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Isoform-Selective Activation of Human Constitutive Androstane Receptor by *Ginkgo biloba* Extract: Functional Analysis of the SV23, SV24, and SV25 Splice Variants

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Running Title: *G. biloba* and hCAR Splice Variants

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ABBREVIATIONS: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; DEHP, di-(2-ethylhexyl)phthalate; DMSO, dimethyl sulfoxide; hCAR, human constitutive androstane receptor; hRXRα, human retinoid X receptor alpha; hSRC-1, human steroid receptor coactivator-1; hSRC-2, human steroid receptor coactivator-2; hSRC-3, human steroid receptor coactivator-3; mCAR, mouse constitutive androstane receptor; RXRα, retinoid X receptor alpha; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

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ABSTRACT

Naturally-occurring splice variants of human constitutive androstane receptor (hCAR) exist, including hCAR-SV23 (insertion of amino acids SPTV), hCAR-SV24 (APYLT), and hCAR-SV25 (SPTV and APYLT). An extract of *G. biloba* was reported to activate hCAR-SV24 and the wild-type (hCAR-WT). However, it is not known whether it selectively affects hCAR splice variants, how it activates hCAR isoforms, and which chemical is responsible for the effects of the extract. Therefore, we evaluated the impact of *G. biloba* extract on the functionality of hCAR-SV23, hCAR-SV24, hCAR-SV25, and hCAR-WT, and compared it to that of phenobarbital, di-(2-ethylhexyl)phthalate (DEHP), 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO), and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in cell-based reporter gene assays. Among the hCAR splice variants investigated, only hCAR-SV23 was activated by *G. biloba* extract, and this required co-transfection of a retinoid X receptor-α (RXRα) expression plasmid. The extract activated hCAR-SV23 to a lesser extent than hCAR-WT, but ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, or bilobalide was not responsible for the effects of the extract. CITCO activated hCAR-SV23, hCAR-SV24, and hCAR-WT. By comparison, phenobarbital activated hCAR-WT, whereas DEHP activated hCAR-SV23, hCAR-SV24 (with exogenous RXRα supplementation), and hCAR-WT. TCPOBOP did not affect the activity of any of the isoforms. *G. biloba* extract and phenobarbital did not bind or recruit coactivators to the ligand-binding domains of hCAR-WT and hCAR-SV23, whereas positive results were obtained with the controls (CITCO for hCAR-WT and DEHP for hCAR-SV23). In conclusion, *G. biloba* extract activates hCAR in an isoform-selective manner, and hCAR-SV23, hCAR-SV24, and hCAR-WT have overlapping but distinct sets of ligands.

Introduction

The constitutive androstane receptor (CAR), also referred to as MB67 (Baes et al., 1994), belongs to the superfamily of nuclear hormone receptors. It is designated as NR1I3 (Germain et al., 2006). CAR regulates the expression of multiple genes involved not only in xenobiotic metabolism, transport, and toxicity, but also in various other functions, including gluconeogenesis, lipogenesis, and thyroid hormone homeostasis (Gao and Xie, 2010). It can be activated by a mechanism that involves direct binding of an agonist to the ligand-binding domain of the receptor and recruitment of coactivators to the ligand-receptor complex (Timsit and Negishi, 2007). Chemicals shown to activate CAR by this mode of action are chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime (CITCO), which is an agonist of human CAR (hCAR) (Maglich et al., 2003), and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), which is an agonist of mouse CAR (mCAR) (Tzameli et al., 2000). In contrast, phenobarbital activates CAR not by binding to the ligand-binding domain of the receptor (Moore et al., 2000), but by cellular and molecular signaling mechanisms in which the intricate details are still not well-understood (Timsit and Negishi, 2007).

Naturally-occurring splice variants of hCAR have been identified, including hCAR-SV23, hCAR-SV24, and hCAR-SV25 (Lamba et al., 2005; Lamba, 2008). Table 1 shows the various nomenclatures of these splice variants and the reference isoform or the wild-type (hCAR-WT). Tissue distribution studies have shown that these hCAR splice variants and hCAR-WT are expressed in the liver (Savkur et al., 2003; Auerbach et al., 2003; Lamba et al., 2004; Jinno et al., 2004; Ross et al., 2010). Hepatic hCAR-SV23, hCAR-SV24, and hCAR-SV25 mRNA have been reported to be expressed at levels of approximately 6-30%, 20-42%, and 2-10% of total

hCAR transcripts, respectively (Savkur et al., 2003; Jinno et al., 2004; DeKeyser et al., 2009; Ross et al., 2010). As depicted in Fig. 1, the hCAR-SV23 splice variant has a 12-nucleotide insert between exons 6 and 7, resulting in the addition of 4 amino acids (SPTV), whereas the hCAR-SV24 splice variant has a 15-nucleotide insert between exons 7 and 8, leading to the addition of 5 amino acids (APYLT) (Auerbach et al., 2003). By comparison, the hCAR-SV25 splice variant has both the 12-nucleotide insert (identical to hCAR-SV23) and the 15-nucleotide insert (identical to hCAR-SV24) (Savkur et al., 2003; Auerbach et al., 2003). A limited number of functional studies have identified ligands of these splice variants. The only known hCAR-SV23 activators to date are di-(2-ethylhexyl)phthalate (DEHP) (DeKeyser et al., 2009), several other phthalates (DeKeyser et al., 2011), and CITCO (DeKeyser et al., 2009). hCAR-SV24 activators include CITCO (Auerbach et al., 2005; Faucette et al., 2007) and artemisinin (Faucette et al., 2007). To date, only two chemicals have been studied for their effects on hCAR-SV25 function. However, neither clotrimazole (Auerbach et al., 2003) nor CITCO (Jinno et al., 2004) activated this isoform in those studies.

