Garcinoic Acid Is a Natural and Selective Agonist of Pregnane X Receptor

Desirée Bartolini, Francesca De Franco, Pierangelo Torquato, Rita Marinelli, Bruno Cerra, Riccardo Ronchetti, Arne Schön, Francesca Fallarino, Antonella De Luca, Guido Bellezza, Ivana Ferri, Angelo Sidoni, William G. Walton, Samuel J. Pellock, Matthew R. Redinbo, Sridhar Mani, Roberto Pellicciari, Antimo Gioiello, Francesco Galli

Submitted date: 19/12/2019 • Posted date: 23/12/2019
Licence: CC BY-NC-ND 4.0
Citation information: Bartolini, Desirée; De Franco, Francesca; Torquato, Pierangelo; Marinelli, Rita; Cerra, Bruno; Ronchetti, Riccardo; et al. (2019): Garcinoic Acid Is a Natural and Selective Agonist of Pregnane X Receptor. ChemRxiv. Preprint. https://doi.org/10.26434/chemrxiv.11409396.v1

Pregnane X receptor (PXR) is a master xenobiotic-sensing transcription factor with a key role in drug metabolism and disposition. Its activity regulates a number of physiological processes in the liver and intestine, and it is now a validated target for human diseases associated with inflammation and dysregulation of the immune system. The identification of chemical probes to investigate the therapeutic relevance of the receptor is still highly desired. In fact, currently available PXR ligands are not highly selective and can exhibit toxicity and/or potential off-target effects. In this study, we have identified the naturally-occurring garcinoic acid as a selective and efficient PXR agonist. The properties of garcinoic acid as a specific PXR agonist was demonstrated using different approaches - screening on a panel of nuclear receptors, physical and thermodynamic evaluation of binding affinity, and co-crystallization study. Cytotoxicity assays, transcriptional and functional experiments were carried out in human liver cells, in mouse liver and gut tissue to prove compound activity and target engagement. Taken together, these data support the conclusion that garcinoic acid efficiently activates PXR and may prove to be an amenable lead toward the development of differentially acting PXR regulating compounds.
Garcinoic Acid Is a Natural and Selective Agonist of
Pregnane X Receptor

Desirée Bartolini,§‡ Francesca De Franco,§‡ Pierangelo Torquato,§ Rita Marinelli,§ Bruno Cerra,§ Riccardo Ronchetti,§ Arne Schon,§ Francesca Fallarino,Ω Antonella De Luca,Ω Guido Bellezza,§ Ivana Ferri,‡ Angelo Sidoni,‡ William G. Walton,¶ Samuel J. Pellock,¶ Matthew R. Redinbo,¶ Sridhar Mani,∞ Roberto Pellicciari,† Antimo Gioiello,*§ Francesco Galli,*§

§ Department of Pharmaceutical Sciences, University of Perugia, 06122 Perugia, Italy. † TES Pharma, Taverne di Corciano, 06073 Perugia, Italy. ¶ The Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA. Ω Department of Experimental Medicine, University of Perugia, 06129 Perugia, Italy. ≠ Section of Anatomic Pathology and Histology, Department of Experimental Medicine, University of Perugia, 06129 Perugia, Italy. ¶ Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, USA. ∞ The Departments of Biochemistry, Medicine, Genetics and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA.

KEYWORDS. Garcinoic acid, Pregnane X receptor, vitamin E, tocopherols, tocotrienols, X-ray crystal structure, nuclear receptors, pharmacology.
ABSTRACT. Pregnane X receptor (PXR) is a master xenobiotic-sensing transcription factor with a key role in drug metabolism and disposition. Its activity regulates a number of physiological processes in the liver and intestine, and it is now a validated target for human diseases associated with inflammation and dysregulation of the immune system. The identification of chemical probes to investigate the therapeutic relevance of the receptor is still highly desired. In fact, currently available PXR ligands are not highly selective and can exhibit toxicity and/or potential off-target effects. In this study, we have identified the naturally-occurring garcinoic acid as a selective and efficient PXR agonist. The properties of garcinoic acid as a specific PXR agonist was demonstrated using different approaches - screening on a panel of nuclear receptors, physical and thermodynamic evaluation of binding affinity, and co-crystallization study. Cytotoxicity assays, transcriptional and functional experiments were carried out in human liver cells, in mouse liver and gut tissue to prove compound activity and target engagement. Taken together, these data support the conclusion that garcinoic acid efficiently activates PXR and may prove to be an amenable lead toward the development of differentially acting PXR regulating compounds.
1. Introduction

Pregnane X receptor (PXR or NR subfamily 1, group I, member 2, NR1I2) is universally recognized as a master regulator of key xenobiotic and drug metabolizing genes,\textsuperscript{1,2} such as cytochrome P450 isoform 3A4 (CYP3A4), several phase II genes and the multidrug resistance protein 1 (MDR1).\textsuperscript{3,4} The PXR gene cloned in 1998 by two independent groups\textsuperscript{5,6} is principally expressed in the liver and intestine.\textsuperscript{7,8} Its activation can be elicited by a wide number of ligands\textsuperscript{3,4} that include endogenous steroids such as lithocholic acid and many other cholesterol metabolites, several classes of drugs and natural products, such as antibiotics, anticancer agents, the antihypertensive nifedipine, and antifungals such as clotrimazole, herbal medicines as the antidepressant hyperforin from St. John’s wort,\textsuperscript{9} gut microflora products, such as indole 3-propionic acid,\textsuperscript{10,11} some dietary phenolics and phytosterols that include the fat-soluble vitamin E.\textsuperscript{12,13} At the same time, a limited number of PXR antagonists and activity inhibitors have also been identified,\textsuperscript{4,14} and include the drugs ketoconazole and A-792611, and the natural compounds sulforaphane, sesamine, coumestrol and camptothecin, some of which have been investigated in overcoming drug resistance.

Besides transcriptional regulation of drug metabolizing genes, PXR activity and its interaction with other nuclear receptors (NRs) and transcription factors appear to influence important physiological processes of the liver and small intestine, such as the regulation of inflammatory and metabolic pathways, and the preservation of intestinal wall integrity.\textsuperscript{15,17}

Over the years, an intense research has been devoted to investigate the potential of PXR as pharmacological target of human pathologies, such as cholestatic liver disease,\textsuperscript{18} inflammatory bowel disease (IBD)\textsuperscript{19,20} and dyslipidemia.\textsuperscript{21} However, ligand promiscuity is a major obstacle in
the pharmacological approach to such NRs as most of known PXR modulators have other primary targets, and PXR itself may be considered an off-target in drug development.22

Studies on the antibiotic rifaximin (Xifaxan),19 a PXR agonist also used to treat diarrhea23 and hepatic encephalopathy,24 demonstrated the efficacy of this drug in the treatment of irritable bowel syndrome (IBS). Such an application received FDA approval in May 201525 and further studies demonstrated therapeutic effects that were independent of its antibiotic activity on microbiota.26 As a consequence, PXR has emerged as a molecular target for the treatment of this syndrome. Therefore, the identification of novel and selective PXR agonists holds great potential in the development of more efficient therapeutic protocols.

