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RESEARCH PAPER

Differential effects of clinically used derivatives and metabolites of artemisinin in the activation of constitutive androstane receptor isoforms

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BACKGROUND AND PURPOSE

Widespread resistance to antimalarial drugs requires combination therapies with increasing risk of pharmacokinetic drug–drug interactions. Here, we explore the capacity of antimalarial drugs to induce drug metabolism via activation of constitutive androstane receptors (CAR) by ligand binding.

EXPERIMENTAL APPROACH

A total of 21 selected antimalarials and 11 major metabolites were screened for binding to CAR isoforms using cellular and *in vitro* CAR-coactivator interaction assays, combined with *in silico* molecular docking. Identified ligands were further characterized by cell-based assays and primary human hepatocytes were used to elucidate induction of gene expression.

KEY RESULTS

Only two artemisinin derivatives arteether and artemether, the metabolite deoxyartemisinin and artemisinin itself demonstrated agonist binding to the major isoforms CAR1 and CAR3, while arteether and artemether were also inverse agonists of CAR2. Dihydroartemisinin and artesunate acted as weak inverse agonists of CAR1. While arteether showed the highest activities *in vitro*, it was less active than artemisinin in inducing hepatic CYP3A4 gene expression in hepatocytes.

CONCLUSIONS AND IMPLICATIONS

Artemisinin derivatives and metabolites differentially affect the activities of CAR isoforms and of the pregnane X receptor (PXR). This negates a common effect of these drugs on CAR/PXR-dependent induction of drug metabolism and further provides an explanation for artemisinin consistently inducing cytochrome P450 genes *in vivo*, whereas arteether and artemether do not. All these drugs are metabolized very rapidly, but only artemisinin is converted to an enzyme-inducing metabolite. For better understanding of pharmacokinetic drug–drug interaction possibilities, the inducing properties of artemisinin metabolites should be considered.

Abbreviations

ACT, artemisinin combination therapy; AD, activation domain; ADME, absorption distribution metabolism excretion; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime; CYP, cytochrome P450; DBD, DNA-binding domain; DRIP205, vitamin D receptor interacting protein 205; HAART, highly active antiretroviral treatment; LBD, ligand-binding domain; LBP, ligand-binding pocket; PXR, pregnane X receptor; RID, receptor interaction domain; SRC, steroid receptor coactivator

Introduction

Malaria, an infectious disease caused by parasitic protozoans of the genus *Plasmodium*, is one of the major global health problems, resulting in about 225 million cases in the year 2009, of whom an estimated 780 000 were killed by the disease (WHO, 2010b). In the past, malaria parasites, especially the clinically most relevant *Plasmodium falciparum*, have increasingly developed resistance against common antimalarial drugs such as chloroquine, sulfadoxine/pyrimethamine and mefloquine (White, 2004), thereby forcing the need for combination therapy of several drugs. Currently, artemisinin combination therapies (ACT) are recommended for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2010a), consisting of an artemisinin-type drug with short half-life and a partner drug, which is slowly eliminated. In malaria-endemic areas, especially in sub-Saharan Africa, HIV infections are also highly prevalent, thereby putting a substantial part of the population at risk of co-infection. Malaria and HIV infection interact and worsen each other's clinical outcome (see Skinner-Adams *et al.*, 2008). Because the HIV/AIDS standard therapy by highly active antiretroviral treatment (HAART) is also a combination of several drugs, the potential risk of pharmacokinetic interactions between antiretroviral and antimalarial drugs by inhibition and/or induction of drug metabolism and transport appears to be clinically relevant. Pharmacokinetic drug–drug interactions between both treatments mostly involve antiretroviral protease inhibitors and non-nucleoside reverse transcriptase inhibitors, which have been shown to alter the drug metabolism of antimalarials by inhibition and/or induction of distinct metabolizing enzymes (Khoo *et al.*, 2005; Skinner-Adams *et al.*, 2008). However, much less is known about the equivalent capacity of antimalarial drugs, especially regarding their potential to induce drug metabolism and/or transport.

Induction of drug metabolism and transport is mainly mediated by the activation of two xenosensing nuclear receptors, the pregnane X receptor (PXR, NR1I2) and the constitutive androstane receptor (CAR, NR1I3; nomenclature follows Alexander *et al.*, 2011), which upon their activation transcriptionally regulate genes involved in absorption, distribution, metabolism and excretion (ADME), among them most prominently cytochrome P450 (CYP) genes and ABCB1, encoding the drug efflux pump MDR1/P-glycoprotein (di Masi *et al.*, 2009). Whereas PXR is thought to be exclusively activated by ligand binding of a structurally diverse array of xeno- and endobiotics, among them many therapeutic agents (di Masi *et al.*, 2009), CAR is also activated indirectly. Phenobarbital-type activators of CAR do not bind to the receptor as ligands (Moore *et al.*, 2000). They indirectly activate CAR by inducing the translocation of the receptor from the cytoplasm to the nucleus (Kawamoto *et al.*, 1999), via a mechanism involving a kinase-signalling cascade (Blättler *et al.*, 2007). Direct binding of a ligand has been previously regarded to be only of minor importance for CAR activation, as only few ligands were known. Furthermore, these exert only small effects on the transcriptional activity of reference variant CAR1 in reporter gene assays (Maglich *et al.*, 2003; Xu *et al.*, 2004; Burk *et al.*, 2005), as overexpression of the receptor by transfection of transformed cells already results in spontaneous nuclear localization (Kawamoto *et al.*, 1999) and strong constitutive transcriptional activity (Baes *et al.*, 1994). However, recent studies have demonstrated the existence of numerous CAR ligands (Dring *et al.*, 2010). The existence of the splice variant CAR3, also called CAR-SV2, which, because of an insertion of the five amino acids APYLT into the ligand-binding domain (LBD), is completely dependent on ligand binding for transcriptional activation (Arnold *et al.*, 2004; Auerbach *et al.*, 2005), and which accounts for about 50% of CAR transcripts encoding functional proteins (Ross *et al.*, 2010), further emphasizes the previously underestimated role of ligands in CAR physiology.

In a recent systematic analysis, seven out of 16 antiretroviral drugs, most of them currently used in HAART, have been shown to activate the xenosensors PXR and/or CAR (Svärd *et al.*, 2010). In contrast, the capacity of antimalarial drugs to activate these two xenosensing nuclear receptors is largely unknown and has not yet been systematically investigated. It has only been demonstrated that artemisinin activates PXR and CAR1 by agonist binding (Burk *et al.*, 2005; Simonsson *et al.*, 2006). A class effect of artemisinin-type compounds in induction of drug metabolism has been suggested by clinical data (Asimus *et al.*, 2007) but this has not been analysed on the level of nuclear receptor activation. By using a combination of cellular and *in vitro* assays, as well as *in silico* molecular modelling, we have investigated whether drugs currently used in malaria therapy and/or their respective major metabolites were ligands of CAR isoforms, consequently inducing ADME genes with a potential risk for drug–drug interactions. Given the published clinical data on the induction of drug metabolism *in vivo*, special attention was paid to artemisinin-type compounds.

Methods

Cell culture, transient transfections and reporter gene assays

COS1 and Caco-2 TC7 cells were cultivated as described previously (Arnold *et al.*, 2004). The origin and culture of HepG2 cells have been described by Hoffart *et al.* (2012). One day before transfection, cells were plated in 24-well plates at the following densities: COS1, 3×10^4 cells per well; Caco-2 TC7,

4.2×10^4 cells per well; and HepG2, 1.5×10^5 cells per well. Transfections were performed in triplicate using Effectene™ transfection reagent (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. Mammalian two-hybrid CAR coactivator interaction and assembly assays, as well as CAR-dependent promoter reporter gene assays, were performed as described (Arnold *et al.*, 2004; Burk *et al.*, 2005), using expression plasmids and firefly luciferase reporter gene plasmids as specified in the respective Figure legends. β-Galactosidase reference plasmid pCMVβ (20 ng) was always co-transfected. If necessary, respective empty expression vectors or pUC18 were used to fill up to a total amount of 200 ng of plasmid DNA per well. Subsequently, cells were treated for 40 h (if not stated otherwise) with the indicated chemicals dissolved in DMSO or with an equivalent amount of DMSO (final 0.1%). Due to the lower affinity of CAR3 for 6-(4-chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime (CITCO), this compound had to be used at 10 μM. Luciferase and β-galactosidase activities were analysed as described (Burk *et al.*, 2002). Luciferase activity was normalized with respect to transfection efficiency using the corresponding β-galactosidase activity.

Molecular docking analysis

X-ray crystals structures of human CAR (Xu *et al.*, 2004) were obtained from the Protein Data Bank (PDB) (Berman *et al.*, 2002). Both CAR chains of PDB entries 1XV9 and 1XVP were prepared using MOE (Chemical Computing Group Inc., Montreal, Canada) and AutoDock Tools (Sanner, 1999). Three-dimensional coordinates of the antimalarials were either obtained from the Cambridge Structural Database (The Cambridge Crystallographic Data Centre, Cambridge, UK) or generated within MOE. All structures were minimized prior docking using the MMFF94x force field (Halgren, 1996). Molecular docking was performed using the Lamarckian genetic algorithm as implemented in AutoDock 4.2 (Morris *et al.*, 1998; Huey *et al.*, 2007). Atom type grid maps ($46 \times 46 \times 50$ points, spacing of 0.375 Å, grid centre at Leu206:CD2) were pre-calculated using AutoGrid. For each ligand, 10 docking runs were performed. Ligands were docked into all four human CAR ligand-binding pockets (LBPs; chains B and D of both PDB entries). Resulting docking poses were clustered based on a root mean square deviation criterion of 2.0 Å. In order to take the coherence of the AutoDock score and the molecular weight into account, AutoDock scores were normalized by dividing the score over the square-root of the number of heavy atoms (Pan *et al.*, 2003). Scores of all four docking approaches were averaged and multiplied by 10.