Ginkgo biloba is often used by consumers for the self-treatment of various medical conditions, including dementia (Weinmann et al., 2010). It contains bioactive terpene trilactones, such as ginkgolides and bilobalide (van Beek and Montoro, 2009), and their chemical structures are shown in Fig. 2. Commercial preparations of *G. biloba* extract contain approximately 6% w/w terpene trilactones and 24% w/w flavonol glycosides, although considerable variability exists (Kressmann et al., 2002). As determined in a cell-based reporter gene assay (Li et al., 2009), a single concentration (100 µg/ml) of a *G. biloba* extract of undefined abundance of terpene trilactones and flavonol glycosides was reported to activate hCAR1 (also known as hCAR-WT; Table 1) and hCAR3 (also referred to as hCAR-SV24; Table 1) by ≤ 2-fold over the

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control group. However, it remains to be investigated whether *G. biloba* extract selectively activates hCAR splice variants, how it activates hCAR isoforms, and which chemical constituent(s) is responsible for the hCAR-activating effect of *G. biloba* extract.

In the current study, we compared the effects of *G. biloba* extract on the activity of hCAR-SV23, hCAR-SV24, hCAR-SV25, and hCAR-WT in cell-based reporter gene assays and evaluated the role of five individual terpene trilactones (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) in the extract. As a way to determine whether the extract exhibits receptor agonism, additional experiments were performed to assess whether it transactivates the ligand-binding domain of the receptor and promotes recruitment of coactivators. For comparative purposes, we also characterized the effects of phenobarbital, DEHP, CITCO, and TCPOBOP in our cell-based assays. Overall, our novel results demonstrate selective activation of hCAR splice variants by *G. biloba* extract and isoform-specific ligand-activation profiles for hCAR-SV23, hCAR-SV24, and hCAR-WT.

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Materials and Methods

***G. biloba* Extract, Chemicals, and Reagents**. Five individual lots of *G. biloba* extract, designated as Lot A, Lot B, Lot C, Lot D, and Lot E, were supplied in a dry powder form by Indena S.p.A. (Milan, Italy). The quantity of terpene trilactones and flavonol glycosides in each individual lot is shown in Table 2. Ginkgolide A (CAS #15291-75-5), ginkgolide B (CAS #15291-77-7), ginkgolide C (CAS #15291-76-6), and (-)-bilobalide (CAS #33570-04-6) were obtained from LKT Laboratories (St. Paul, MN), and ginkgolide J (CAS #107438-79-9) was from ChromaDex (Irvine, CA). Sodium phenobarbital (CAS #57-30-7), di-(2-ethylhexyl)phthalate (DEHP; CAS #117-81-7), 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP; CAS #76150-91-9), and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide (PK11195; CAS #85532-75-8) were purchased from Sigma-Aldrich (St Louis, MO). 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO; CAS #338404-52-7) was obtained from Enzo Life Sciences, Inc. (Plymouth Meeting, PA), and 5α-androstan-3α-ol (androstanol; CAS #7657-50-3) was from Steraloids (Newport, RI). Charcoal-stripped, heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) was bought from Thermo Fisher Scientific, Inc. (Nepean, ON, Canada). Opti-MEM and all other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Laval, QC, Canada) and Dual-Luciferase Reporter Assay System was from Promega (Madison, WI).

Plasmids. pCMV6-XL4-hCAR-WT, pCMV6-neo-hCAR-SV23, pCMV6-XL4-hCAR-SV24, pCMV6-XL5-hCAR-SV25, pCMV6-XL4-hRXRα, pCMV6-XL4, pCMV6-neo, and pCMV6-XL5 were purchased from OriGene Technologies, Inc. (Rockville, MD). *Renilla reniformis* luciferase pGL4.74[*hRluc*/TK] was obtained from Promega. A pGL3-basic-CYP2B6-

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PBREM/XREM-luc reporter was constructed as described previously (Wang et al., 2003). The pVP16 and pM empty vectors were provided in the Matchmaker Mammalian Two-Hybrid Assay Kit (Clontech, Mountain View, CA). PathDetect pFR-luc *trans*-reporter plasmid was purchased from Stratagene (La Jolla, CA). To construct the pVP16-hCAR-WT-LBD (Gln-105 to Ser-348) and pM-hCAR-WT-LBD (Gln-105 to Ser-348) plasmids, the ligand-binding domain (Gln-105 to Ser-348) (Burk et al., 2005) of hCAR-WT was amplified from pCMV6-XL4-hCAR-WT and inserted into the pVP16 or pM vector. To construct the pVP16-hCAR-SV23-LBD (Gln-105 to Ser-352) and pM-hCAR-SV23-LBD (Gln-105 to Ser-352) plasmids, the ligand-binding domain (Gln-105 to Ser-352) (Arnold et al., 2004) of hCAR-SV23 was amplified from pCMV6-neo-hCAR-SV23 and inserted into the pVP16 or pM vector. To construct the pM-hSRC1-RID plasmid, the receptor-interacting domain of hSRC-1 containing three consensus LXXLL motifs (Asp-621 to Asn-765) (Chang et al., 1999) was amplified from pCMV6-XL5-NCOA1 (OriGene Technologies, Inc.) and cloned into the pM vector. To construct the pM-hSRC2-RID plasmid, the receptor-interacting domain of hSRC-2 containing three consensus LXXLL motifs (Lys-583 to Thr-779) (Arnold et al., 2004) was amplified from human liver QUICK-Clone cDNA (Clontech) and cloned into the pM vector. To construct the pM-hSRC3-RID plasmid, the receptor-interacting domain of hSRC-3 containing three consensus LXXLL motifs (Ser-582 to Asp-782) (Arnold et al., 2004) was amplified from human liver QUICK-Clone cDNA (Clontech) and cloned into the pM vector. The primers used to construct the plasmids are shown in Table 3. The plasmids were sequenced (Nucleic Acid Protein Service Unit at the University of British Columbia, Vancouver, BC, Canada), and the identity of plasmids was confirmed by comparing their sequence with published sequence.