Recent studies suggested that some forms of vitamin E (vitamers or metabolites) might fulfil this aim. Podsun et al.27 demonstrated in LS 180 human colorectal adenocarcinoma cells that α-tocopherol 13’-carboxylic acid (2), a long-chain metabolite (LCM) of α-tocopherol (3),28,29 and δ-tocotrienol (4) (Chart 1),30,31 show PXR agonist function up-regulating the PXR-dependent gene p-glycoprotein. Other studies by some of us32 demonstrated an increased PXR expression in mouse brain and in vitro in embryonal cortical astrocytes treated with a natural analogue namely garcinoic acid (GA, 1) (Chart 1), firstly described as a bioactive compound by Mazzini and colleagues.33 This is a plant δ-tocotrienol derivative with different African ethno-medicine applications.33,34 With this premise, herein we aim to explore the activity of GA (1) as PXR agonist. To pursue this aim, we will realize the isolation of GA (1) from Garcinia Kola seeds and use the pure compound for metabolite synthesis and characterization (Chart 1). Most importantly, binding assays, gene expression profile, co-crystallization and in vivo experiments, will be carried out to prove the efficacy of GA (1) as PXR agonist and target engagement.
2. Results

2.1 Isolation of Garcinoic Acid and Metabolite Synthesis

Initially, we aimed to revise standard extraction procedures from *Garcinia kola* seeds for isolating sufficient amount of pure GA (1) for compound characterization and metabolite synthesis. Current extraction methods are based on Terashima protocol and consist on the alcoholic extraction of the seed followed by silica gel chromatography. Using our optimized approach, we were able to increase the purified GA (1) on multigram scale. Three different
parameters have been investigated to improve yield extraction: seeds/solvent ratio (1:1 or 2:1, w/v), temperature (25 °C, 40 °C, 60 °C), and solvent (MeOH or EtOH) (see Table S2, Supporting Information). The best conditions were obtained using MeOH as the solvent, at 25 °C and with a seeds/solvent ratio 1:1, w/v (Entry 3, Table S2). Using the optimized protocol, 1.2 Kg of finely ground *G. Kola* seeds were extracted affording 9.4 g of garcinoic acid (1) (0.78% yield w/w).

The synthesis of metabolites 2, 5-7 has been realized according to Scheme 1A. In particular, the hydrogenolysis reaction of GA (1) under flow conditions using a packed Pd/C cartridge afforded compound 5 in quantitative yield. Next, reduction of the carboxylic group by means of LiAlH₄ in anhydrous THF at r.t. gave 13’-hydroxy-δ-tocopherol (7) in 87% isolated yield. 13’-carboxy-δ-tocopherol (5) was also reacted with (CH₂O)ₙ in the presence of SnCl₂ and HCl 12 M in Et₂O at 70 °C to obtain 13’-hydroxy-α-tocopherol (2) (92% yield) readily reduced to the corresponding alcohol derivative 6 with LiAlH₄ in anhydrous THF (62% yield). The glucuronyl metabolite of GA (8) was prepared in a four step synthetic sequence (Scheme 1B). Thus, GA ester (9), prepared by reacting 1 with CH₂N₂ in Et₂O at -78 °C (89%), was coupled with methyl-1-bromo-2,3,4-tri- O-acetyl-α-D-glucuronate (10) and Fetizon reagent in toluene to give 11 in 19% overall yield after silica gel purification. Finally, mild basic hydrolysis (Na₂CO₃/MeOH, r.t.) furnished the desired glucuronide 8 in 17% isolated yield over three steps after flash chromatography.
Scheme 1. Synthesis of metabolites 2, 5-7. Reagents and conditions: (a) 10% Pd/C cartridge (s-cart, 30 × 4 mm i.d.), 1 bar (full H2 mode), 25 °C, 1 mL min⁻¹; (b) LiAlH4, THF, 0 °C -> r.t.; (c) SnCl2, HCl 12 M, Et2O, (CH2O)n, 70 °C; (d) CH2N2, Et2O, 0 °C -> r.t.; (e) methyl-1-bromo-2,3,4-tri-O-acetyl-α-D-glucuronate (10), Fetizon reagent, molecular sieves, toluene; (f) Na2CO3, MeOH, r.t.

2.2 Identification of garcinoic acid as a selective PXR agonist

First, ligand binding activity of GA (1) was screened by AlphaScreen technology over a panel of NRs that beside to hPXR (Figure 1A) included major human endocrine and metabolic receptors
such as the retinoid X receptor (RXR), constitutive androstan receptor (CAR), farnesoid X receptor (FXR), vitamin D receptor (VDR), liver X receptor (LXR) form α and β, and the PPAR isoforms α, δ, and γ (see Figure S1, Supporting Information). GA (1) was compared with its analogue δ-tocotrienol (4), the vitamers 3 and γ-tocopherol, their short chain metabolites (SCMs) α-CEHC and γ-CEHC, and the LCMs 2 and 7 (Chart 1). The test was performed in the presence of reference standard agonists for the different NRs. Among vitamin E compounds, only GA showed significant PXR agonist activity (see Figure S1A, Supporting Information). Much lower was the response of δ-tocotrienol (4), while all the other vitamin E compounds were almost completely inactive at the receptor. Most importantly, GA (1) showed a high selectivity for PXR, with only a low activity at the LXRβ receptor (see Figure S1B, Supporting Information). None of the other vitamin E compounds exhibited any appreciable agonist activity on this receptor.
Figure 1. PXR agonist activity and binding properties of GA. Binding activity of GA (1) was assessed by AlphaScreen test at 10 µM compound concentration (A) and during a dose-dependent experiment (B). Comparisons were made with a series of vitamin E compounds and the PXR agonist 0.05 µM T0901317 (see Table S1, Supporting information). Calorimetric titrations of GA binding to PXR-LBD (C) was investigated in comparison with 2 and 4 compounds dissolved in DMSO and then further diluted to 60 µM final concentration in 25 mM Hepes buffer, pH 7.5, containing 150 mM NaCl. The final concentration of PXR-LBD in the reaction mixture was 3 µM, and DMSO was 8 % v/v.
The activity of GA as PXR agonist was then evaluated in dose-response experiments in comparison with T0901317\textsuperscript{38} (Figure 2B); the resulting EC\textsubscript{50} values were 1.3 and 0.015 µM, respectively. Comparisons were also made with physiological LCM analogues 2, 5-8 (Table 1).

**Table 1.** Activity of GA (1) and metabolites 2, 5-8 on PXR as assessed by AlphaScreen test.\textsuperscript{a}

| Test molecule | EC\textsubscript{50} (µM) | Efficacy (%) |
|---------------|------------------------|-------------|
| T0901317     | 0.050                  | 100         |
| GA (1)       | 1.3                    | 103         |
| 2             | 3.3                    | 11          |
| 5             | 3                      | 18          |
| 6             | 1.5                    | 14          |
| 7             | 1.6                    | 24          |
| 8             | 17                     | 14          |

\textsuperscript{a}Data represent mean values ± SDs of at least three independent experiments.