Coactivator-dependent receptor ligand assay (CARLA)

Escherechia coli BL21 (DE3) plysS, transformed with the bacterial expression plasmid encoding glutathione-S-transferase (GST)/steroid receptor coactivator 1 (SRC-1) receptor interaction domains (RID) fusion protein, were grown at 29°C for 3 h after induction of recombinant protein expression by 0.5 mM isopropyl-β-*D*-thiogalactopyranoside. Soluble recombinant protein was prepared by ultra-centrifugation of crude bacterial lysate, which was generated by the disruption of cells, suspended in NETN buffer (composition: 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 20 mM Tris-Cl pH 8.0, 0.5% (v/v) Nonidet P40), through one freeze-thaw cycle and subsequent sonication. Protein quantification was done by SDS-PAGE of an aliquot and staining the gel with Coomassie.

The TNT T7 quick coupled transcription/translation system was used to translate *in vitro* ^{35}S-labelled full-length human CAR1 protein in a 50 μL reaction, containing 1 μg of the respective expression plasmid and 20 μCi ^{35}S-methionine.

The CARLA was performed essentially as described by Krey *et al.* (1997). Briefly, 1 mL reactions were set up in NETN buffer with 0.5% (w/v) skimmed milk powder, using 3–5 μg of GST-tagged SRC-1 RID protein, bound to glutathione-Sepharose 4B beads (25 μL bed volume), 2 μL of ^{35}S-labelled CAR1 protein, and the respective chemicals or 1% solvent DMSO only. After overnight incubation at 4°C with constant rotation, beads were washed three times in NETN buffer, supplemented with the respective chemicals. Bound GST/SRC-1 fusion protein/CAR1 complexes were extracted from the beads by boiling in SDS-protein sample buffer and separated on 10% SDS-polyacrylamide gels, which were subsequently stained with Coomassie, dried and exposed to BAS-IP MS 2325 imaging plates (Fuji, Kanagawa, Japan). CAR1 protein bound to SRC-1 was detected by reading the image plates in a phosphor-storage scanner BAS-1800II (Fuji) and quantified by densitometric scanning of the image, using AIDA software (Raytest, Straubenhardt, Germany). Coomassie staining of the protein gels demonstrated the use of equal amounts of GST/SRC-1 fusion protein in each reaction. Respective control experiments, which had been set up with GST protein only, demonstrated negligible binding of CAR1 to the GST moiety of the GST/SRC-1 fusion protein.

Surface plasmon resonance

Expression of soluble CAR1-LBD and SRC-1-RID His-tag fusion proteins has been described previously (Hoffart *et al.*, 2012). Measurement of protein–protein interaction was performed by surface plasmon resonance using the Biacore 3000 instrument (GE Healthcare, Freiburg, Germany), as previously described (Hoffart *et al.*, 2012). Briefly, CAR1-LBD protein, which has been pre-incubated with chemicals for 30 min at room temperature, was injected onto SRC-1-RID protein, bound on CM5 sensor chips. Both association and dissociation was measured for 1 min.

Primary human hepatocytes

These procedures were approved by the local ethical committees of the Charité, Humboldt University Berlin, Germany. Tissue samples from human liver resections were obtained from patients undergoing partial hepatectomy because of primary or secondary liver tumours. Experimental procedures were performed according to the institutional guidelines for liver resections of tumour patients with primary or secondary liver tumours including the patient's consent. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (Nussler *et al.*, 2009). Only cell preparations with a viability > 80%, as determined by Trypan blue exclusion, were used for experiments. The isolated cells were seeded at a density of 1.5

$\times 10^6$ cells per·well into collagen type I-coated 6-well plates. Hepatocytes were cultivated and treated with chemicals as described previously (Hoffart *et al.*, 2012). Besides CAR, hepatocytes also express PXR. Thus, CITCO was used at 1 μM to ensure activation of CAR only.

Quantitative real-time PCR analysis

Total RNA and first-strand cDNA were prepared as previously described (Burk *et al.*, 2002). The integrity of RNA samples was confirmed by formaldehyde agarose gel electrophoresis. PCR reactions were set up with cDNA corresponding to 25 pg (18S rRNA) or 25 ng (all other assays) of total RNA and the qPCR MasterMix Plus Low ROX. Gene expression levels were quantified by TaqMan real-time quantitative PCR using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The experiments were performed in a final volume of 25 μL using the default settings of the 7500 Real-Time PCR system. Assays were done in triplicate. The CYP3A4 assay was done as described by Wolbold *et al.* (2003), whereas CYP2B6 and ABCB1 assays were performed as described previously by Burk *et al.* (2005). 18S rRNA levels were determined as described by Hoffart *et al.* (2012). Serial dilutions of respective linearized cDNA plasmids were used to create the calibration curves, ranging from 30 to 3×10^7 copies. The respective gene expression levels were normalized to the corresponding 18S rRNA levels and calculated as copies per 10^7 copies of 18S rRNA.

Data analysis

The mean values of at least three independent experiments were used for statistical analysis. Multiple comparisons were generally performed using one-way ANOVA with post-tests as shown in the respective figure legends. Comparisons with a hypothetical mean were performed using one sample *t*-test, with *P*-values were adjusted by the method of Bonferroni. Statistical analysis of hepatocyte experiments (Figure 8) was performed similarly, if the data were normally distributed. If this could not be tested due to small sample size or was not applicable, multiple comparisons were performed using Friedman's test with Dunn's multiple comparison post-test. All calculations were made using InStat 3.1 (GraphPad Software, La Jolla, CA, USA).

Plasmids

The following plasmids, which have been used in mammalian two-hybrid assays, have been described previously: the expression plasmid encoding the fusion protein of VP16-activation domain (AD) and the human CAR3, there called CAR-SV2, LBD (CAR3 amino acids 105–353), the Gal4-dependent reporter gene construct pGL3-G5 and the expression plasmids encoding fusion proteins of the GAL4 DNA-binding domain (DBD) and RID of human coactivators SRC-1 (NCOA1, amino acids 583–783), and vitamin D receptor interacting protein 205 (DRIP205) (MED1, amino acids 527–774) (Arnold *et al.*, 2004); the expression plasmid encoding the fusion protein of VP16-AD and part of the human CAR1-LBD (CAR1 amino acids 151–348), the expression plasmid encoding the fusion protein of GAL4-DBD and the helix 1 part of human CAR-LBD (amino acids 105–150) (Burk *et al.*, 2005).

The expression plasmids encoding VP16-AD/mouse CAR-LBD amino acids 95–358 and VP16-AD/human CAR2-LBD amino acids 151–352 fusion proteins were constructed by amplifying the respective sequences by PCR using appropriate primers out of pCR3-mCAR or pcDhCAR(SV3), respectively, and cloning into vector pVP-16 AD (Clontech, Mountain View, CA, USA).

Enhancer/promoter reporter gene plasmids pGL3-CYP3A4(-7830/Δ7208-364) (Hustert *et al.*, 2001), pB-1.6k/PB/XREM (Wang *et al.*, 2003) and p-7971(Δ7012-227)MDR (Burk *et al.*, 2005) were here referred to as CYP3A4, CYP2B6 and ABCB1, respectively. Expression plasmids encoding human CAR1 (Burk *et al.*, 2002), CAR2 and CAR3 [previously called CAR-SV3 and CAR-SV2, respectively, in Arnold *et al.*, (2004)] and RXRα (Hoffart *et al.*, 2012) have been described. Mouse CAR expression plasmid pCR3-mCAR was kindly provided by M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The β-galactosidase expression plasmid pCMVβ was purchased from Clontech.

The bacterial expression plasmid encoding GST/SRC-1 RID fusion protein was constructed by amplifying the sequences encoding human SRC-1 amino acids 583–783 by PCR, using appropriate primers, out of human liver cDNA and cloning into vector pGEX-6P1 (GE Healthcare). The plasmid encodes SRC-1 RID as an N-terminal GST fusion protein. Bacterial expression plasmids, encoding both N-terminal His-tagged human CAR1-LBD and human SRC-1 RID were described previously (Hoffart *et al.*, 2012). The identities of all PCR-amplified DNA fragments were verified by sequencing.

Other materials

Antimalarial drugs and drug metabolites were purchased from Sigma-Aldrich (Taufkirchen, Germany), if not indicated otherwise. Carboxymefloquine, cycloguanil, dapsone, dapsone hydroxylamine, deoxyartemisinin, deoxyarteether, desethylchloroquine, didesethylchloroquine, isoquinine and N-desethylamodiaquine were purchased from Toronto Research Chemicals (North York, ON, Canada), whereas artemisinin, dihydroartemisinin, arteether, artemether and artesunate were kindly provided by Dafra Pharma (Turnhout, Belgium). Piperaquine, pyronaridine were from AvaChem Scientific (San Antonio, TX, USA), lumefantrine and desbutyl-lumefantrine were kindly provided by Novartis (Basel, Switzerland). Chlorcycloguanil was obtained from GlaxoSmithKline (Stevenage, UK). DMSO, PK11195 and 5α-androst-16-en-3α-ol (androstenol) were purchased from Sigma-Aldrich. CITCO was purchased from BIOMOL/Enzo Life Sciences (Plymouth Meeting, PA, USA).