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HepG2 Cell Culture. HepG2 human hepatocellular carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as described previously (Lau et al., 2010).

Transient Transfection and Reporter Gene Assays. As reported previously, DEHP, which is a known activator of hCAR-SV23, is present in fetal bovine serum, but it is removable by charcoal treatment (DeKeyser et al., 2009). In the present study, all the assays were conducted using HepG2 cells cultured in 10% v/v charcoal-stripped, heat-inactivated fetal bovine serum. HepG2 cells were seeded onto 24-well microplates at a density of 100,000 cells/well and in a volume of 0.5 ml of culture medium. hCAR-WT-, hCAR-SV23-, hCAR-SV24-, and hCAR-SV25-dependent reporter gene assays were conducted as follows. At 5 h after plating, HepG2 cells were transfected for 24 h with 20 μl of a transfection master mix containing FuGENE 6 transfection reagent (either 3 μl/μg of DNA or 0.4 μl/well, as specified in the figure legend), serum-free Opti-MEM (20 μl/well), pCMV6-XL4-hRXRα (10 ng/well, unless specified otherwise in the figure legend), pGL4.74[*hRluc*/TK] internal control vector (5 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), and a hCAR expression plasmid (50 ng/well) or its respective empty vector (50 ng/well). The full-length hCAR expression plasmids were pCMV6-XL4-hCAR-WT, pCMV6-neo-hCAR-SV23, pCMV6-XL4-hCAR-SV24, and pCMV6-XL5-hCAR-SV25. In the hCAR-WT-LBD and hCAR-SV23-LBD assays, HepG2 cells were transfected with pM-hCAR-WT-LBD (Gln-105 to Ser-348; 40 ng/well), pM-hCAR-SV23-LBD (Gln-105 to Ser-352; 40 ng/well), or pM empty vector (40 ng/well) along with pCMV6-XL4-hRXRα (10 ng/well for hCAR-SV23-LBD assay and none for the hCAR-WT-LBD assay), pGL4.74[*hRluc*/TK] internal control plasmid (5 ng/well), and pFR-luc reporter plasmid (100

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ng/well), using FuGENE 6 transfection reagent (3 μl/μg of DNA) diluted in 20 μl of serum-free Opti-MEM.

Transfected HepG2 cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing *G. biloba* extract (50-800 μg/ml), ginkgolide A (8.8 μg/ml), ginkgolide B (2.4 μg/ml), ginkgolide C (11.2 μg/ml), ginkgolide J (4.8 μg/ml), bilobalide (22.4 μg/ml), sodium phenobarbital (1000 μM), CITCO (10 μM), DEHP (10 μM), TCPOBOP (0.25 μM), or vehicle, as detailed in each figure legend. The chemicals were dissolved in 100% DMSO and diluted with culture medium to give a final DMSO concentration of 0.1% v/v. *G. biloba* extract was suspended directly in culture medium containing 0.1% v/v DMSO. In the hCAR-WT-dependent reporter gene assay, androstanol (10 μM), which is a hCAR-WT inverse agonist (Moore et al., 2000), was added to each treatment group to reduce the constitutive activity (Burk et al., 2005). In the hCAR-WT-LBD assay, PK11195 (10 μM) was used as the inverse agonist (Li et al., 2008) because androstanol was found to be ineffective. At the end of the treatment period, transfected HepG2 cells were lysed and firefly luciferase and *R. reniformis* luciferase activities were determined using a Dual-Luciferase Reporter Assay System. Luminescence was measured using a GloMax 96 microplate luminometer (Promega Corporation). Luciferase activity was expressed as a ratio of firefly luciferase to *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector (pCMV6-XL4, pCMV6-neo, or pCMV6-XL5).

Mammalian Two-Hybrid Assay. A hCAR-dependent mammalian two-hybrid assay was performed as described in detail previously (Lau et al., 2011). At 5 h after plating, HepG2 cells were co-transfected with pVP16-hCAR-WT-LBD (40 ng/well), pVP16-hCAR-SV23-LBD (40 ng/well), or pVP16 empty vector (40 ng/well) along with a coactivator expression plasmid

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(10 ng/well), pCMV6-XL4-hRXRα (10 ng/well), pGL4.74[*hRluc*/TK] internal control plasmid (10 ng/well), and pFR-luc reporter plasmid (100 ng/well). The coactivator expression plasmids were pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID. At 24 h after transfection, cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing *G. biloba* extract, sodium phenobarbital, CITCO, DEHP, TCPOBOP, or the corresponding vehicle, as detailed in each figure legend. In the hCAR-WT-dependent mammalian two-hybrid assay, androstanol (10 μM; hCAR-WT inverse agonist) (Moore et al., 2000) was added to each treatment group to reduced the constitutive activity (Burk et al., 2005).

Statistical Analysis. Data were analyzed by two-way analysis of variance, and when significant differences were detected, the Student Newman-Keuls multiple comparison test was performed (SigmaPlot 11.0, Systat Software, Inc., San Jose, CA). The level of statistical significance was set *a priori* at $P < 0.05$.