All these compounds showed EC\textsubscript{50} values similar to GA (1) (between 1.5 and 3.3 µM) with the exception of the glucuronide 8 that showed an EC\textsubscript{50} of 17 µM, suggesting an interfering role of
phase II derivatization for GA agonist activity. The percentage of efficacy compared with T0901317 was 103% for GA and ≤ 24% for the other LCMs (Table 1); worth of note, the δ-configuration of the chromanol ring was associated with higher agonist efficacy compared with α-configuration.

Specificity of GA agonist activity for PXR was demonstrated by siRNA technique in HepG2 cells. When PXR gene was transiently inhibited, a reduction of both the PXR and CYP3A4 gene transcription response to GA treatment was confirmed (Figure 2A and 2B, respectively).

2.3 Calorimetric titration of garcinoic acid binding to the PXR ligand binding domain

To obtain direct proof of interaction with hPXR ligand binding domain (LBD), we performed an isothermic titration calorimetry (ITC) experiment using His-tagged hPXR LBD protein in solution. (Figure 1C). ITC measures the affinity, $K_a$, and Gibbs energy ($\Delta G = -RT\ln K_a$) and the changes in enthalpy, $\Delta H$, and entropy, $\Delta S$, associated with the binding of the FKK compounds ($\Delta G = -RT\ln K = \Delta H - T\Delta S$). Enthalpic and entropic contributions to binding affinity define the nature of the forces that drive the binding reaction. GA (1) binds to a single site in LBD ($K_a = 330$ nM, $\Delta G = -8.8$ kcal/mol, $\Delta H = -6.4$ kcal/mol, $-T\Delta S = -2.4$ kcal/mol, $N = 0.9$) (Figure 1C, left panel). In contrast, the other PXR inactive analogs, δ-tocotrienol (4) and α-tocopherol 13'-carboxylic acid (2), do not show any detectable binding to PXR LBD in this assay (Figure 1C, middle and right panel, respectively).
Figure 2. PXR and CYP3A4 expression in HepG2 cells treated with GA. siRNA technique was used to transiently inhibit PXR (A) or CYP3A4 (B) gene expression [* p<0.05; ** p<0.01 vs. WT or Ctr test; # p<0.01 vs. Rifampicin (RIF)]. PXR (C) and CYP3A4 (D) protein and mRNA (E) expression were also assessed in cells treated for 24 hrs with 1 and 25 μM GA. MDR1 mRNA expression (F) was evaluated at concentrations between 1 and 50 μM GA. P-glycoprotein activity (G) was measured in the presence of GA between 50 nM and 25 μM (circles) and Verapamil (triangles) was used as a control. t-test: control vs treatments, *p<0.05; **p<0.01.
2.4 Crystal structure of PXR-garcinoic acid complex

To determine the structural basis of GA-PXR binding interaction, crystals of the LBD of hPXR incubated with GA (I) were grown successfully. The resultant crystals diffracted x-rays to 2.3 Å resolution and revealed GA bound in a single orientation within the PXR ligand binding pocket of LBD (Figure 3; Table S2, Supporting Information). The fused ring moiety of GA contacts a set of three aromatic residues (F288, W299, and Y306) in the PXR LBD, forming an array of van der Waals contacts including face-to-face (F288) and edge-to-face (W299) π-π interactions. Other specific contacts include hydrogen bonds with the endocyclic oxygen and phenolic hydroxyl of GA with Q285 and the backbone of S247, respectively (Figure 3). Lastly, the carboxylate moiety of GA (I) forms an ionic interaction with H407. Taken together, the hPXR LBD specifically recognizes GA with numerous contacts that corroborate the potent binding of this ligand.
Figure 3. Crystal structure of hPXR-garcinoic acid complex. (A) Overview of hPXR LBD-garcinoic acid complex. (B) 2.3 Å resolution x-ray diffraction data of crystals demonstrated that GA (gray) binds in a single orientation within the ligand binding pocket of hPXR, contacted by four amino acid side chains (cyan) and one main chain region (M246-S247; cyan). Distances noted are in Å.

2.5 In vitro and in vivo target engagement

In vitro data. In order to confirm the activity of GA as a PXR agonist, PXR protein expression was investigated in tumoral (HepG2) and non-tumoral (HepaRG) human liver cell lines that were preliminarily investigated for compound toxicity (see Figure S2, Supporting Information). GA
increased in a dose-dose-dependent manner PXR protein expression in HepG2 cells (Figure 2C). CYP3A4 protein and mRNA expression (Figure 2D and 2E, respectively), and MDR1 gene expression and activity (Figure 2F and 2G, respectively) also increased in HepG2 cells.

Even higher was the response of PXR protein to GA treatment in non-tumoral HepaRG cells (see Figure S3A, Supporting Information). This activity of GA was significantly higher compared with that of the α-T OH metabolites 2 and 6 (see Figure S3A, Supporting Information). Moreover, GA reverts the PXR binding and antagonist effect of sulforaphane (see Figure S3C, Supporting Information). Immunoblot analysis in undifferentiated and differentiated (murine secreting) HT29 intestinal cells (Figure S5, Supporting Information) further confirmed that GA stimulates the protein expression of both PXR and CYP3A4.

The GA-induced stimulation of PXR protein expression was investigated for its functional consequences on the CYP450-mediated ω-oxidation and subsequent catabolism of α-T OH side chain. In HepG2 cells, GA treatment did not modify the levels of α-tocopherol (3) that was rapidly taken up compensating the depletion of this vitamin observed under standard culture conditions (see Figure S4A, Supporting Information). On the contrary, GA stimulated both the CYP450-mediated production and efflux of α-tocopherol metabolites (see Figure S4A-C, Supporting Information). The latter effect is in agreement with an effect of GA as activator of the PXR-dependent membrane transporter MDR1, which has been already described for other LCMs, such as 2.

**In vivo data.** GA toxicity and efficacy in activating PXR were investigated in mice after acute administration of increasing doses of GA in a single bolus. At observation (24 hrs after the treatment), mortality was 100 % in the group treated with 100 mg, and 33% in the group that
received 50 mg GA. Doses of ≤ 25 mg were well tolerated as suggested by the gross appearance
and behavior of the treated animals, and postmortem examination of liver tissue and all main
organs that revealed complete absence of signs of damage. A dose-dependent stimulation effect of
GA on PXR protein and mRNA was observed in the liver tissue in interval of dosage between 5
and 25 mg (Figure 4A and 4B, respectively). PXR mRNA also increased in intestine upon GA
treatment (Figure 5A). Such transcriptional response, however, was not sufficient to significantly
increase PXR protein expression in this tissue (not shown), possibly due to the much lower PXR
levels compared with liver tissue (Figure 4A). GA also increased CYP3A4 protein expression in
both these two tissues (Figure 4B and 5B).