Further reagents were purchased as indicated: glutathione sepharose 4B and Biacore CM5 sensor chips (GE Healthcare), TNT quick coupled transcription/translation system (Promega, Madison, WI, USA), ^{35}S-methionine with specific activity of 1175 Ci·mmol^{-1} and radioactive concentration of 10 μCi·μL^{-1} (MP Biomedicals, Santa Ana, CA, USA), qPCR MasterMix Plus Low ROX (Eurogentec, Seraing, Belgium). Oligonucleotide primers and TaqMan probes were custom-synthesized by Biomers (Ulm, Germany) and Applied Biosystems respectively.

Results

Screening of antimalarial drugs and drug metabolites for ligand binding to human CAR3

The ligand-dependent splice variant CAR3 has been proposed as a sensitive tool for the identification of CAR ligands (Faucette *et al.*, 2007), as the insertion of the five amino acids APYLT was predicted to not change the LBP of the receptor (Auerbach *et al.*, 2005). Additionally, CAR3 is of importance by itself, as its expression even exceeds that of the reference variant. Thus, we initially screened antimalarial drugs and drug metabolites for CAR activation, using a mammalian two-hybrid CAR3-coactivator interaction assay (Arnold *et al.*, 2004). Figure 1 shows that the prototypical human CAR ligand CITCO strongly induced the interaction of CAR3 with coactivator DRIP205 (MED1). Significant dose-dependent induction was also observed with the artemisinin-type drugs arteether, artemether and artemisinin itself, as well as with the metabolites deoxyarteether and deoxyartemisinin, if used at 10 and 100 μM (Figure 1). Artemisinin, which was previously shown to activate human CAR1 by binding as an agonist (Burk *et al.*, 2005), turned out to be less efficient and potent than the derivatives arteether and artemether. The pharmacologically inactive deoxy metabolites demonstrated similar efficacy and potency to those of artemisinin (Table 1). In summary, these artemisinin-type compounds represent novel putative CAR ligands. When tested at 10 μM, none of the other antimalarials showed induction (Figure 1). However, cytotoxicity prevented the analysis of atovaquone, desbutyllumefantrine, hydroxychloroquine, piperaquine, pyrimethamine and pyronaridine. Treatment with 100 μM resulted in cytotoxic effects of even more compounds and none of the remaining ones showed induction (Supporting Information Table S1).

Molecular docking of antimalarials into the human CAR LBP

Due to cytotoxicity, most antimalarials outside the artemisinin class could not be tested at concentrations higher than 10 μM. Thus, we performed *in silico* molecular docking analyses using the published human CAR X-ray crystal structures (Xu *et al.*, 2004). All antimalarial drugs and drug metabolites, as well as the reference ligand CITCO, were successfully docked into the LBP of human CAR. Normalized average docking scores are shown in Supporting Information Table S2. Artemisinin-type chemicals, which have been identified as putative human CAR ligands in the mammalian two-hybrid assay, scored highest among all compounds. Surprisingly, dihydroartemisinin, which showed no effect in the two-hybrid assay, also scored among the putative agonists. Several other antimalarials demonstrated high docking scores; however, these did not reach the levels of the best-scored artemisinin-type compounds.

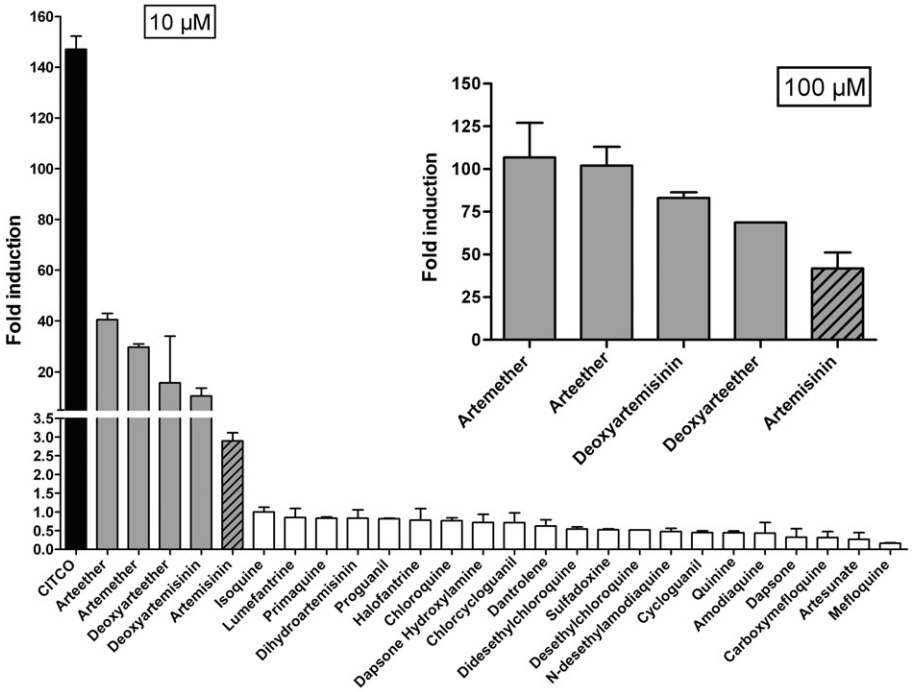

Figure 1

Antimalarial drugs of the artemisinin class induce the interaction of CAR3 with coactivator DRIP205 (MED1). Mammalian two-hybrid coactivator interaction assays in COS1 cells, co-transfected with expression plasmids encoding GAL4-DBD/DRIP205-RID (527–774) and VP16-AD/hCAR3-LBD (105–353) fusion proteins. Cells were treated with 0.1% DMSO or 10 μM of the indicated compounds (100 μM in inset). Columns show mean fold induction (±SD) of the respective normalized activity of co-transfected reporter plasmid pGL3-G5 by chemical treatment, as compared with treatment with solvent DMSO only, which was designated as unity.

Table 1

EC_{50} values for CAR3 activation by antimalarials

Compound	EC_{50} (μM)	I_{max} (f.c.)	C at I_{max} (μM)	C range (μM)
Artemisinin	>60	60.2	300	0.1–300
Arteether	10.4	243.8	100	0.1–300
Artemether	12.6	100.8	100	0.1–300
Deoxyartemisinin	61	66.9	300	0.3–300
Deoxyarteether	49	82.3	300	1–300
CITCO	1.3	237.9	10	0.01–30

COS-1 cells, transfected as described in Figure 1, were treated with compounds at the indicated ranges of concentrations in half-log steps. Calculations were done with means of three independent experiments. I_{max}, maximal induction; f.c., fold change; C, concentration.

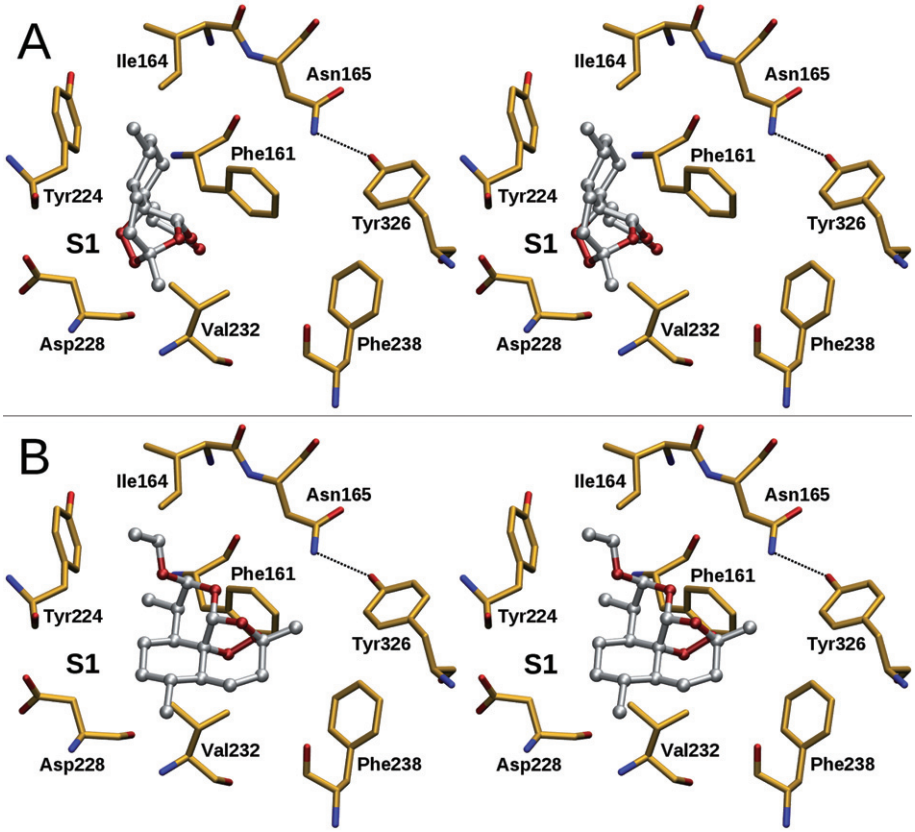

Figure 2

Docking of artemisinin-type drugs into human CAR. Stereoscopic views of the best-ranked conformation of artemisinin (A) and arteether (B) within the LBP of human CAR. The hydrogen bond connecting Asn^{165} and Tyr^{326} is indicated as a dotted line. Ligands are shown in ball and stick representation. Selected amino acids of the LBP, including the S1 sub-pocket, are displayed as capped sticks.