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Results

***G. biloba* Extract Selectively Activates hCAR Isoforms: Comparison with Phenobarbital, DEHP, CITCO, and TCPOBOP.** A cell-based reporter gene assay (without co-transfecting a hRXRα expression plasmid) was conducted to evaluate the effect of *G. biloba* extract (800 μg/ml) on the activity of hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25. The extract increased hCAR-WT activity (Fig. 3A), but not that of hCAR-SV23 (Fig. 3B), hCAR-SV24 (Fig. 3C), or hCAR-SV25 (Fig. 3D), when compared with both the corresponding empty vector-transfected, *G. biloba* extract-treated group and the hCAR-WT-transfected, vehicle-treated control group. By comparison, phenobarbital (1000 μM) activated hCAR-WT (Fig. 3A), but not the other hCAR isoforms (Fig. 3B-3D). As expected, among the hCAR isoforms investigated, DEHP (10 μM) increased only hCAR-WT (Fig. 3A) and hCAR-SV23 (Fig. 3B), whereas CITCO (10 μM) elevated the activity of hCAR-WT (Fig. 3A), hCAR-SV23 (Fig. 3B), and hCAR-SV24 (Fig. 3C). TCPOBOP at a maximal mCAR-activating concentration (0.25 μM; Tzameli et al., 2000) did not influence the activity of any of these hCAR isoforms.

Another finding in the experiment shown in Fig. 3A-3D was that *G. biloba* extract, but not TCPOBOP, phenobarbital, DEHP, or CITCO, was capable of increasing luciferase activity in cells transfected with an empty vector (i.e. pCMV6-XL4, pCMV6-neo, or pCMV6-XL5). Therefore, to further explore the basis of this effect, an experiment was performed with various combinations of plasmids. As shown in Fig. 4, the reporter construct (pGL3-basic-CYP2B6-PBREM/XREM-luc) was responsible for the increase in luciferase activity in cells transfected with an empty vector and treated with *G. biloba* extract (800 μg/ml). Therefore, the design of all subsequent experiments included additional control groups in which the cells were transfected with the corresponding empty vector.

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Co-Transfection of a Retinoid X Receptor-α (RXRα) Expression Plasmid Leads to Activation of hCAR-SV23 but not hCAR-SV24 or hCAR-SV25 by *G. biloba* Extract. The lack of an effect by *G. biloba* extract on the hCAR splice variants SV23 (Fig. 3B), SV24 (Fig. 3C), and SV25 (Fig. 3D) may reflect a need for exogenous supplementation of HepG2 cells with RXRα. Therefore, a concentration-response experiment was conducted in which the cells were co-transfected with a human RXRα (hRXRα) expression plasmid (pCMV6-XL4-hRXRα; 1-50 ng/well). Co-transfection of pCMV6-XL4-hRXRα led to activation of hCAR-SV23 (Fig. 5C), but not hCAR-SV24 (Fig. 5E) or hCAR-SV25 (Fig. 5G), and it further elevated the extent of hCAR-WT activation by *G. biloba* extract (800 µg/ml; Fig. 5A). By comparison, co-transfection of a hRXRα expression plasmid resulted in enhanced activation of hCAR-SV23 (Fig. 5D) by DEHP (10 µM) and CITCO (10 µM) and of hCAR-SV24 by CITCO (10 µM) and DEHP (10 µM) (Fig. 5F). In contrast, it did not have any effect in hCAR-SV23-transfected cells treated with TCPOBOP (0.25 µM) or phenobarbital (1000 µM) (Fig. 5D), or in hCAR-SV24-transfected cells treated with TCPOBOP or phenobarbital at the above concentrations (Fig. 5F). Similarly, exogenous hRXRα supplementation did not influence hCAR-WT (Fig. 5B) or hCAR-SV25 (Fig. 5H) activity in cells treated with each of these individual chemicals, as analyzed by two-way ANOVA. Given that hRXRα supplementation was necessary in hCAR-WT and hCAR-SV23 activation by *G. biloba* extract, subsequent reporter gene assays were conducted in cells co-transfected with the hRXRα expression plasmid (10 ng/well).

Multiple Lots of *G. biloba* Extract Activate hCAR-WT and hCAR-SV23, But Not hCAR-SV24 or hCAR-SV25. We also compared the effect of five individual lots of *G. biloba* extract on hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 activities. As shown in Fig. 6A and 6B, each of the lots increased hCAR-WT and hCAR-SV23 activities to a comparable

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extent. In contrast, none of the lots affected hCAR-SV24 (Fig. 6C) or hCAR-SV25 (Fig. 6D) activity. Based on the similarity of results among multiple lots of extract, subsequent experiments were conducted using Lot A. Given that the extract had no effect on hCAR-SV24 or hCAR-SV25 activity, the remaining experiments focused only on hCAR-WT and hCAR-SV23.

Concentration-Response Relationship in hCAR-WT and hCAR-SV23 Activation by *G. biloba* Extract. A detailed concentration-response experiment was conducted to compare the effect of *G. biloba* extract (50-800 µg/ml) on hCAR-WT and hCAR-SV23 activities. Concentrations greater than 800 µg/ml of extract were not investigated because of solubility problems. Our previous data indicated that *G. biloba* extract at concentrations up to 800 µg/ml did not increase the release of lactate dehydrogenase (a marker of cytotoxicity) in HepG2 cells (Lau et al., 2010). As shown in Fig. 7A, the extract at concentrations of 50, 100, and 200 µg/ml had no effect on hCAR-WT activity, whereas at 400, 600, and 800 µg/ml, it increased hCAR-WT activity. By comparison, the extract at concentrations of 50, 100, 200, and 400 µg/ml had no effect on hCAR-SV23 activity, whereas at 600 and 800 µg/ml, it increased hCAR-SV23 activity (Fig. 7B). Overall, hCAR-WT was activated to a greater extent than hCAR-SV23 by *G. biloba* extract.