The response of PXR protein to GA treatment in both these two tissues was confirmed by IHC
evaluation. In the hepatic parenchyma (Figure 4C), PXR was mainly localized in sinusoidal
endothelial cells. In mice treated with 25 mg GA, PXR staining also localized in hepatocytes,
particularly around centrolobular veins (zone 3) and the lobular midzonal area (zone 2). In small
bowel from control group mice, PXR expression localized exclusively in the lamina propria,
lymphocytes and plasma cells of intestinal villi, without any significant staining of enterocytes
(Figure 5C). On the contrary, in mice treated with GA a strong and diffuse PXR expression was
observed in villous epithelium (enterocytes) and focally also in the glandular epithelium of crypts
at the base of mucosa. Furthermore, a weak staining was present on the brush border of enterocytes
in mice treated with 10 mg of GA (Figure 5C).
Figure 4. PXR and CYP3A4 expression in the liver of mice treated with GA. Mice were treated with increasing doses of GA from 5 to 25 mg administered as a single bolus and PXR mRNA (A) and protein expression (B, left panel) were measured in liver samples 24 hr post-treatment. CYP3A4 protein was also investigated (B, right panel); *p<0.05; **p<0.01. Liver histology was examined by hematoxylin and eosin (H&E) staining and IHC for PXR antigen (C).
Figure 5. PXR and CYP3A4 expression in the intestine of mice treated with GA. PXR mRNA (A), CYP3A4 immunoblot (B) IHC of PXR antigen (C) in gut samples of mice treated with increasing doses of GA. Treatments and histology were as for Figure 4.
3. Discussion

In this study we demonstrate that GA (1) is a selective and efficient PXR agonist. To this aim, we have firstly optimized the extraction method to isolate 1 in sufficient amount and high purity to conduct metabolite synthesis and get insights into compound binding and activity at PXR. These properties of GA were demonstrated with different approaches including screening on a panel of NRs, physical and thermodynamic (calorimetric) evaluation of binding affinity, co-crystallization study, and then transcriptional and functional evaluation that was carried out in human liver cells and in mouse liver and gut tissue. Compared with the reference agonist T0901317, crystallographic data were indicative of GA binding potency that can be explained by the presence of several and stable interactions (Figure 3). These include van der Waals contacts between the chroman ring of GA and three aromatic residues (F288, W299, and Y306), hydrogen bonds established by the endocyclic oxygen and phenolic hydroxyl group of GA with residue Q285 and the backbone of S247, and the crucial ionic interaction of the carboxylate moiety of GA with H407 (Figure 4).

The transcriptional function of PXR affects genes encoding drug-metabolizing enzymes and transporters to essentially detoxify and eliminate xenobiotics and endotoxins.\textsuperscript{1,2,15} This role has been explained on the basis of interactions with other NRs and regulatory proteins; upon activation, PXR typically heterodimerizes with the retinoid X receptor (RXR) to recruit co-activators, instead of co-repressors, that modulate the binding of the heterodimer with specific responsive elements in the promoter regions of target genes. For example, in the proximal promoter region of CYP3A4, a repeat of half hexamers divided by six nucleotides, was recognized as a proximal PXR responsive element (2169/2152).\textsuperscript{6} Additionally, the CYP3A4 promoter, includes the xenobiotic responsive enhancer module (XREM), a distal enhancer sequence containing two extra PXR responsive elements.\textsuperscript{44,45}
**MDR1** (or P-gp) is also a PXR reporter gene in liver cells and recent reports showed the hepatic metabolite 2 as able to stimulate this gene,\(^{27}\) consistent with the a role of PXR not only as master regulator, but also as a sensor of vitamin E metabolism and biotransformation products. These aspects exemplify the concept of xenosensor proposed for this NR (recently reviewed elsewhere),\(^{15}\) which may extend to vitamin E also suggesting a role for PXR as candidate receptor for this family of natural products,\(^{13,29}\) According with this latter role and with preliminary data obtained in HepG2 cells\(^ {46}\) and further described in,\(^ {13}\) GA is a potent stimulator of PPAR\(\gamma\) and CYP4F2 protein expression. Together with **CYP3A4**, this CYP450 isoform is believed to sustain the hepatic metabolism of vitamin E as well as that of long-chain fatty acids downstream of transcriptional elements such as PPAR\(\gamma\) and other NRs, including PXR.\(^ {47}\) According with a PXR-mediated mechanism for these responses to GA treatment, in this study GA was confirmed to stimulate PXR and CYP3A4 expression of human liver cells, thus resulting in a higher \(\omega\)-oxidation activity on the cellular vitamin E to form \(\alpha\)-tocopherol (3) (Supporting Information, Figure S3). The efflux of this \(\omega\)-oxidation product was also increased exemplifying another important and PXR-mediated detoxification step of vitamin E molecules by the transport function of **MDR1** and other phase III genes.\(^ {27,29}\)

Important enough, screening the GA binding over a panel of NRs, we were able to demonstrate the selective agonist profile at PXR (see Figure S1, Supporting Information). No response to GA and other analogues with tocol-like structures was observed on PPARs and FXR, RAR and RXR. CAR, another NR originally proposed to help explain the CYP3A-dependent metabolism of vitamin E in human hepatocytes\(^ {30,48}\) and characterized by constitutive activation, was partially inhibited during affinity binding experiments with GA. These findings support selective action of GA for PXR and shed light on future applications of GA in targeted chemoprevention and therapy.
protocols of PXR-expressing organs, such as the liver, intestine and to a slighter extent the kidney. In fact, the role of PXR as transcriptional regulator of xenobiotic and drug metabolizing genes with a broad substrate promiscuity has represented the major drawback for the pharmacological approach to the receptor. In this respect, GA represents a novel and selective PXR agonist, holding great potential in the development of therapeutic agents for a range of human pathologies that include cholestatic liver disease, dyslipidemia, IBD, and recently rifaximin has received FDA approval for IBS treatment.

Our in vivo data unequivocally demonstrate that both intestinal and liver PXR respond to the agonist activity of GA thus providing mechanistic support for the investigation of this natural compound in PXR-related diseases of the liver and gut. Furthermore, in a recent study we demonstrated a PXR agonist effect of GA in the mouse brain as well as in isolated astrocytes, compatible with the role of this NR in Apo-mediated detoxification of Alzheimer disease related molecule Aβ peptide.