Artemisinin, the top-ranked compound, was docked into the rear of the LBP without any contacts to amino acids directly interacting with residues of the ligand-dependent activation function located on the C-terminal helix 12 (H12) (Figure 2A). The ligand partially occupied the S1 sub-pocket, which is expected to be addressed by the Phe^{161} side chain in un-liganded receptor (Jyrkkärinne *et al.*, 2008; Windshügel and Poso, 2011). Besides displacing Phe^{161} from the S1 sub-pocket, artemisinin restricted the conformational flexibility of Phe^{161} by embracing the phenyl side chain, thereby enhancing van der Waals interactions between it and Leu^{336}, Met^{340}, Asn^{165} as well as Tyr^{326}. This is expected to better maintain the helical conformation of the activation function. Although structurally very similar, the binding modes of arteether and artemether differ significantly from that determined for artemisinin (Figure 2B).

Arteether showed only marginal S1 occupation. However, the molecule revealed extensive van der Waals contacts with Phe^{161}, Phe^{238} and Tyr^{326}, respectively. The aromatic side chain of Tyr^{326} directly contacts the activation helix, which is considered as important for the basal activity of CAR. By interacting with Tyr^{326}, arteether restricts the conformational freedom of the Tyr^{326} side chain and thus improves Tyr^{326}-H12 contacts, which in turn limit H12 movement and maintain its α-helical fold. Most binding modes of artemether also revealed vdW interactions with Phe^{238} and/or Tyr^{326}, respectively. In contrast to arteether and artemether, the binding mode of artemisinin did not show any interaction with either Phe^{328} or Tyr^{326}.

Artesunate, the only drug of the artemisinin class scoring lower, was found to be placed in various binding modes within the LBP (Supporting Information Table S2). Several docking poses revealed the hydrogen succinate moiety orientated towards the LBD–H12 interface, which may disturb the hydrogen bond between Asn^{165} and Tyr^{326}, thus destabilizing Tyr^{326}-H12 interactions and finally causing receptor inactivation by interfering with keeping H12 in the active conformation. The binding mode of dihydroartemisinin demonstrated to be very similar to that of artemisinin and did not offer any explanation for its lack of agonist activity (data not shown).

In vitro *coactivator interaction assays confirm deoxyartemisinin, arteether and artemether as CAR1 agonists*

Several antimalarials, which did not prove to be CAR3 ligands, nevertheless demonstrated high docking scores *in silico*, thereby indicating that they may be CAR1 ligands. Thus, we further analysed *in vitro* ligand binding to CAR1, using a CARLA. The assay relies on the ligand-dependent interaction of ^{35}S-methionine labelled full-length reference variant CAR1 with the bacterially expressed RID of coactivator SRC-1 (NCOA1). All antimalarials were tested at 100 μM, with some of them further analysed at 300 μM. CAR1 already showed constitutive interaction with SRC-1. This interaction was enhanced by agonist ligands, as demonstrated by CITCO. CARLA confirmed the agonist binding of all artemisinin derivatives and metabolites, which were already identified as CAR3 ligands in the two-hybrid assay (Figure 3A, Supporting Information Table S1). Artemisinin itself showed a two-fold increase in coactivator recruitment and thus demonstrated weaker activity than its metabolite and the derivatives. The rank order was artemisinin < deoxyartemisinin < artemether < arteether. In contrast, dihydroartemisinin and artesunate, even at 300 μM, did not show any significant induction of coactivator interaction, indicating that these two artemisinin-type compounds were not agonists of CAR1. Similarly, none of the other antimalarials were CAR1 agonists in CARLA (Supporting Information Table S1). The non-ligand CAR activator phenobarbital did not show any effect, thereby demonstrating the specificity of the CARLA assay. However, it was clearly limited to the identification of agonists, as the human CAR1 inverse agonists, PK11195 and clotrimazole, did not show significant reduction of the basal interaction between SRC-1 and CAR1 (Supporting Information Table S1).

To further confirm agonist binding of the clinically used derivatives arteether and artemether, as well as of the parent drug artemisinin, a Biacore surface plasmon resonance assay was applied. Figure 3B shows that all three compounds and CITCO significantly increased the interaction of purified CAR1 LBD with immobilized SRC-1 RID ($P < 0.05$; one-sample *t*-test, *P*-values Bonferroni-adjusted) as compared with treatment with vehicle DMSO only. This increase was significantly diminished by concomitant treatment with the human CAR1 inverse agonist clotrimazole (Figure 3B). Relative activities of compounds were similar to those identified with CARLA.

The artemisinin metabolite deoxyartemisinin and derivatives arteether and artemether demonstrate ligand binding to and activation of human CAR1/3 and mouse CAR

Binding of a ligand to a nuclear receptor does not only result in changes of protein–protein interactions, but also in intramolecular conformational shifts. Among others, helix 1 of the LBD reorientates with respect to the remainder of the LBD after ligand binding. By separately expressing helix 1 and the remainder of the LBD, this conformational change can be used to create a nuclear receptor assembly assay for ligand binding (Pissios *et al.*, 2000). Here, we used the mammalian two-hybrid assembly assay for human CAR1 (Burk *et al.*, 2005) to confirm in a cell-based assay that arteether, artemether and deoxyartemisinin also act as ligands of the reference variant CAR1 (Figure 4A), besides recruiting coactivators to the ligand-dependent splice variant CAR3 (see Figure 1). Deoxyarteether, an arteether metabolite that is not detected in mammals (Lee and Hufford, 1990), was not further analysed. Activities of the derivatives and the artemisinin metabolite significantly exceeded the activity of artemisinin itself. Ligand binding of these artemisinin-type compounds was not restricted to human CAR, as they also induced the interaction of mouse CAR-LBD with SRC-1 RID in a mammalian two-hybrid coactivator interaction assay (Figure 4B).

To demonstrate induction of CAR transcriptional activity by the newly identified agonists of the artemisinin class, promoter reporter gene analyses were performed. First, we analysed activation of the ligand-dependent splice variant CAR3 using promoter reporter genes derived from three different CAR target genes. Arteether, artemether and deoxyartemisinin significantly induced the transcriptional activity of CAR3 at the ABCB1 (Figure 5A), CYP2B6 (Figure 5B) and CYP3A4 (Figure 5C) enhancer/promoters, as did the prototypical agonist CITCO. Arteether demonstrated significantly stronger activation of CAR3 than the parent drug artemisinin, which showed the weakest activity at all three reporter genes. At the CYP2B6 reporter gene, deoxyartemisinin also displayed a significantly stronger effect than artemisinin (Figure 5B).

In contrast to CAR3, the reference variant shows strong constitutive activity in promoter reporter gene assays (Arnold *et al.*, 2004 and Figure 6A). To demonstrate activation by ligands, its intrinsic activity has first to be inhibited by an inverse agonist. Figure 6A shows that treatment with the inverse agonist PK11195 reduced the constitutive activity of CAR1 to 13%. Concomitant treatment with artemisinin, its derivatives arteether and artemether, and its metabolite