Ginkgolide A, Ginkgolide B, Ginkgolide C, Ginkgolide J, and Bilobalide Do Not Contribute to the Activation of hCAR-WT or hCAR-SV23 by *G. biloba* Extract. To investigate whether any of the five individual terpene trilactones contributes to the activation of hCAR-WT and hCAR-SV23 by the extract, a reporter gene assay was conducted on transfected HepG2 cells treated with each of the chemicals at a level equivalent to those present in a hCAR-WT- or hCAR-SV23-activating concentration of the extract. Based on the concentration-

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response curves, an 800 μg/ml concentration of the extract was chosen because it was a concentration that activated both hCAR-WT (Fig. 7A) and hCAR-SV23 (Fig. 7B). Our results indicated that at a level present in an 800 μg/ml concentration of the extract, ginkgolide A (8.8 μg/ml), ginkgolide B (2.4 μg/ml), ginkgolide C (11.2 μg/ml), ginkgolide J (4.8 μg/ml), and bilobalide (22.4 μg/ml) did not activate hCAR-WT (Fig. 7C) or hCAR-SV23 (Fig. 7D). The terpene trilactones are present together in the *G. biloba* extract (Table 2). Therefore, to investigate the possibility of pharmacological synergism, we determined the effect of a combination of the five individual terpene trilactones at the above concentrations. However, it also had no effect, whereas in the same experiment, *G. biloba* extract (800 μg/ml) did activate hCAR-WT (Fig. 7C) and hCAR-SV23 (Fig. 7D).

***G. biloba* Extract Does Not Transactivate the Ligand-Binding Domains of hCAR-WT or hCAR-SV23.** To determine whether *G. biloba* extract transactivates the ligand-binding domains of hCAR-WT and hCAR-SV23, a reporter gene assay was conducted on HepG2 cells transfected with pM-hCAR-WT-LBD (Gln-105 to Ser-348) or pM-hCAR-SV23-LBD (Gln-105 to Ser-352). Whereas CITCO (10 μM) transactivated hCAR-WT-LBD (Fig. 8A) and DEHP (10 μM) transactivated hCAR-SV23-LBD (Fig. 8B), *G. biloba* extract (800 μg/ml), phenobarbital (1000 μM), and TCPOBOP (0.25 μM) had no effect (Fig. 8A and 8B). Control analysis indicated that none of the treatment groups affected the activity in cells transfected with the pM empty vector (Fig. 8A and 8B).

***G. biloba* Extract Does Not Promote Recruitment of Coactivators to hCAR-WT or hCAR-SV23.** A mammalian two-hybrid assay was performed to determine whether *G. biloba* extract is capable of recruiting coactivators (hSRC-1, hSRC-2, and hSRC-3) to the ligand-binding domains of hCAR-WT and hCAR-SV23. In HepG2 cells co-transfected with pVP16-

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hCAR-WT-LBD and pM-hSRC1-RID (Fig. 9A), pM-hSRC2-RID (Fig. 9B), or pM-hSRC1-RID (Fig. 9C), *G. biloba* extract (800 µg/ml) did not affect the luciferase activity, whereas an increase was obtained with CITCO (10 µM; a positive control). In contrast to DEHP (10 µM; a positive control), a lack of effect was obtained with the extract in cells co-transfected with pVP16-hCAR-SV23-LBD and pM-hSRC1-RID (Fig. 9D), pM-hSRC2-RID (Fig. 9E), or pM-hSRC1-RID (Fig. 9F). Phenobarbital (1000 µM) and TCPOBOP (0.25 µM) also had no effect on hSRC-1, hSRC-2, or hSRC-3 recruitment to hCAR-WT (Fig. 9A-9C) or hCAR-SV23 (Fig. 9D-9F). Control analysis indicated a lack of an effect of the extract and chemicals in cells co-transfected with pVP16 (empty vector) and pM-hSRC1-RID, pM-hSRC2-RID, or pM-hSRC3-RID (Fig. 9A-9F).

Discussion

The 4-amino acid (SPTV) insertion in hCAR-SV23 has been postulated to extend the loop between helices 6 and 7, modify the properties of the ligand-binding pocket, and alter the ligand-binding specificity (Auerbach et al., 2003; DeKeyser et al., 2011). A novel finding in the present study is that among the hCAR splice variants investigated, hCAR-SV23 was the only isoform activated by *G. biloba* extract, and this was verified with multiple lots of the extract. To date, *G. biloba* extract is one of the few activators of hCAR-SV23. The others are CITCO (DeKeyser et al., 2009 and present study) and some of the phthalates, such as DEHP (DeKeyser et al., 2009 and present study) and di-isononyl phthalate (DeKeyser et al., 2011). As shown for the first time in the present study, phenobarbital and TCPOBOP at concentrations known to activate hCAR-WT (Fig. 3A) and mCAR (Tzameli et al., 2000), respectively, were not capable of activating hCAR-SV23, as evaluated in our cell-based reporter gene assays. In contrast to DEHP, *G. biloba* extract did not transactivate the ligand-binding domain of hCAR-SV23 or promote recruitment of coactivators (hSRC-1, hSRC-2, and hSRC-3), indicating that the extract did not act as an agonist of this hCAR isoform. These results provide the first demonstration of an indirect mechanism of ligand-mediated activation of hCAR-SV23.