In view of other important roles of GA and its vitamin E-derived analogues as allosteric inhibitor of 5-lipooxygenase, and PPAR-γ and CYP450 activators, our findings suggest that GA can be exploited as a valuable chemical probe and lead compound for medicinal chemistry explorations to disclose novel therapeutic agents for the prevention and treatment of lipotoxicity and chronic inflammatory diseases, such as atherosclerosis, and non-alcoholic fatty liver disease and steatohepatitis. Current studies are directed towards the preparation of GA (1) derivatives with improved potency, properties and metabolic stability whose results will be reported in due course.
4. Experimental Procedures

**Isolation of garcinioic acid (1).** Finely ground seeds (1.2 kg) of *Garcinia Kola* were suspended in MeOH (1.2 L) and stirred at the 25 °C for 6.5 h. The resulting suspensions were filtered off under vacuo and the resulting solid residue was re-suspended in MeOH (1.2 L) and stirred at 25 °C for further 17.5 h. The suspension was filtered again under vacuo affording a crude brownish oil (56 g). The crude was purified by silica gel flash chromatography (Eluent: CH$_2$Cl$_2$/MeOH, from 100:0 to 90:10, v/v) affording 9.4 g (yield 0.78%, w/w) of pure garcinioic acid (1) as yellow-green oil.$^{52}$

$^1$H-NMR (400 MHz, CDCl$_3$): δ 1.27 (3H, s), 1.53-1.61 (1H, m), 1.63 (6H, m), 1.75-1.79 (2H, m), 1.84 (3H, s), 1.99-2.01 (2H, m), 2.06-2.13 (9H, m), 2.20 (3H, s), 2.29-2.30 (2H, m), 2.70 (2H, t, $J= 6.49$ Hz), 5.13-5.14 (2H, m), 6.41 (1H, d, $J= 2.51$ Hz), 6.50 (1H, d, $J= 2.52$ Hz), 6.88-6.92 (1H, m). $^{13}$C-NMR (100.6 MHz, CDCl$_3$): δ 12.0, 15.8, 15.9, 16.0, 22.1, 22.4, 24.0, 26.4, 27.5, 31.3, 38.0, 39.5, 42.0, 77.3, 112.6, 115.7, 121.2, 124.4, 125.1, 126.9, 127.3, 133.7, 134.8, 145.0, 145.9, 147.7, 173.3.

**Cloning, expression and purification of PXR-LBD/SRC-1p.** Tethered PXR-LBD/SRC-1p was synthesized by GenScript. The gene product was subsequently sub-cloned into the pLIC-His pMCSG7 expression vector. Site directed mutagenesis of the N-terminal tag was performed to replicate the construct used for a previously determined PXR crystal structure (PDB: 3CTB).$^{53}$

The final expression vector containing the His-tagged PXR-LBD/SRC-1p construct was transformed into E. coli BL21(DE3) cells. A 100 mL overnight culture supplemented with 100 μg/mL ampicillin was used to inoculate a 1.5 L culture supplemented with 100 μg/mL ampicillin at 37°C until OD600 was approximately 0.6. The temperature was reduced to 18°C and expression was induced with the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside and continued overnight. The next day, cells were harvested and frozen at -80 °C prior to purification. Protein
was purified by Ni affinity chromatography and size exclusion chromatography using the following buffers: Ni A - 25 mM HEPES, pH 7.9, 5% v/v glycerol, 150 mM NaCl, 1 mM DTT, 10 mM imidazole. Ni B - 25 mM HEPES, pH 7.9, 5% v/v glycerol, 150 mM NaCl, 1 mM DTT, 250 mM imidazole. GF - 25 mM HEPES, pH 7.9, 5% v/v glycerol, 150 mM NaCl, 5 mM DTT.

**Crystallization, x-ray diffraction data collection, structure refinement.** PXR-LBD-SRC-1p was incubated with 1 mM GA and then crystallized in 30% (v/v) MPD and 100 mM imidazole/HCl pH 7.0 by the hanging drop vapor diffusion method at 4°C. Diffraction data was collected at 100 K at APS Beamline 23-ID-D. Data was reduced in XDS\textsuperscript{54,55} and scaled in Aimless.\textsuperscript{55} The tethered PXR-LBD/SRC-1p-GA structure was solved via molecular replacement in Phenix using the tethered PXR-LBD/SRC-1p apo structure (PDB: 3CTB). The model was refined using Phenix with manual adjustments using Coot as necessary. Authors will release the atomic coordinates and experimental data upon article publication.

**AlphaScreen assay of nuclear receptor binding.** Activity of nuclear receptors was determined using AlphaScreen technology, a recruitment coactivator assay previously described.\textsuperscript{56}

**Expression and purification of His-tagged PXR Ligand Binding Domain (LBD) protein.**
The expression and purification of His-tagged PXR Ligand Binding Domain (LBD) protein was performed as published with minor modifications.\textsuperscript{57} For the expression of the protein, Luria-Bertani (LB) media was inoculated with a saturated culture of BL21-Gold cells transformed with HIS-LIC plasmid containing the PXR LBD construct. The mixture was then allowed to shake at 37°C until the cells reached an OD\textsubscript{600} ~ 0.6. The temperature was then reduced to 18 °C, at which time IPTG was added (final concentration of 0.1 mM) to induce protein expression. For
purification of PXR-LBD, the His-tag was not removed and the un-cleaved protein was loaded onto the gel filtration column with buffer [HEPES (25 mM, pH 7.5) and NaCl (150 mM)].

**Isothermal Titration Calorimetry (ITC).** ITC was performed using a VP - ITC microcalorimeter from MicroCal/Malvern Instruments (Northampton, MA, USA). The protein and the ligands (in DMSO, 8% vol/vol final concentration) were prepared in 25 mM Hepes, pH 7.5 with 150 mM NaCl. In all the experiments, the ligand solution was injected in 10-µL aliquots into the calorimetric cell containing PXR-LBD [3 µM] at 37 °C. The respective concentrations of Garcinoic acid, δ-tocotrienol (4), and UPF-2547 in the syringe were 60 µM each. The heat evolved upon each injection of the ligands was obtained from the integral of the calorimetric signal. The heat associated with binding to PXR-LBD in the cell was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted against the molar ratio, and the enthalpy change (ΔH), association constant (K_a = 1/K_d), and the stoichiometry were obtained by nonlinear regression of the data.

**Cell studies.** HepG2 (human hepatoma cell line; ATCC® HB-8065™, Manassas, VA, USA) and HepaRG (HPRGC10, Thermo Fisher Scientific) cells were maintained in culture as previously described. Experiments were performed between the passage 2 and 10. Cell viability, clonogenic activity and apoptotic cell death were assessed as previously described (see Figure S2, Supporting Information).

**Immunoblots.** HepG2 and HepaRG cells were harvested and lysed in ice-cold Cell Lysis Buffer (Cell Signaling Technology), and 20 µL/mL protease inhibitor cocktail (Roche). Murine tissues (10 mg each) were weighed and resuspended in 300 µL PBS supplemented with NP40 (1:50) and a cocktail of protease inhibitors (Pierce, Thermo Fisher Scientific). After preliminary
homogenation in a 1-ml potter, sonication was performed in ice (3 cycles of 15 s each with 1 min intervals). After incubation in ice for 1 h and centrifugation (12,000 rpm for 20 min at 4 °C), the supernatant was recovered and total proteins were quantified by BCA assay kit (Pierce, Thermo Fisher Scientific). 20 µg of proteins were resolved by 10-12% SDS-PAGE, and immunoblot was performed using anti-PXR (H-160, 1:200 dilution; sc-25381, Santa Cruz Biotechnology, Inc.), anti-PXR (1:1000 dilution, ab192579, abcam), anti-CYP3A4 (1:1000 dilution; TA324142, OriGene Technologies, Inc., Rockville, MD), anti-GAPDH (D16H11, 1:1000 dilution; #5174, Cell Signaling Technology, Beverly, MA, USA;) as primary antibodies. A horseradish peroxidase–conjugated anti-rabbit IgG (1:2000 dilution; #7074, Cell Signaling Technology, Beverly, MA, USA;) was used as secondary antibody. Band intensity was analyzed using Gel Pro analyzer Software.