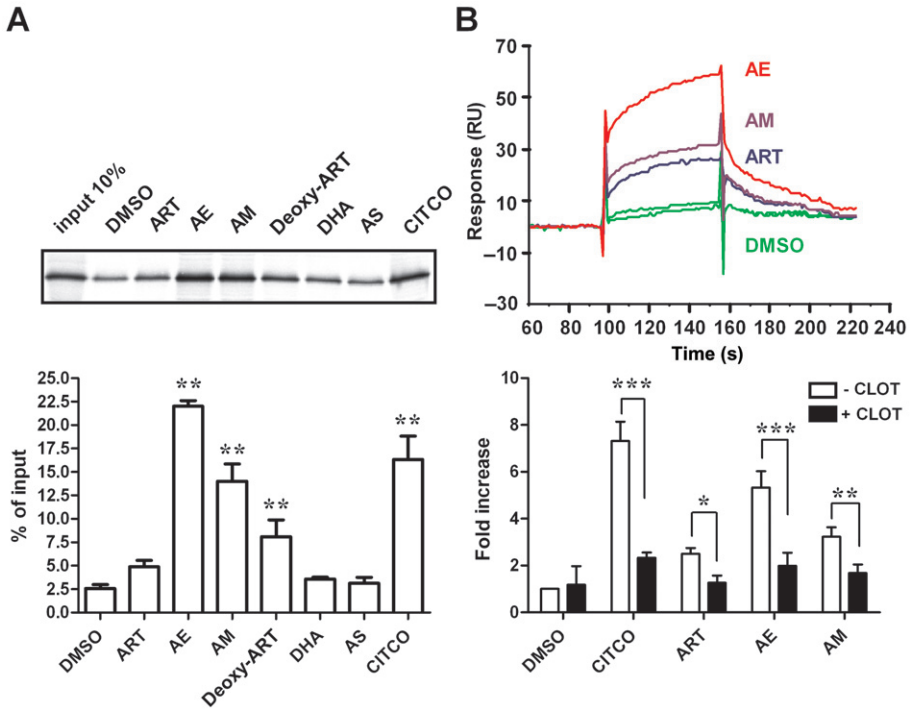

Figure 3

Artemisinin derivatives and metabolites demonstrate differential ligand binding to CAR1 in co-activator interaction assays *in vitro*. (A) CARLA. Ligand-dependent induction of the interaction of bacterially expressed GST/SRC-1 RID with ^{35}S-Met labelled full-length human CAR1 protein was analysed by GST pull-down and subsequent protein gel electrophoresis. Quantification of CAR protein, bound to SRC-1, was performed by phosphor storage scanning. The assay was performed in the presence of solvent DMSO only (1%), 10 μM CITCO or 100 μM of the indicated artemisinin-type compounds. Upper panel, scanning image of a representative experiment; lower panel, quantitative analysis with columns showing means ± SD of three to four independent experiments. $^{**}P < 0.01$, significantly different from DMSO only; one-way ANOVA with Dunnett's multiple comparisons test. (B) Biacore analysis of ligand-induced co-activator interaction. CAR1-LBD protein, pre-incubated with 10 μM CITCO, 100 μM of the indicated artemisinin-type compounds or 1% DMSO only, in the absence (–CLOT) or presence (+CLOT) of 100 μM clotrimazole, was injected onto immobilized SRC-1 RID protein. Upper panel, individual sensorgrams of a representative experiment in the absence of clotrimazole; lower panel, quantitative analysis with columns showing means ± SD of three to four independent experiments. Binding of CAR to SRC-1, in the presence of DMSO only, was designated as unity. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from no clotrimazole (-CLOT); one-way ANOVA with Bonferroni multiple comparisons test. (A–B) ART, artemisinin; AE, arteether; AM, artemether; Deoxy-ART, deoxy-artemisinin; DHA, dihydroartemisinin; AS, artesunate.

deoxyartemisinin re-induced the CAR-dependent transactivation of the CYP2B6 reporter gene promoter. With 35% of basal levels, artemisinin demonstrated significantly weaker effects than arteether, which achieved 75% (Figure 6A). The constitutive activity of mouse CAR was reduced by the inverse agonist androstenol to 6% (Figure 6B). Concomitant treatment with the same artemisinin-type compounds also significantly restored mouse CAR activity. The ranking of compounds in the activation of mouse CAR was different from the one observed with human CAR. With 54% of constitutive activity, artemisinin was significantly stronger as an activator of mouse CAR than arteether and deoxyartemisinin, which both achieved about 34% (Figure 6B).

Artemisinin derivatives and metabolites as inverse agonists of CAR1 and CAR2

The initial cellular and *in vitro* screening assays both proved to be suitable for the detection of agonists of the respective isoforms, but failed to detect inverse agonists (Supporting Information Table S1). In contrast, the CAR1 assembly assay showed no bias in that respect (Hoffart *et al.*, 2012). However, dihydroartemisinin and artesunate, which were not CAR1/3 agonists (see Figure 1 and Supporting Information Table S1), weakly induced the assembly of the human CAR1 LBD, thereby indicating ligand binding (Figure 7A). Both compounds further demonstrated dose-dependent inhibition of the constitutive transcriptional activity of CAR1, indicating inverse agonism (Figure 7B).

Besides CAR1 and CAR3, which together represent 80–95% of functional CAR transcripts in human liver (Ross *et al.*, 2010), the minor isoform CAR2 has been demonstrated to exhibit distinct ligand-binding specificity (Auerbach *et al.*, 2007). Using a CAR2 assembly assay, arteether and artemether also emerged as putative ligands of this splice variant, whereas the other derivatives and metabolites did not (Figure 7C). Surprisingly, all derivatives and metabolites, with the exception of deoxyartemisinin, significantly inhibited the constitutive activity of CAR2, suggesting that they act as CAR2 inverse agonists (Figure 7D).

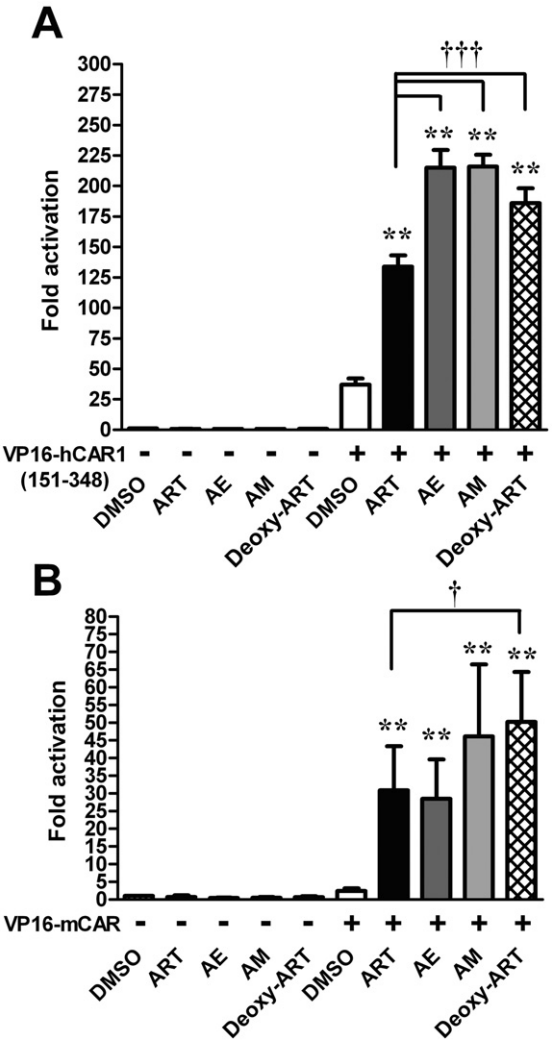

Figure 4

Artemisinin derivatives and the deoxy-metabolite induce the assembly of human CAR1 and the interaction of mouse CAR with coactivator SRC-1. (A) COS1 cells, co-transfected with expression plasmids encoding GAL4-DBD/hCAR-LBD (105–150) and VP16-AD/hCAR1-LBD (151–348) fusion proteins (+) or empty vector pVP16-AD (–), were treated with 0.1% DMSO or 100 μM of the indicated compounds. Mean fold activation (±SD) of the normalized activity of co-transfected reporter plasmid pGL3-G5 by treatment with the indicated compounds is shown. The corresponding activity of cells, transfected with GAL4-DBD/hCAR-LBD (105–150) expression plasmid and pVP16-AD, treated with DMSO only, was designated as unity. (B) COS1 cells, co-transfected with expression plasmids encoding GAL4-DBD/SRC1-RID (583–783) and VP16-mCAR-LBD (95–358) (+) or empty vector pVP16-AD (–) were treated as described earlier. Mean fold activation (±SD) of the normalized activity of co-transfected reporter plasmid pGL3-G5 by treatment with the indicated chemicals is shown. The corresponding activity of cells, transfected with GAL4-DBD/SRC1-RID (583–783) expression plasmid and pVP16-AD, treated with DMSO only, was designated as 1. (A–B) $^{**}P < 0.01$, significantly different from DMSO only; repeated measures one-way ANOVA with Dunnett's multiple comparisons test. †$P < 0.05$; †††$P < 0.001$, significant differences between individual treatments; one-way ANOVA with Bonferroni's multiple comparisons test (selected pairs). ART, artemisinin; AE, arteether; AM, artemether; Deoxy-ART, deoxy-artemisinin; DHA, dihydroartemisinin; AS, artesunate.