Our result indicating activation of hCAR-WT by *G. biloba* extract is in agreement with the only previous finding in which a 2-fold increase was obtained with an 100 μg/ml concentration of an extract (Li et al., 2009). However, based on two-way ANOVA of our dose-response data, statistically significant activation of hCAR-WT was not obtained until the extract concentrations were 400, 600, and 800 μg/ml. In contrast to CITCO but the same as phenobarbital, *G. biloba* extract activates hCAR-WT by an indirect mechanism rather than by receptor agonism. This conclusion is based on the ability of CITCO and inability of

phenobarbital and the extract to transactivate the ligand-binding domain of hCAR-WT and promote the recruitment of coactivators (hSRC-1, hSRC-2, and hSRC-3). Overall, it appears that *G. biloba* extract is a phenobarbital-type rather than a CITCO-type of hCAR-WT activator. The mechanism of CAR activation by phenobarbital is still not well-understood. Based on the cumulative experimental evidence obtained for this drug, a key step is facilitating the translocation of the cytoplasmic receptor to the nucleus (Timsit and Negishi, 2007), and this has been shown to be associated with dephosphorylation of an amino acid residue in CAR (threonine 38 in hCAR and threonine 48 in mCAR) (Mutoh et al., 2009).

The *G. biloba* extract used in our study contained known quantities of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide. As assessed by reporter gene assays, these five individual terpene trilactones, when test individually or in combination, were shown not to be responsible for the activation of hCAR-WT or hCAR-SV23 by the extract. In a previous study, the same experimental approach led to the identification of ginkgolide A as a partial contributor to human pregnane X receptor agonism by *G. biloba* extract (Lau et al., 2010). Currently, it is not known whether other chemical constituents in the extract contribute to the hCAR-WT and hCAR-SV23 activation. Flavonol glycosides, such as those of quercetin, kaempferol, and isorhamnetin, are another class of chemicals present in *G. biloba* extract (van Beek and Montoro, 2009). However, it remains to be investigated whether they contribute to the hCAR-WT and hCAR-SV23-activating effects of the extract, but it has been shown that quercetin aglycone (10 μM) does not activate hCAR-WT (Yao et al., 2010).

The 5-amino acid (APYLT) insertion in hCAR-SV24 has been hypothesized to expand the loop between helices 8 and 9, cause steric hindrance during heterodimerization of hCAR-SV24 and hRXRα, and render little or no influence on the structure of the ligand-binding domain

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(Auerbach et al., 2003). In the present study, *G. biloba* extract did not activate hCAR-SV24 even in cells supplemented with exogenous hRXRα. However, in a previous study, the extract was shown to minimally (< 2-fold) activate hCAR-SV24 in a cell-based reporter gene assay in which the empty vector-transfected, *G. biloba* extract-treated group was not included (Li et al., 2009). A possible reason for the apparent discrepancy may be related to our finding that *G. biloba* extract was capable of increasing the background luciferase activity in empty vector-transfected cells. In our study performed in cultured HepG2 cells, the increase could be attributed to the interaction between *G. biloba* extract and an endogenous receptor(s) that is functionally compatible with our reporter plasmid (pGL3-basic-CYP2B6-PBREM/XREM-luc). However, the identity of that endogenous receptor is not pregnane X receptor even though a small amount of it may be present in HepG2 cells. This proposal is based on our data showing that ginkgolide A and phenobarbital, which are agonists of human pregnane X receptor (Moore et al., 2000; Lau et al., 2010), did not increase the reporter activity in cells transfected with the empty vector (i.e. in the absence of a receptor expression plasmid; Fig. 3A-3D). Overall, our results highlight the importance of conducting reporter gene assays with all the appropriate control groups.

hCAR-WT and hCAR-SV24 have common ligands, including CITCO (Maglich et al., 2003; Faucette et al., 2007), artemisinin (Burk et al., 2005; Faucette et al., 2007), and phenytoin (Wang et al., 2004; Faucette et al., 2007). However, *G. biloba* extract (present study), DEHP (DeKeyser et al., 2011 and present study), and resveratrol (Dring et al., 2010) activate hCAR-WT, but not hCAR-SV24. Conversely, other chemicals, such as 7-(acetoxy)-6-(*p*-methoxyphenyl)pyrrolo-[2,1-d][1,5]benzothiazepine (also referred to as NF49), nonylbenzene, doxylamine, and pheniramine, activate hCAR-SV24, but not hCAR-WT (Dring et al., 2010;

Anderson et al., 2011). Collectively, these results indicate that hCAR-WT and hCAR-SV24 have overlapping but distinct sets of ligands, suggesting that the use of hCAR-SV24 in cell-based reporter gene assays as a screening tool (Faucette et al., 2007) may lead to false-positive and false-negative identification of hCAR-WT ligands.

hCAR-SV25 contains both the 4-amino acid (SPTV) and 5-amino acid (APYLT) insertions (Auerbach et al., 2003). Previously, only two chemicals were investigated for their potential interaction with hCAR-SV25, but both clotrimazole (Auerbach et al., 2003) and CITCO (Jinno et al., 2004) were shown not to activate hCAR-SV25. Our data confirmed the lack of effect of CITCO and showed for the first time that *G. biloba* extract, DEHP, phenobarbital, and TCPOBOP are not capable of activating hCAR-SV25 even in the presence of exogenous hRXRα. Overall, the experimental data indicate that the insertion of both the SPTV and APYLT amino acids leads to a loss of ligand recognition by hCAR-SV25, in agreement with predictions based on computer modeling studies (Savkur et al., 2003; Auerbach et al., 2003).