**P-glycoprotein activity assay.** To evaluate the effects of GA on recombinant human Pgp in a cell membrane fraction, we used the Pgp-Glo assay system (Promega, Madison Wisconsin USA), according to manufacturer’s instruction. The assay relies on the ATP dependence of the light-generating reaction of firefly luciferase.

**Gene Expression and siRNA PXR transfection.** RNA was extracted using RNeasy Plus Kit (QIAGEN, Valencia, CA) and quantified with Implen NanoPhotometer (GmbH Germany) and cDNA synthesis was carried out using SuperScript IV (Thermo Fisher Scientific, MA). For siRNA PXR transfection, HepG2 cells were transfected with siRNAs PXR using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo-Fischer). Reverse transfection was also carried out and after the silencing procedure (24 hrs) HepG2 cells were treated with GA for further 24 h.
**In vivo studies.** 6–7 weeks old C57BL/6 wild-type male mice from Charles River were kept under standard environmental conditions (22 °C, 35% relative humidity, 12 h dark/ light cycle) with free access to tap water and standard diet. After shipping, mice were allowed to adapt to the new environment before initiating the experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Perugia and all surgical and treatment. Animals were divided into six treatment groups (3 animals each): control (vehicle = olive oil) and GA treated groups at the dosage of 5, 10, 25, 50 and 100 mg. GA was administration by oral gavage as a single bolus and the animals were sacrificed after 24 hrs according to institutional guidelines to collect organs for histology and biochemistry evaluation as described in the other sections.

**IHC assay.** Liver and gut mouse specimens were formalin-fixed and paraffin-embedded. Four micrometer sections were mounted on polarized glass slides and stained with haematoxylin and eosin (Sigma-Aldrich). Immunohistochemistry analysis of PXR (polyclonal antibody; dilution 1:200; ab217375, abcam) was performed using Bond III (Vision BioSystems, Buffalo Grove, IL, USA) automated slide preparation system and a Polymer Refine Detection (Vision BioSystems) was used as antibody detection system. 3,3’-Diaminobenzidine (DAB) was used as chromogen. The section was then counterstained with haematoxylin (Sigma-Aldrich) and mounted with DPX (06522, Sigma-Aldrich).

**Statistical Analysis.** Statistically significant differences were determined using one-way analysis of variance followed by Tukey’s multiple comparison test as the post hoc test or t-test and differences were considered statistically significant at p < 0.05.
ASSOCIATED CONTENT

**Supporting Information.** Supplementary figures including general methods, synthetic procedures, analytical and spectroscopic characterization of compounds 2, 5-8, NMR spectra and HPLC-HRMS copies, ligand binding activity of garcinoc acid and other vitamin E analogues on a series of nuclear receptors, cell-based tests and in vivo assays, crystallographic data collection and refinement statistics, are available free of charge at http://pubs.acs.org.

AUTHOR INFORMATION

**Corresponding Author**

*To whom correspondence should be addressed. Antimo Gioiello, Department of Pharmaceutical Sciences, University of Perugia, Via del Liceo 1, Perugia, Italy. E-mail address: antimo.gioiello@unipg.it. Tel: +39 075 585 2318/5182.

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

**Funding Sources**

Part of this research was supported by Italian Ministry of University grant program, National Technology Agrifood Cluster, Health and Nutrition program, PROS.IT project (CTN01_00230_413096; completed). A.G. and F.G. have also been supported by the “Ricerca di base” grant program of the University of Perugia (Completed). At the time of this research, D.B. was a post-doc fellow of the FIRC-AIRC young investigator’ grant program. S.M. was funded by
NIH grants R01 CA127231 (completed); CA 161879 (completed); CA222469 and Department of Defense Partnering PI (W81XWH-17-1-0479; PR160167); R01 ES030197, as well as R43DK105694 (PI: Jay Wrobel, completed), P30DK041296 (PI: Alan Wolkoff) (Pilot Award completed).

**ABBREVIATIONS**

CAR, constitutive androstane receptor; FXR, farnesoid x receptor; GA, garcinoic acid; IBD, intestinal bowel disease; IBS, irritable bowel syndrome; ITC, isothermal titration calorimetry; LBD, ligand binding domain; LCM, long-chain metabolite; LXR, liver x receptor; NR, nuclear receptor; MDR1, multidrug resistance protein 1; PPAR: peroxisome proliferator-activated receptor; PXR, pregnane x receptor; RXR, retinoid x receptor; SCM, short-chain metabolite; \(\alpha\)-TOH, alpha-tocopherol; \(\delta\)-T, gamma-tocotrienol.

**References**

(1) Kandel, B. A.; Thomas, M.; Winter, S.; Seehofer, D.; Burk, O.; Schwab, M.; Zanger, U. M. Genomewide comparison of the inducible transcriptomes of nuclear receptors CAR, PXR and PPARalpha in primary human hepatocytes. *Biochim. Biophys. Acta* **2016**, *1859*, 1218-1227.

(2) Kliewer, S. A.; Goodwin, B.; Willson, T. M. The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr. Rev.* **2002**, *23*, 687-70.

(3) Chen, Y.; Tang, Y.; Guo, C.; Wang, J.; Boral, D.; Nie, D. Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochem. Pharmacol.* **2012**, *83*, 1112-1126.
(4) Banerjee, M.; Chen, T. Differential regulation of CYP3A4 promoter activity by a new class of natural product derivatives binding to pregnane X receptor. *Biochem. Pharmacol.* **2013**, 86, 824-835.

(5) Kliewer, S. A.; Moore, J. T.; Wade, L.; Staudinger, J. L.; Watson, M. A.; Jones, S. A.; McKee, D. D.; Oliver, B. B.; Willson, T. M.; Zetterström, R. H.; Perllmann, T.; Lehmann, J. M. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **1998**, 92, 73-82.

(6) Blumberg, B.; Sabbagh, W. Jr.; Juguilon, H.; Bolado, J. Jr.; van Meter, C. M.; Ong, E. S.; Evans, R. M. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **1998**, 12, 3195-3205.

(7) Fagerberg, L.; Hallström, B. M.; Oksvold, P.; Kampf, C.; Djureinovic, D.; Odeberg, J.; Habuka, M.; Tahmasebpoor, S.; Danielsson, A.; Edlund, K.; Asplund, A.; Sjöstedt, E.; Lundberg, E.; Szigyarto, C. A.; Skogs, M.; Takanen, J. O.; Berling, H.; Tegel, H.; Mulder, J.; Nilsson, P.; Schwenk, J. M.; Lindskog, C.; Danielsson, F.; Mardinoglu, A.; Sivertsson, A.; von Feilitzen, K.; Forsberg, M.; Zwahlen, M.; Olsson, I.; Navani, S.; Huss, M.; Nielsen, J.; Ponten, F.; Uhlén, M. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol. Cell. Proteomics* **2014**, 13, 397-406.