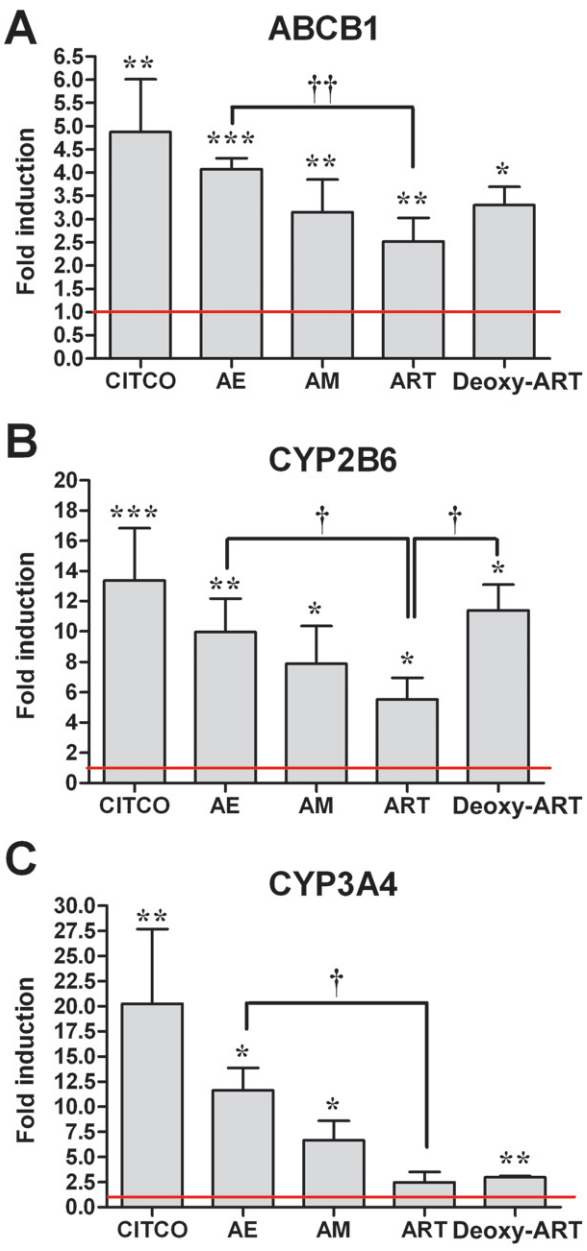

Figure 5

Artemisinin derivatives and the deoxy-metabolite induce the transcriptional activity of CAR3. Caco-2 TC7 cells, co-transfected with expression plasmids encoding human CAR3 and RXRα, together with the enhancer/promoter reporter gene plasmids of ABCB1 (A), CYP2B6 (B) and CYP3A4 (C), were treated with 0.1% DMSO, 10 μM CITCO, 30 μM AE or AM, 100 μM ART or deoxy-ART. Mean fold induction (±SD) of the normalized activity of respective co-transfected reporter plasmids by treatment with the indicated compounds is shown. The respective activity in the presence of DMSO only was designated as unity. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from DMSO only; one-sample *t*-test, with *P*-values adjusted by the method of Bonferroni †$P < 0.05$; ††$P < 0.01$, significant differences between ART and the other artemisinin-type compounds; one-way ANOVA with Bonferroni multiple comparisons test (selected pairs). ART, artemisinin; AE, arteether; AM, artemether; Deoxy-ART, deoxy-artemisinin; DHA, dihydroartemisinin; AS, artesunate.

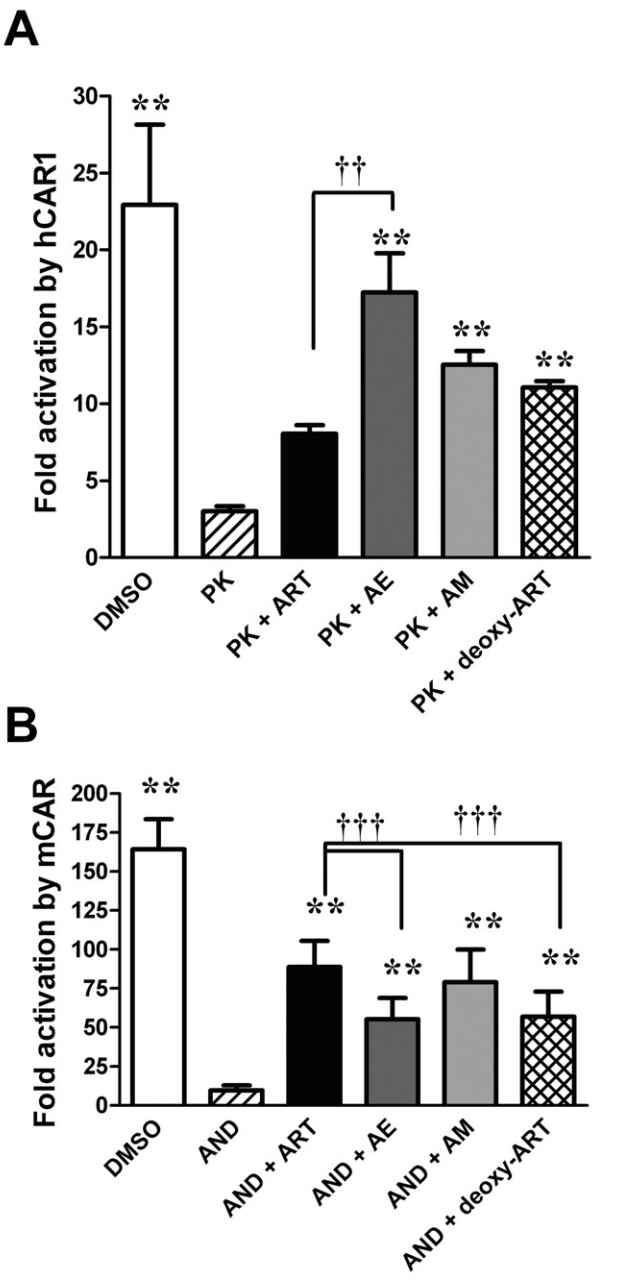

Figure 6

Artemisinin derivatives and the deoxy-metabolite differentially activate human CAR1 and mouse CAR. COS1 cells were co-transfected with the CYP2B6 enhancer/promoter reporter gene and expression plasmids encoding (A) human CAR1 or (B) mouse CAR. Columns show mean fold activation (±SD) by the respective CAR proteins in the presence of the indicated compounds, alone or in combination: 0.15% DMSO (DMSO); 5 μM PK11195 (PK); 5 μM androstenol (AND); 100 μM artemisinin (ART); 100 μM arteether (AE); 100 μM artemether (AM); 100 μM deoxyartemisinin (deoxy-ART). The activity in the presence of empty expression vector pcDNA3 and treatment with DMSO only, was designated as unity. $^{**}P < 0.01$ significantly different from empty vector and DMSO only; repeated measures one-way ANOVA with Dunnett multiple comparisons test. $^{††}P < 0.01$; $^{†††}P < 0.001$, significant differences between ART and the other artemisinin-type compounds; repeated measures one-way ANOVA with Bonferroni's multiple comparisons test (selected pairs).

Artemisinin-type compounds as PXR agonists

As artemisinin is a mixed CAR and PXR agonist (Burk *et al.*, 2005), its metabolite and derivatives were further tested for ligand binding to and activation of PXR. With the exception of dihydroartemisinin and artesunate, all artemisinin-type compounds induced PXR LBD assembly, suggesting that they bind as ligands (Supporting Information Figure S1). Deoxyartemisinin, arteether and artemether further induced PXR-dependent transcriptional activation in a reporter gene assay, with respective EC_{50} values in the same range as observed for CAR3 (Supporting Information Table S3).

Clinically used derivatives and metabolites of artemisinin differentially induce the expression of cytochrome P450 and ABCB1 genes in primary human hepatocytes

Artemisinin has been shown to induce the expression of CAR/PXR target genes in primary human hepatocytes (Burk *et al.*, 2005). Thus, we analysed whether the newly identified CAR1/3 agonists of the artemisinin class, among which the derivatives arteether and artemether show higher affinity to CAR and PXR than artemisinin, may exert similar effects. Additionally, dihydroartemisinin and artesunate were tested. Figure 8 shows that arteether, artemether and deoxyartemisinin demonstrated induction of CYP3A4 (Figure 8A) and CYP2B6 (Figure 8B) expression in primary human hepatocytes. However, with the exception of artemether, induction by artemisinin appeared to be stronger than induction by the other compounds, even if statistical significance was not always achieved, most likely due to the high interindividual variability of induction between hepatocyte donors. Arteether, artemether and artemisinin induced the expression of ABCB1 to a similar extent (Figure 8C). In contrast, dihydroartemisinin and artesunate did not induce the expression of the two cytochrome P450 genes. CYP3A4 expression was even significantly down-regulated in cells treated with dihydroartemisinin. A similar tendency was observed for CYP2B6 by treatment with dihydroartemisinin and for CYP3A4 by treatment with artesunate.

Discussion and conclusions

The combination of CAR1 and CAR3 screening assays unequivocally showed that most antimalarial drugs were not agonists of the two major human CAR isoforms, together accounting for 80–95% of functional CAR transcripts (Ross *et al.*, 2010). Thus, we may conclude that CAR-dependent drug–drug interactions should not be anticipated, in their therapeutic use. However, we cannot exclude the possibility that at least some of them may activate the constitutively active isoform CAR1 indirectly, by a phenobarbital-like mechanism.

Here we provide the first evidence that arteeether, artemether and the deoxy metabolite of artemisinin were novel agonists of CAR1 and CAR3. In contrast, dihydroartemisinin and artesunate were weak inverse agonists of CAR1. Regarding the minor isoform CAR2, all artemisinin-type compounds may act as inverse agonists, although ligand binding to CAR2

