activate human and mouse PXR was evaluated in an in vitro transient transfection system. When using a synthetic liver x receptor response element (LXRE)3-luciferase reporter construct (30), genistein, equol, and daidzein all significantly activated full-length wild-type mouse PXR to a level comparable to or less than that with PCN, an established mouse PXR agonist. Both genistein and equol had dose-dependent activation, with a greater effect due to genistein. These responses were dependent on PXR, because there was no activation when we used the mouse PXR mutant, E424K, which contains a mutation in the AF2 domain and is therefore defective in activation when we used the human PXR mutant, E427K, which contains a mutation in the AF2 domain (Fig. 1A). In addition, no transactivation occurred when the pSG5 empty vector was used in place of the pSG5-mouse PXR plasmid (data not shown). Consistent results were also obtained using HepG2 cells (data not shown).

Equol and genistein significantly transactivated full-length, wild-type human PXR when using the (LXRE)3-luciferase reporter, although to a lesser degree than RIF, a well-established human PXR agonist. The activation was dependent on PXR, because no activation occurred when the human PXR mutant, E427K, which contains a mutation in the AF2 domain (Fig. 2), or the empty pSG5 vector was used (data not shown). Consistent results were also obtained using HepG2 cells (data not shown).

Recruitment of the coactivator steroid receptor coactivator-1 to PXR. To further assess the ability of the individual isoflavones to act as ligands for either human or mouse PXR, a mammalian 2-hybrid assay was used to test their ability to induce coactivator recruitment. In this assay, Gal4 is fused with the coactivator steroid receptor coactivator-1 (SRC-1) and either mouse (Fig. 3A) or human PXR (Fig. 3B) was fused with the transactivator VP16. The ability of genistein, equol, daidzein, and PCN to direct high levels of luciferase expression in a dose-dependent manner indicates that they promote interaction of mouse PXR with the coactivator (Fig. 3A). Differences were observed for isoflavone concentration and isoflavone treatments (P < 0.001). For example, equol potently recruited SRC-1 to human PXR in a dose-dependent manner equivalent to that with RIF. However, the ability of genistein to recruit SRC-1 to human PXR was much weaker than that of RIF and equol (Fig. 3B).
The native human CYP3A4 promoter. The responsiveness of the human CYP3A4 promoter, a known human PXR target gene, was analyzed. Genistein, daidzein, and equol all significantly induced the activity of the human CYP3A4 luciferase reporter (Fig. 4). Equol induced luciferase-driven promoter activity in a dose-dependent manner and was the most potent activator of the endogenous human PXR in these cells, with induction comparable to that with RIF.

CYP3A4 mRNA and protein levels in primary human hepatocytes. Primary human hepatocytes were chosen to further study the effects of isoflavones on endogenous CYP3A4 mRNA and immunoreactive protein levels. RIF was used as a positive control while equol, genistein, and daidzein were tested for their ability to induce CYP3A4 expression. In agreement with previous studies, RIF significantly induced CYP3A4 mRNA levels to 370 ± 51.8% of the control, whereas equol was the only isoflavone to significantly induce CYP3A4 mRNA expression to 145 ± 11.2% of the control, normalized to 18s (n = 5). CYP3A4 mRNA levels were unaffected by treatment with genistein or daidzein (data not shown). Consistent with these observations, RIF induced CYP3A4 immunoreactive protein expression (4100 ± 200% of the control; P < 0.05) normalized to β-actin expression (n = 5). Equol was the only isoflavone to induce CYP3A4 protein expression: 1030 ± 350% of the control (P < 0.05); genistein: 630 ± 320% of the control; daidzein: 340 ± 170% of the control (n = 5).

Cyp3A11 mRNA expression in wild-type and PXR knockout primary mouse hepatocytes. To further assess the effect of isoflavones on Cyp3A11 mRNA expression levels in the mouse, we used primary hepatocytes isolated from both wild-type and PXR-null mice. Hepatocytes were treated with DMSO, 10 μmol/L PCN, genistein, daidzein, or equol for 24 h. After 24 h, total RNA was isolated and real-time quantitative PCR was performed to measure Cyp3A11 mRNA expression, using...
and human PXR. Transient transfection studies using a synthetic sequence differences between PXR in different mammalian species also observed activation of the LBD of mouse PXR.