Heterodimerization of RXRα with CAR is a key step in the mammalian CAR activation pathway (Timsit and Negishi, 2007). A conclusion from the current study on the functionality of hCAR-SV23, hCAR-SV24, and hCAR-WT is that the necessity of exogenous RXRα supplementation in HepG2 cells depends on the specific ligand and hCAR isoform under investigation. This conclusion is based on the following patterns obtained in our experiments: 1) exogenous RXRα must be added in order to elicit receptor activation (e.g. *G. biloba* extract/hCAR-SV23 and DEHP/hCAR-SV24); 2) exogenous RXRα is not obligatory, but the additional input further enhances the activity (e.g CITCO/hCAR-SV23, DEHP/hCAR-SV23, CITCO/hCAR-SV24, and *G. biloba* extract/hCAR-WT); and 3) exogenous RXRα is not required, and supplementation does not further enhance the activity (e.g. phenobarbital/hCAR-WT,

DEHP/hCAR-WT, and CITCO/hCAR-WT). Therefore, prior to a study, preliminary experiments should be conducted to assess whether it is necessary to co-transfect a hRXRα expression plasmid in the cell type of interest when investigating ligand-activation of hCAR isoforms in reporter gene assays.

In conclusion, *G. biloba* extract interacts with hCAR in an isoform-selective manner. It activated hCAR-WT and hCAR-SV23, but not hCAR-SV24 or hCAR-SV25. These effects of the extract were not due to ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, or bilobalide. In contrast to CITCO and DEHP (DeKeyser et al., 2009), *G. biloba* extract did not activate hCAR-WT or hCAR-SV23 by receptor agonism, but by an indirect mechanism that did not involve binding to the ligand-binding domain of the receptor or stimulating the recruitment of coactivators. The demonstration of hCAR activation by *G. biloba* extract may provide a mechanistic insight for the neuroprotective effect of this herbal medicine against β-amyloid toxicity, which has been reported in various experimental models (Luo et al., 2002). As shown in *in vitro* and *ex vivo* experiments, chemical activation of CAR up-regulates P-glycoprotein expression in rodent brain capillaries (Wang et al., 2010), and P-glycoprotein is known to reduce the accumulation of brain β-amyloid (Hartz et al., 2010), which is believed to play a role in the etiology of Alzheimer's disease (Zlokovic, 2005). Finally, our data along with those reported recently (Dring et al., 2010; Anderson et al., 2011) support the conclusion that hCAR-SV23, hCAR-SV24, and hCAR-WT have overlapping but distinct sets of ligands.

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Authorship Contributions

Participated in research design: Lau and Chang.

Conducted experiments: Lau and Yang

Contributed new reagents or analytic tools: N/A

Performed data analysis: Lau.

Wrote or contributed to the writing of the manuscript: Lau and Chang.

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Footnotes

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Figure Legends

Fig. 1. Schematic representation of hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25. Each numbered box represents an exon (top panel). The shaded boxes represent the insertion of 12 nucleotides between exon 6 and exon 7 or the insertion of 15 nucleotides between exon 7 and exon 8 (Auerbach et al., 2003). The number of base pairs (bp) is according to the August 14, 2011 updated information in the National Center for Biotechnology Information (NCBI) database. The bottom panel shows the amino acid (aa) sequences of hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 (NCBI database). The 4-amino acid (SPTV) and 5-amino acid (APYLT) insertions are indicated in bold.

Fig. 2. Chemical structures of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide (van Beek and Montoro, 2009).

Fig. 3. Comparative effect of *G. biloba* extract, TCPOBOP, phenobarbital, DEHP, and CITCO on hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 activities. Cultured HepG2 cells were transfected with pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), and a hCAR expression plasmid (50 ng/well) or its corresponding empty vector control plasmid (50 ng/well). Transfected cells were treated with vehicle, *G. biloba* extract (800 μg/ml; Lot A), TCPOBOP (0.25 μM), sodium phenobarbital (1000 μM), DEHP (10 μM), or CITCO (10 μM). In the hCAR-WT-dependent reporter gene assay, androstanol (10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three to five independent

experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector *and* the vehicle-treated control group transfected with the same hCAR expression plasmid ($P < 0.05$). Androstanol reduced hCAR-WT activity in the vehicle-treated control group by 65 ± 1%.

Fig. 4. Effect of various plasmid combinations on luciferase activity in cultured HepG2 cells treated with *G. biloba* extract. Cells were transfected with pGL4.74[*hRluc*/TK] (5 ng/well), pCMV6-XL4 (empty vector; 50 ng/well), pCMV6-XL4-hCAR-SV24 (50 ng/well), pGL3-basic (50 ng/well), and/or pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), using FuGene 6 (0.4 µl/well). Transfected cells were treated with vehicle or *G. biloba* extract (800 µg/ml; Lot A). Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding vehicle-treated control group *and* the *G. biloba* extract-treated group transfected only with pGL4.74[*hRluc*/TK] ($P < 0.05$).