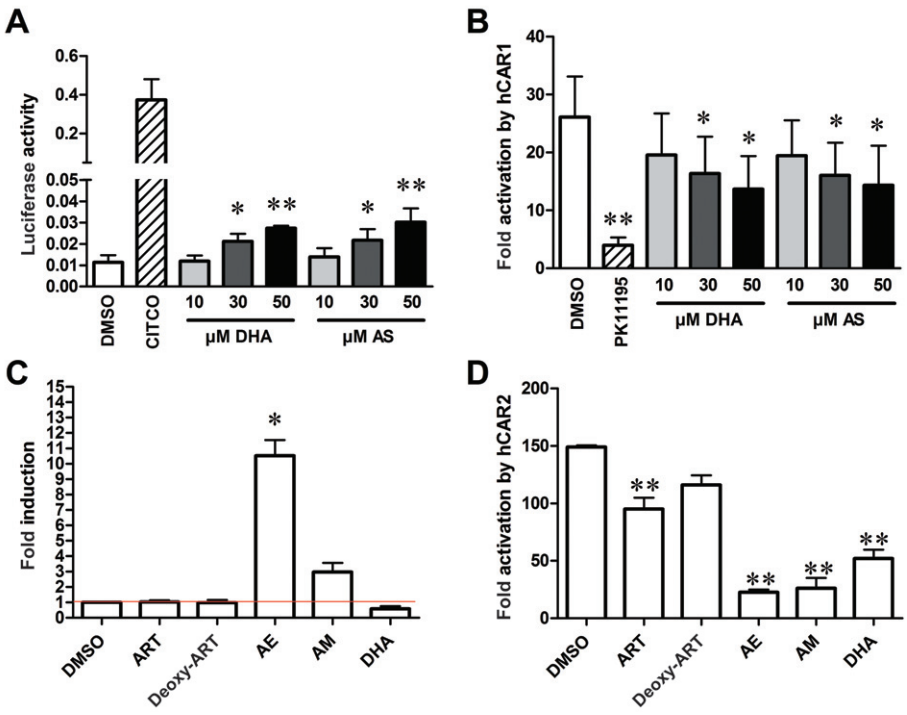

Figure 7

Artemisinin derivatives and metabolites as inverse agonists of CAR1 and CAR2. (A) HepG2 cells, co-transfected with expression plasmids encoding GAL4-DBD/hCAR-LBD (105–150) and VP16-AD/hCAR1-LBD (151–348) fusion proteins were treated with 0.1% DMSO, 1 μM CITCO, dihydroartemisinin (DHA) or artesunate (AS) at the indicated concentrations for 24 h. Mean (±SD) normalized activity of co-transfected reporter plasmid pGL3-G5 is shown. (B) HepG2 cells, co-transfected with the CYP2B6 enhancer/promoter reporter gene and human CAR1 expression plasmid, were treated as described earlier or with 10 μM PK11195 for 24 h. Columns show mean fold activation (±SD) by CAR1 in the presence of the indicated compounds. The activity in the presence of empty expression vector pcDNA3 and treatment with DMSO only was designated as unity. (C) HepG2 cells, co-transfected with expression plasmids encoding GAL4-DBD/hCAR-LBD (105–150) and VP16-AD/hCAR2-LBD (151–352) fusion proteins were treated with 100 μM AE, AM, ART, deoxy-ART, 30 μM DHA or 0.1% DMSO for 24 h. Columns show mean fold induction (±SD) of normalized activity of co-transfected reporter pGL3-G5 by the respective treatment. The activity in the presence of DMSO only was designated as unity. *$P < 0.05$, significantly different from DMSO only; one-sample *t*-test, with *P*-values adjusted by the method of Bonferroni. (D) HepG2 cells, co-transfected with the CYP2B6 enhancer/promoter reporter gene plus human CAR2 and RXRα expression plasmids, were treated as described in C. Columns show mean fold activation (±SD) by CAR2/RXRα in the presence of the indicated compounds. The activity of cells transfected with RXRα only and treated with DMSO, was designated as unity. (A, B, D) *$P < 0.05$; **$P < 0.01$, significantly different from DMSO only; one-way ANOVA with Dunnett's multiple comparisons test.

was only shown for arteether and artemether. Thus, interindividual variability of *in vivo* induction of drug metabolism by these compounds may be co-determined by the relative amounts of CAR isoforms.

The concentrations of artemisinin-type compounds, which were required to activate CAR1/3, as suggested by the EC_{50} values of the CAR3-coactivator interaction assay, greatly exceed the maximal plasma levels at clinically used oral doses, ranging between 0.5 and 2 μM for the different drugs (calculated with data compiled by Kyle *et al.*, 1998). However, ligands seem to bind with lower affinity to CAR3 than to CAR1. The EC_{50} value of 1.3 μM, which we obtained for CITCO induction of CAR3 interaction with coactivator DRIP205, is 25-fold higher than the one reported for CITCO induction of CAR1 coactivator interaction (Maglich *et al.*, 2003). Thus, it is assumed that the assay may underestimate the affinity with which the agonists of the artemisinin class bind to CAR1. Additionally, intrahepatic concentrations achieved *in vivo* are anticipated to exceed maximal plasma levels, given the lipophilicity of artemisinin-type drugs, which show logP values up to 3.60 (Gautam *et al.*, 2009). Accordingly, accumulation in tissues, including liver, has been demonstrated for artemether and arteether in animals (see Navaratnam *et al.*, 2000 and references therein).

Cellular and *in vitro* assays both demonstrated that arteether and artemether activated CAR1 and CAR3 more strongly than the parent drug artemisinin and its metabolite deoxyartemisinin. The difference cannot be attributed to the higher lipophilicity of the derivatives (Gautam *et al.*, 2009), or to differential transmembrane transport, as the *in vitro* CAR-coactivator interaction data also demonstrate significantly stronger effects of the derivatives. Although artemisinin revealed the highest docking score among all antimalarials, it did not overall interact favourably with CAR, as its carbonyl moiety did not share any hydrogen bond with amino acid residues of the LBP. The more potent arteether and artemether adopt different binding modes and show interactions with amino acids important for basal and ligand-

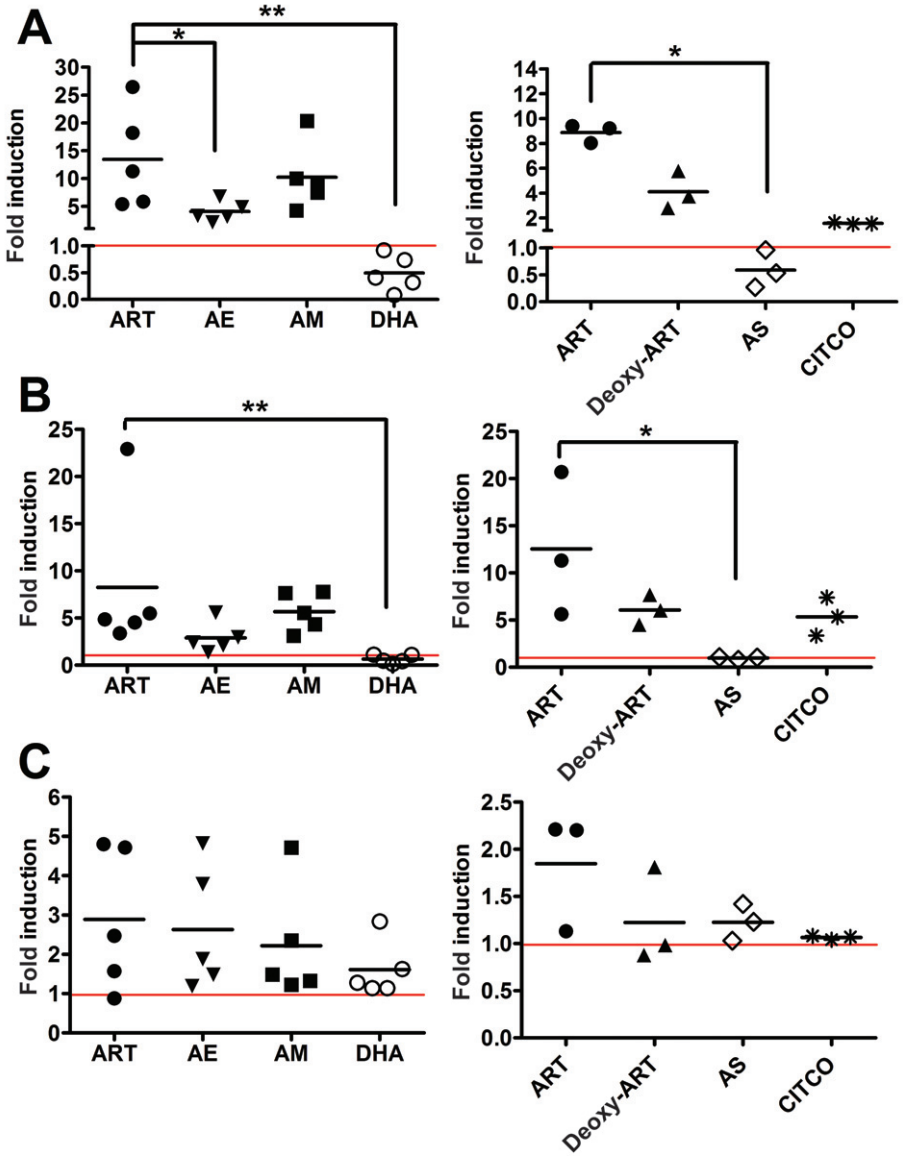

Figure 8

Artemisinin derivatives and metabolites differentially induce the expression of cytochrome P450 and ABCB1 genes in primary human hepatocytes. Total RNA was prepared after 48 h of treatment of primary human hepatocyte cultures with 100 μM of the indicated artemisinin-type compounds, 1 μM CITCO or 0.1% DMSO only, followed by analysis of (A) CYP3A4; (B) CYP2B6; and (C) ABCB1 mRNA expression by TaqMan real-time RT-PCR. Expression levels were normalized with respect to the corresponding expression of 18S rRNA. Data are shown as fold induction by treatment with compounds, as compared with the treatment with DMSO only, which was designated as unity, and are presented as scatter plots with means indicated by lines. *$P < 0.05$; **$P < 0.01$, significant differences between induction by ART and the other artemisinin-type compounds; repeated measures one-way ANOVA with Bonferroni multiple comparisons test (A, left panel) or by Friedman test with Dunn's multiple comparison post-test (A, right panel and B). Left and right panels show the results of two different series of experiments, performed with $n = 5$ (left) and $n = 3$ (right) individual cultures of hepatocytes. ART, artemisinin; AE, arteether; AM, artemether; Deoxy-ART, deoxy-artemisinin; DHA, dihydroartemisinin; AS, artesunate.

induced activity, which may explain their higher efficacy and potency as CAR ligands.