(8) [https://www.ncbi.nlm.nih.gov/gene/8856#gene-expression](https://www.ncbi.nlm.nih.gov/gene/8856#gene-expression)

(9) Hogle, B. C.; Guan, X.; Folan, M. M.; Xie, W. PXR as a mediator of herb-drug interaction. *J. Food Drug Anal.* **2018**, 26, S26-S31.
(10) Wikoff, W. R.; Anfora, A. T.; Liu, J.; Schultz, P. G.; Lesley, S. A.; Peters, E. C.; Siuzdak, G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 3698-3703.

(11) Venkatesh, M.; Mukherjee, S.; Wang, H.; Li, H.; Sun, K.; Benechet, A. P.; Qiu, Z.; Maher, L.; Redinbo, M. R.; Phillips, R. S.; Fleet, J. C.; Kortagere, S.; Mukherjee, P.; Fasano, A.; Le Ven, J.; Nicholson, J. K.; Dumas, M. E.; Khanna, K. M.; Mani, S. Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. *Immunity* **2014**, *41*, 296-310.

(12) Galli, F. Interactions of polyphenolic compounds with drug disposition and metabolism. *Curr. Drug Metab.* **2007**, *8*, 830-838.

(13) Galli, F.; Azzi, A.; Birringer, M.; Cook-Mills, J. M.; Eggersdorfer, M.; Frank, J.; Cruciani, G.; Lorkowski, S.; Özer, N. K. Vitamin E: emerging aspects and new directions. *Free Radic. Biol. Med.* **2017**, *102*, 16-36.

(14) Mani, S.; Dou, W.; Redinbo, M. R. PXR antagonists and implication in drug metabolism. *Drug Metab. Rev.* **2013**, *45*, 60-72.

(15) Oladimeji, P. O.; Chen, T. PXR: more than just a master xenobiotic receptor. *Mol. Pharmacol.* **2018**, *93*, 119-127.

(16) Cave, M. C.; Clair, H. B.; Hardesty, J. E.; Falkner, K. C.; Feng, W.; Clark, B. J.; Sidey, J.; Shi, H.; Aqel, B. A.; McClain, C. J.; Prough, R. A. Nuclear receptors and nonalcoholic fatty liver disease. *Biochim. Biophys. Acta* **2016**, *1859*, 1083-1099.
(17) Lopez-Velazquez, J. A.; Carrillo-Cordova, L. D.; Chavez-Tapia, N. C.; Uribe, M.; Mendez-Sanchez, N. Nuclear receptors in nonalcoholic fatty liver disease. *J. Lipids* **2012**, 2012, 139875.

(18) Kakizaki, S.; Takizawa, D.; Tojima, H.; Horiguchi, N.; Yamazaki, Y.; Mori, M. Nuclear receptors CAR and PXR; therapeutic targets for cholestatic liver disease. *Front. Biosci.* **2011**, *16*, 2988-3005.

(19) Cheng, J.; Shah, Y. M.; Gonzalez, F. J. Pregnan e X receptor as a target for treatment of inflammatory bowel disorders. *Trends Pharmacol. Sci.* **2012**, *33*, 323-330.

(20) Shah, Y. M.; Ma, X.; Morimura, K.; Kim, I.; Gonzalez, F. J. Pregnan e X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-kappaB target gene expression. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *292*, G1114-1122.

(21) Gao, J.; Xie, W. Targeting xenobiotic receptors PXR and CAR for metabolic diseases. *Trends Pharmacol. Sci.* **2012**, *33*, 552-558.

(22) Lau, A. J.; Yang, G.; Yap, C. W.; Chang, T. K. Selective agonism of human pregnane X receptor by individual ginkgolides. *Drug Metab. Dispos.* **2012**, *40*, 1113-1121.

(23) DuPont, H. L. Therapy for and prevention of traveler's diarrhea. *Clin. Infect. Dis.* **2007**, *45* Suppl. 1, S78-84.

(24) Lawrence, K. R.; Klee, J. A. Rifaximin for the treatment of hepatic encephalopathy. *Pharmacotherapy* **2008**, *28*, 1019-1032.

(25) Administration USFDA (2015) FDA approves two therapies to treat IBS-D. ed Release FN (U.S. Food and Drug Administration, Silver Spring, MD).
(26) Sartor, R. B. Review article: the potential mechanisms of action of rifaximin in the management of inflammatory bowel diseases. *Aliment. Pharmacol. Ther.* **2016**, *43*, Suppl. 1, 27-36.

(27) Podszun, M. C.; Jakobi, M.; Birringer, M.; Weiss, J.; Frank, J. The long chain alpha-tocopherol metabolite alpha-13'-COOH and gamma-tocotrienol induce P-glycoprotein expression and activity by activation of the pregnane X receptor in the intestinal cell line LS 180. *Mol. Nutr. Food Res.* **2017**, *61*, 1600605.

(28) Galli, F.; Polidori, M. C.; Stahl, W.; Mecocci, P.; Kelly, F. J. Vitamin E biotransformation in humans. *Vitam. Horm.* **2007**, *76*, 263-280.

(29) Schubert, M.; Kluge, S.; Schmölz, L.; Wallert, M.; Galli, F.; Birringer, M.; Lorkowski, S. Long-chain metabolites of vitamin E: metabolic activation as a general concept for lipid-soluble vitamins? *Antioxidants* **2018**, *7*, pii: E10.

(30) Birringer, M.; Drogan, D.; Brigelius-Flohe, R. Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation. *Free Radic. Biol. Med.* **2001**, *31*, 226-232.

(31) Birringer, M.; Pfluger, P.; Kluth, D.; Landes, N.; Brigelius-Flohe, R. Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *J. Nutr.* **2002**, *132*, 3113-3118.

(32) Marinelli, R.; Torquato, P.; Bartolini, D.; Mas-Bargues, C.; Bellezza, G.; Gioiello, A.; Borras, C.; Fallarino, F.; Mani, S.; Sidoni, A.; Viña, J.; Galli, F. *manuscript in preparation*.

(33) Mazzini, F.; Betti, M.; Netscher, T.; Galli, F.; Salvadori, P. Configuration of the vitamin E analogue garcinoic acid extracted from Garcinia Kola seeds. *Chirality* **2009**, *21*, 519-524.
(34) Wallert, M.; Bauer, J.; Kluge, S.; Schmölz, L.; Chen, Y. C.; Ziegler, M.; Searle, A. K.; Maxones, A.; Schubert, M.; Thürmer, M.; Pein, H.; Koeberle, A.; Werz, O.; Birringer, M.; Peter, K.; Lorkowski, S. The vitamin E derivative garcinoic acid from Garcinia kola nut seeds attenuates the inflammatory response. *Redox Biol.* **2019**, *24*, 101166.

(35) Birringer, M.; Lington, D.; Vertuani, S.; Manfredini, S.; Scharlav, D.; Glei, M.; Ristow, M. Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress. *Free Rad. Biol. Med.* **2010**, *49*, 1315-1322.

(36) Terashima, K.; Takaya, Y.; Niwa, M. Powerful antioxidative agents based on garcinoic acid from *Garcinia kola*. *Bioorg. Med. Chem.* **2002**, *10*, 1619-1625.