Following this initial observation, we subsequently determined the specificity of individual soy isoflavones to activate the LBD of PPAR and PPAR using a Gal4-based transient transfection system (22). The isoflavones daidzein and genistein activate mouse PXR. Genistein was the most potent treatment with genistein and daidzein induced Cyp3A11 mRNA expression to 5-fold (P < 0.05) and 3-fold (P < 0.05) of the DMSO control, respectively. Equol treatment did not significantly induce Cyp3A11 mRNA levels (Fig. 5). All observed increases in Cyp3A11 mRNA expression were abolished in hepatocytes obtained from PXR null mice.

**Murine hepatic Cyp3A11 gene expression in vivo.** Wild-type (SV/129) mice were fed a diet with either a LIS or HIS diet for 6 wk (29). The effects on hepatic expression of Cyp3A11 mRNA and another PXR-target gene, organic anion-transporting polypeptide c (Oatpc; also known as Oatp1b2, slco1b2) (31), were measured. The expression of both of these genes was induced in the livers of mice following the HIS diet: Cyp3A11: 5.7 ± 0.4-fold of the LIS control diet; Oatpc: 3.2 ± 1.1-fold of the LIS control diet (P < 0.05), normalized to 18s (n = 7–9).

**Discussion**

Bioactive properties of dietary soy have been suggested in numerous studies, but there is still no clear consensus regarding the soy component(s) that are responsible for the observed effects. Soy isoflavones exist as glycines in nature and following ingestion are hydrolyzed by intestinal glucosidases, producing the aglycones genistein, daidzein, and glycitein. Based on the hypothesis that soy isoflavones may activate NHR and that this could be one underlying mechanism behind the bioactivity of soy, we previously confirmed activation of PPAR by soy isoflavones (9). Following this initial observation, we subsequently determined the specificity of individual soy isoflavones to activate the LBD of other NHR using a Gal4-based transient transfection system (22). In addition to activation of LBD of both PPARα and PPARγ, we also observed activation of the LBD of mouse PXR.

Because variations in xenobiotic response result from sequence differences between PXR in different mammalian species (12,14), we tested the ability of isoflavones to activate mouse and human PXR. Transient transfection studies using a synthetic (LXRE)2-luciferase reporter demonstrated that genistein, equol, and daidzein activate mouse PXR. Genistein was the most potent inducer, with a dose-dependent induction comparable to that of PCN, a potent ligand of mouse PXR. The responses in all cases were dependent on PXR activation, because they were not observed when using a mutant form of the receptor (Fig. 1) or when using the pSG5 empty vector (not shown). The E424K mutation is located in the AF2 transactivation domain, where ligand binding and coactivator recruitment occurs. Abolishment of transactivation when using the AF2 mutant clearly indicates the absolute requirement of PXR for the observed transactivation. In comparison, human PXR was activated more significantly by equol than genistein or daidzein under the conditions tested.

Ligand-dependent recruitment of coactivators to NHR plays an important role in transcriptional activation of target genes. Consequently, using a mammalian 2-hybrid assay, we assessed the ability of the soy isoflavones to recruit the coactivator SRC-1 to PXR. Genistein, equol, and daidzein recruited SRC-1 to mouse PXR, although less potently than PCN (Fig. 3A). In comparison, equol recruited SRC-1 to human PXR as robustly as RIF (Fig. 3B). The observation that isoflavones recruit coactivator is consistent with their function as agonist ligands for PXR. Although the concentrations of isoflavones used in these particular assay systems are above physiological levels observed following dietary intake, it is not unusual to test drugs and other potential ligands at higher concentrations in reporter gene assay systems (32,33), particularly when using a synthetic reporter such as the one used in this case. For example, RIF is generally used at a concentration of 10 μmol/L to elicit a significant in vitro response.

When the native CYP3A4 promoter was tested, a robust, dose-dependent activation by equol was observed (Fig. 4), which was greater than that seen when using the synthetic LXRE-response element reporter (Fig. 2). The enhanced activation observed at lower doses of isoflavones when using the CYP3A4 promoter compared with the synthetic LXRE-reporter, may relate to the presence of both the distal enhancer module (XREM) and elements located in the proximal promoter region that direct human PXR-mediated transactivation of CYP3A4 (26). This may more closely resemble in vivo gene regulation. Activation of the CYP3A4 promoter with 10 μmol/L equol was comparable to that seen with 10 μmol/L RIF under the conditions tested (Fig. 4), indicating that equol is capable of inducing CYP3A4 expression. Reports suggest that serum levels of isoflavones could reach...
1 μmol/L or more following intake of dietary soy supplements (34) and the concentrations of isoflavones tested in the present study are consistent with previous work assessing the activation of PPAR in transient transfection studies (8,9).