In contrast, artemisinin demonstrated higher activity than arteether and similar activity as artemether in the induction of CAR/PXR target gene expression in primary human hepatocytes. Stronger activation of PXR by artemisinin cannot explain the difference, as the respective EC_{50} values demonstrate that this drug is also less effective in PXR activation than arteether or artemether. However, arteether is primarily metabolized by CYP3A4 (Grace *et al.*, 1998), whereas artemisinin and artemether are primarily metabolized by CYP2B6 (Svensson and Ashton, 1999; Honda *et al.*, 2011). Hepatocytes, as cultured in this study, demonstrate a 10-fold higher expression of CYP3A4 than of CYP2B6 (data not shown). Therefore it may be assumed that arteether is more extensively metabolized than artemisinin and arte-

mether, resulting in more rapid clearance. Alternatively, differential transmembrane transport of arteether in hepatocytes may explain its reduced activity. Inhibition of CYP gene expression by dihydroartemisinin and artesunate may be explained by them acting as inverse agonists of CAR1.

The strong induction of CYP gene expression by artemisinin in primary human hepatocytes confirms *in vivo* observations. In clinical studies, artemisinin most strongly and consistently induced the activities of CYP3A4, CYP2C19 and CYP2B6, whereas arteether and artemether demonstrated much weaker induction, if at all. Dihydroartemisinin and artesunate also slightly affected the activities of CYP2B6 and CYP3A4 (Asimus *et al.*, 2007; Elsherbiny *et al.*, 2008), which, according to the results presented here, cannot be explained by activation of CAR or PXR.

Our data suggest a molecular mechanism which may at least partially explain the differential inductive activities of artemisinin-type drugs *in vivo*. Data obtained *in vitro* using immortalized cell lines, which lack significant drug metabolism, unequivocally demonstrated artemisinin being the weakest CAR/PXR agonist. Nevertheless, it was the strongest inducer in drug metabolism-competent primary human hepatocytes, as well as *in vivo*. Metabolism by CYP enzymes results in metabolites with differential activity towards CAR and PXR: arteether and artemether are both metabolized to dihydroartemisinin, primarily by CYP3A4 and CYP2B6, respectively (Grace *et al.*, 1998; Honda *et al.*, 2011), which here proved not to activate PXR and even to inhibit CAR1. Four different metabolites of artemisinin, including deoxyartemisinin, have been identified in humans (Lee and Hufford, 1990), which may be formed by CYP2B6, as this is the primary isozyme metabolizing artemisinin (Svensson and Ashton, 1999). Here, deoxyartemisinin was shown to be a CAR1/3 and PXR agonist of its own. Thus, metabolism of arteether and artemether results, on the one hand, in rapid disappearance of the inducing parent compounds, and on the other hand, in the formation of a non-inducing, but inhibiting, metabolite. In contrast, artemisinin autoinduction of metabolism gives rise to at least one inducing metabolite, thereby potentially resulting in the continued and increased activation of CAR and PXR. The lower affinity of artemisinin seems to be compensated by formation of at least one metabolite, which also activates both receptors (Figure 9). Participation of metabolites in the induction by artemisinin is further suggested by the persistence of its autoinduction for 5 days after a single dose (Zhang *et al.*, 2001), which cannot be explained by artemisinin itself due to its short elimination half-life of 2–3 h (Giao and de Vries, 2001). Further studies are required to analyse the inducing capacity of the other metabolites of artemisinin and to quantify their respective plasma levels.

ACT is the current state-of-the-art treatment of uncomplicated *P. falciparum* malaria (WHO, 2010a). To reduce the risk of drug–drug interactions due to the induction of drug metabolism and transport, it may be worth considering whether artemisinin-type drugs that do not activate CAR/PXR, such as artesunate and dihydroartemisinin, should be preferred in ACTs. Additionally, our data provide first evidence that the antimalarial drugs amodiaquine, mefloquine, sulfadoxine-pyrimethamine, piperaquine and lumefantrine, which are components of WHO-recommended ACTs (WHO, 2010a), do not induce drug metabolism by ligand activation of CAR. Because these drugs reside for a longer time in the body, in contrast to artemisinin-type drugs, which are rapidly metabolized and characterized by very short elimination half-lives (Giao and de Vries, 2001), this is important information with potential clinical relevance. However, we still do not know if these antimalarial drugs may activate PXR or indirectly CAR1, and thus affect drug metabolism. This question warrants further systematic investigations.

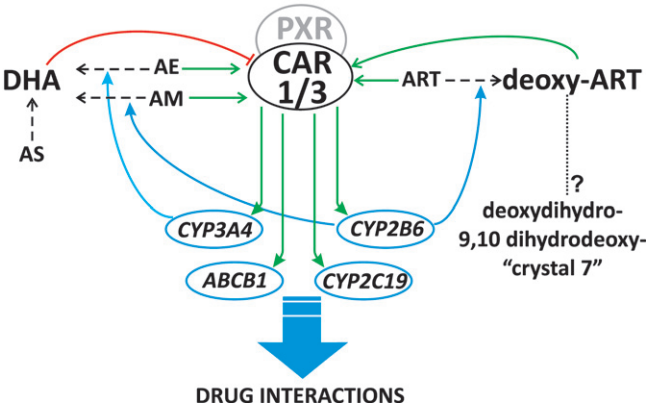

Figure 9

The differential induction by artemisinin-type drugs *in vivo* is proposed to result from the inducing capacity of metabolites. Artemisinin (ART) and its derivatives arteether (AE) and artemether (AM) have been identified as agonists of PXR and major CAR isoforms CAR1 and CAR3. AE and AM, as well as the derivative artesunate (AS), were rapidly metabolized to dihydroartemisinin (DHA), which acts as an inverse agonist of CAR1. On the other hand, ART is rapidly metabolized to deoxyartemisinin (deoxy-ART) and further three metabolites among which deoxy-ART was identified as an agonist of CAR1/3. In contrast to AE, which is primarily metabolized by CYP3A4, ART and AM are primarily metabolized by the polymorphic CYP2B6. The induction of CYP enzymes and further ADME genes, as ABCB1, may result in autoinduction of elimination of artemisinin-type drugs and in drug interactions with concomitant medications. For reasons of simplicity, inhibitory effects on the minor isoform CAR2 are not displayed.

Finally, our data that artemether is an agonist of CAR and PXR may be clinically relevant because Coartem®, a combination of artemether with lumefantrine, is the most frequently procured ACT worldwide (WHO, 2010b). Nowadays, it is also the only WHO-recommended ACT containing an artemisinin-type CAR/PXR agonist (WHO, 2010a). We suggest that drug interactions due to concurrent therapy of malaria by Coartem® and antiretroviral HIV agents should be considered, if the antiretroviral drugs are substrates and competitive inhibitors of CYP2B6, such as efavirenz (Hesse *et al.*, 2001; Ward *et al.*, 2003). In this case, CYP2B6-dependent metabolism of artemether may be inhibited, resulting in higher concentrations of the inducing parent compound. Moreover, genetic variants of CYP2B6 may also affect induction by artemether. No or significantly reduced metabolism of artemether has been demonstrated *in vitro* for several natural loss-of function variants of the enzyme (Honda *et al.*, 2011). Thus, CYP2B6 slow metabolizers may be at risk for increased induction of CYP enzymes, if treated with the

artemether-containing ACT, leading to impairment of the efficacy of the partner drug lumefantrine or of other co-administered drugs. Currently, clinical studies are lacking to support this assumption, but in recent years, there is an increasing awareness of the clinical impact of pharmacogenetics on drug–drug interaction (Kerb and Schwab, 2010). Thus, future studies are warranted.

In conclusion, we here have identified artemisinin-type drugs as novel agonists of the two major CAR isoforms. However, a class effect in xenobiotic induction has not been substantiated, as dihydroartemisinin and artesunate neither activated CAR *in vitro*, nor did they induce CAR/PXR-dependent gene expression in human hepatocytes. The properties of the respective metabolites may determine, at least partially, the differential induction capacity of artemisinin-type drugs *in vivo*. As the overall inducing capacity is made up by the combined properties of parent drugs and metabolites, the individual characteristics of metabolites in activation of xenosensing nuclear receptors should be investigated thoroughly during the drug development of novel artemisinin-based agents.

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Conflicts of Interest

The authors state no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Artemisinin derivatives and the deoxy-metabolite induce the assembly of human PXR. HepG2 cells, co-transfected with expression plasmids encoding GAL4-DBD/hPXR-LBD (132–188) and VP16-AD/hPXR-LBD (189–434) fusion proteins were treated with 0.1% DMSO, 10 μM rifampin (RIF), 100 μM artemisinin (ART), deoxyartemisinin (deoxy-ART), arteether (AE), artemether (AM), 30 μM dihydroartemisinin (DHA) or artesunate (AS) for 24h. Mean fold induction (±SD) of the normalized activity of co-transfected reporter plasmid pGL3-G5 by treatment with the indicated compounds is shown. The corresponding activity of cells, treated with DMSO only, was designated as unity. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significantly different from DMSO only; one-sample *t*-test.

Table S1 Induction of coactivator interaction by antimalarial drugs and drug metabolites

Table S2 Auto dock scores of reference ligand CITCO and antimalarial drugs docked into the CAR LBP. The number of clusters is shown separately for each docking approach.

Table S3 EC_{50} values for PXR activation by artemisinin-type compounds.

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