(37) Mostarda, S.; Filipponi, P.; Sardella, R.; Venturoni, F.; Natalini, B.; Pellicciari, R.; Gioiello, A. Glucuronidation of bile acids under flow conditions: design of experiments and Koenigs–Knorr reaction optimization. *Org. Biomol. Chem.* **2014**, *12*, 9592-9600.

(38) Xue, Y.; Chao, E.; Zuercher, W. J.; Willson, T. M.; Collins, J. L.; Redinbo, M. R. Crystal structure of the PXR-T1317 complex provides a scaffold to examine the potential for receptor antagonism. *Bioorg Med Chem*. **2007**, *15*, 2156-2166.

(39) Dou, W.; Mukherjee, S.; Li, H.; Venkatesh, M.; Wang, H.; Kortagere, S.; Peleg, A.; Chilimuri, S. S.; Wang, Z. T.; Feng, Y.; Fearon, E. R.; Mani, S. Alleviation of gut inflammation by Cdx2/Pxr pathway in a mouse model of chemical colitis. *PLoS One* **2012**, *7*, e36075.

(40) Velazquez-Campoy, A.; Todd, M. J.; Freire, E. HIV-1 protease inhibitors: enthalpic versus entropic optimization of the binding affinity. *Biochemistry* **2000**, *39*, 2201-2207.
(41) Ruben, A. J.; Kiso, Y.; Freire, E. Overcoming roadblocks in lead optimization: a thermodynamic perspective. *Chem. Biol. Drug Des.* **2006**, *67*, 2-4.

(42) Zhou, C.; Poulton, E. J.; Grün, F.; Bammler, T. K.; Blumberg, B.; Thummel, K. E.; Eaton, D. L. The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Mol. Pharmacol.* **2007**, *71*, 220-229.

(43) Leist, M.; Raab, B.; Maurer, S.; Rössick, U.; Brigelius-Flohé, R. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. *Free Radic. Biol. Med.* **1996**, *21*, 297-306.

(44) Goodwin, B.; Hodgson, E.; Liddle, C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol. Pharmacol.* **1999**, *56*, 1329-1339.

(45) Toriyabe, T.; Nagata, K.; Takada, T.; Aratsu, Y.; Matsubara, T.; Yoshinari, K.; Yamazoe, Y. Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. *Mol Pharmacol.* **2009**, *75*, 677-684.

(46) Torquato, P.; Bartolini, D.; Giusepponi, D.; Saluti, G.; Russo, A.; Barola, C.; Birringer, M.; Galarini, R.; Galli, F. a-13'-OH is the main product of a-tocopherol metabolism and influences CYP4F2 and PPAR? Gene expression in HepG2 human hepatocarcinoma cells. *Free Radic. Biol. Med.* **2016**, *96*, S19-S20.

(47) Russo, A.; Bartolini, D.; Torquato, P.; Giusepponi, D.; Barola, C.; Galarini, R.; Birringer, M.; Lorkowski, S.; Galli, F. CYP4F2 repression and a modified alpha-tocopherol (vitamin E)
metabolism are two independent consequences of ethanol toxicity in human hepatocytes. *Toxicol. in Vitro* **2017**, *40*, 124-133.

(48) Landes, N.; Pfluger, P.; Kluth, D.; Birringer, M.; Rühl, R.; Böl, G. F.; Glatt, H.; Brigelius-Flohé, R. Vitamin E activates gene expression via the pregnane X receptor. *Biochem. Pharmacol.* **2003**, *65*, 269-273.

(49) Pein, H.; Ville, A.; Pace, S.; Temml, V.; Garscha, U.; Raasch, M.; Alsabil, K.; Viault, G.; Dinh, C.-P.; Guilet, D.; Troisi, F.; Neukirch, K.; König, S.; Bilancia, R.; Waltenberger, B.; Stuppner, H.; Wallert, M.; Lorkowski, S.; Weinigel, C.; Rummler, S.; Birringer, M.; Roviezzo, F.; Sautebin, L.; Helesbeux, J.-J.; Séraphin, D.; Mosig, A. S.; Schuster, D.; Rossi, A.; Richomme, P.; Werz, P. O.; Koeberle, A. Endogenous metabolites of vitamin E limit inflammation by targeting 5-lipoxygenase. *Nat. Commun.* **2018**, *9*, 3834.

(50) Torquato, P.; Giusepponi, D.; Alisi, A.; Galarini, R.; Bartolini, D.; Piroddi, M.; Goracci, L.; Di Veroli, A.; Cruciani, G.; Crudele, A.; Nobili, V.; Galli, F. Nutritional and lipidomics biomarkers of docosahexaenoic acid-based multivitamin therapy in pediatric NASH. *Sci. Rep.* **2019**, *9*, 2045.

(51) Torquato, P.; Bartolini, D.; Giusepponi, D.; Piroddi, M.; Sebastiani, B.; Saluti, G.; Galarini, R.; Galli, F. Increased plasma levels of the lipoperoxyl radical-derived vitamin E metabolite α-tocopheryl quinone are an early indicator of lipotoxicity in fatty liver subjects. *Free Radic Biol Med.* **2019**, *131*, 115-125.

(52) Terashima, K.; Shimamura, T.; Tanabayashi, M.; Aqil, M.; Akinniyi, J. A.; Niwa, M. Constituents of the seeds of Garcinia Kola: two new antioxidants, garcinoic acid and garcinal. *Heterocycles* **1997**, *45*, 1559-1566.
(53) Wang, W.; Prosise, W. W.; Chen, J.; Taremi, S. S.; Le, H. V.; Madison, V.; Cui, X.; Thomas, A.; Cheng, K. C.; Lesburg, C. A. Construction and characterization of a fully active PXR/SRC-1 tethered protein with increased stability. *Protein Eng. Des. Sel.* **2008**, 21, 425-433.

(54) Kabsch, W. XDS. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, 66, 125-132.

(55) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, 67, 235-242.

(56) Rizzo, G.; Passeri, D.; De Franco, F.; Ciaccioli, G.; Donadio, L.; Rizzo, G.; Orlandi, S.; Sadeghpour, B.; Wang, X.X.; Jiang, T.; Levi, M.; Pruzanski, M.; Adorini, L. Functional characterization of the semisynthetic bile acid derivative INT-767, a dual farnesoid X receptor and TGR5 agonist. *Mol. Pharmacol.* **2010**, 78, 617-630.

(57) Wallace, B. D.; Betts, L.; Talmage, G.; Pollet, R. M.; Holman, N. S.; Redinbo, M. R. Structural and functional analysis of the human nuclear xenobiotic receptor PXR in complex with RXRα. *J. Mol. Biol.* **2013**, 425, 2561-2577.

(58) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, 65, 55-63.

(59) Bartolini, D.; Commodi, J.; Piroddi, M.; Incipini, L.; Sancineto, L.; Santi, C.; Galli, F. Glutathione S-transferase pi expression regulates the Nrf2-dependent response to hormetic diselenides. *Free Radic. Biol. Med.* **2015**, 88, 466-480.