Genistein and daidzein act via PXR in primary mouse hepatocytes to induce Cyp3A11 expression; however, no effect on Cyp3A11 expression by equol was observed (Fig. 5), consistent with the transfection results. In primary human hepatocytes, equol induced CYP3A4 expression, again consistent with transient transfection studies. Overall, the in vitro data presented herein indicates that equol is a more potent inducer of human PXR than the other isoflavones tested, which contrasts with the effects of the isoflavones on mouse PXR. These observations are consistent with species-specific differences in Cytochrome P450-mediated drug and steroid metabolism. Although the presence of dexamethasone in hepatocyte cultures may diminish induction of CYP3A by the isoflavones, as recently proposed (35), we still see an induction by both RIF and equol in human hepatocytes and for genistein and daidzein in wild-type mouse hepatocytes, a response that is clearly lost when using hepatocytes from PXR knockout mice.

To further establish the physiological relevance of our in vitro observations, we fed male wild-type mice a diet containing either low or endogenous levels of soy isoflavones and observed a significant induction in Cyp3A11 and Oatpc (also known as Oatpl1b2, slco1b2) (36) mRNA expression in the liver. The concurrent induction in CYP3A4 and OATP expression would be expected to enhance the transport and metabolism of drugs subject to metabolism by CYP3A4, one example being atorvastatin. It is also possible that isoflavones could affect intestinal CYP3A4 activity, thereby affecting hepatic exposure and should therefore be considered in future studies.

Soy isoflavones are established phytoestrogens, consequently an important question is whether isoflavones may activate both ERβ and PXR in vivo in humans. Based on the combined observations from our in vitro cell based studies and in vivo feeding study in wild-type mice, it is clear that consumption of dietary soy can activate PXR resulting in induction in Cyp3A11 expression. It is understood that there are substantial differences between a rodent diet containing 20% soy protein and a human diet characterized by only occasional intake of soy foods. Therefore, we propose that our results are more pertinent to habitual consumers of soy foods and/or those individuals using isoflavone supplements.

Based on our observations, it is possible that the concurrent intake of soy isoflavones with prescription medication may subsequently affect the pharmacokinetic profile and hence bioavailability of such coadministered drugs. Use of reporter-gene assays is a well-accepted methodology for accurately predicting possible clinically relevant drug-drug interactions. This methodology allows identification of weak PXR activators that in a clinical setting are known to cause drug-drug interactions as a result of CYP3A4 induction, e.g. tamoxifen, cyclophosphamide, and ifosfamide (37). However, bridging the gap between in vitro induction in a cell-based assay and CYP3A4 induction in humans presents serious challenges. Luo et al. (33) showed that data obtained from a human PXR reporter-gene assay correlates well with data showing an induction in CYP3A4 expression in human hepatocytes. Our study demonstrated an association between data from (1) a PXR reporter-gene assay (2), CYP3A4 induction in primary human hepatocytes (3), Cyp3A11 induction in primary mouse hepatocytes, and (4) in vivo following a feeding study. In summary, because PXR regulates drug-metabolizing enzymes, regulation of PXR by bioactive dietary factors is of considerable importance. Consequently, pharmacokinetics and drug disposition could be modulated by dietary factors via interaction with and activation of PXR. Although still controversial, the consumption of soy isoflavones may have a LDL-cholesterol-lowering effect, particularly in individuals with high serum cholesterol concentrations. Considering the fact that many of these individuals may be taking cholesterol-lowering medication along with a soy isoflavone supplement, it is important to understand whether consumption of soy isoflavones alters the half-life and efficacy of such prescription medications. Based on the evidence supporting the physiological and clinical importance of the daidzein metabolite equol and the observations presented herein, we suggest the hypothesis that isoflavone activation of PXR and the subsequent upregulation in CYP3A4 is potentially significant and requires further investigation.

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Literature Cited


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