Fig. 5. Effect of hRXRα on hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 activities in cultured HepG2 cells treated with *G. biloba* extract, TCPOBOP, phenobarbital, DEHP, or CITCO. (A, C, E, G) Cells were transfected with a hCAR expression plasmid (50 ng/well) or its corresponding empty vector control plasmid (50 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), and a varying amount (0, 1, 5, 10, 25, or 50 ng/well) of pCMV6-XL4-hRXRα or pCMV6-XL4 (empty vector). Transfected cells were treated with vehicle or *G. biloba* extract (800 µg/ml; Lot A). (B, D, F, H) Cells were transfected

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with a hCAR expression plasmid (50 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), and with or without pCMV6-XL4-hRXRα (10 ng/well). Transfected cells were treated with vehicle, TCPOBOP (0.25 μM), sodium phenobarbital (1000 μM), DEHP (10 μM), or CITCO (10 μM). In the hCAR-WT-dependent reporter gene assay, androstanol (10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three or four independent experiments performed in triplicate. *, significantly different from the corresponding group transfected with the empty vector *and* the control group (0 ng pCMV6-XL4-hRXRα) transfected with the same hCAR expression plasmid ($P < 0.05$). **, significantly different from the same chemical treatment group not transfected with hRXRα *and* the vehicle-treated control group transfected with hRXRα ($P < 0.05$). Androstanol reduced hCAR-WT activity in the vehicle-treated control group by 56 ± 6% (in cells without pCMV6-XL4-hRXRα) and 43 ± 3% (in cells transfected with 10 ng pCMV6-XL4-hRXRα).

Fig. 6. Effect of multiple lots of *G. biloba* extract on hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 activities. Cultured HepG2 cells were transfected with a hCAR expression plasmid (50 ng/well) or its corresponding empty vector control plasmid (50 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), and pCMV6-XL4-hRXRα (10 ng/well). Transfected cells were treated with vehicle or one of the five lots of *G. biloba* extract (800 μg/ml). In the hCAR-WT-dependent reporter gene assay, androstanol (10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*.

Data are expressed as mean ± S.E.M. for three or four independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector *and* the vehicle-treated control group transfected with the same hCAR expression plasmid ($P < 0.05$). Androstanol reduced hCAR-WT activity in the vehicle-treated control group by 44 ± 3%.

Fig. 7. Concentration-response relationship in hCAR-WT and hCAR-SV23 activation by *G. biloba* extract and the role of terpene trilactones. Cultured HepG2 cells were transfected with a hCAR expression plasmid (50 ng/well) or its corresponding empty vector control plasmid (50 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc (50 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), and pCMV6-XL4-hRXRα (10 ng/well). (A, B) Transfected cells were treated for 24 h with vehicle or *G. biloba* extract (50, 100, 200, 400, 600, or 800 μg/ml; Lot A). (C, D) Transfected cells were treated for 24 h with vehicle, ginkgolide A (GA; 8.8 μg/ml), ginkgolide B (GB; 2.4 μg/ml), ginkgolide C (GC; 11.2 μg/ml), ginkgolide J (GJ; 4.8 μg/ml), bilobalide (BB; 22.4 μg/ml), a combination of these five terpene trilactones (GA + GB + GC + GJ + BB) at the above concentrations, or *G. biloba* extract (800 μg/ml; Lot A). In the hCAR-WT-dependent reporter gene assay, androstanol (10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three or four independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector *and* the vehicle-treated control group transfected with the same hCAR expression plasmid ($P < 0.05$). Androstanol reduced hCAR-WT activity in the vehicle-treated control group by 44 ± 6%.

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Fig. 8. Effect of *G. biloba* extract on the transactivation of the ligand-binding domains of hCAR-WT and hCAR-SV23. (A) Cultured HepG2 cells were transfected with pGL4.74[*hRluc*/TK] (5 ng/well), pFR-luc (100 ng/well), and pM-hCAR-WT-LBD (Gln-105 to Ser-348) (40 ng/well) or pM empty vector (40 ng/well). (B) Cultured HepG2 cells were transfected with pCMV6-XL4-hRXRα (10 ng/well), pGL4.74[*hRluc*/TK] (5 ng/well), pFR-luc (100 ng/well), and pM-hCAR-SV23-LBD (Gln-105 to Ser-352) (40 ng/well) or pM empty vector (40 ng/well). Transfected cells were treated with vehicle, *G. biloba* extract (800 μg/ml; Lot A), TCPOBOP (0.25 μM), sodium phenobarbital (1000 μM), DEHP (10 μM), or CITCO (10 μM). In the hCAR-WT-LBD assay, PK11195 (10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three to six independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector *and* the vehicle-treated control group transfected with the same hCAR expression plasmid ($P < 0.05$). PK11195 reduced hCAR-WT-LBD activity in the vehicle-treated control group by 56 ± 6%.

Fig. 9. Effect of *G. biloba* extract on the recruitment of coactivators to hCAR-WT and hCAR-SV23. Cultured HepG2 cells were transfected with pCMV6-XL4-hRXRα (10 ng/well), pGL4.74[*hRluc*/TK] (10 ng/well), pFR-luc (100 ng/well), a coactivator expression plasmid (10 ng/well), and a hCAR expression plasmid (40 ng/well) or pVP16 empty vector (40 ng/well). Transfected cells were treated with vehicle, *G. biloba* extract (800 μg/ml; Lot A), TCPOBOP (0.25 μM), sodium phenobarbital (1000 μM), DEHP (10 μM), or CITCO (10 μM). Androstanol

(10 μM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly and *R. reniformis* luciferase activities were measured and normalized as described under *Materials and Methods*. Data are expressed as mean ± S.E.M. for three to five independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector *and* the vehicle-treated control group transfected with the same hCAR expression plasmid ($P < 0.05$). Androstanol reduced hCAR-WT activity in the vehicle-treated control group by 54 ± 4%, 51 ± 4%, and 77 ± 2% in cells co-transfected with pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID, respectively.

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TABLE 1

Nomenclature of hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25

GenBank Accession Number[a]	Nomenclature		Reference
	Present Study	Other Studies	
NM_005122	hCAR-WT[b]	Transcript variant 3	NCBI Database[a]
		MB67	(Baes et al., 1994)
		hCAR-REF	(Auerbach et al., 2003)
		hCAR1	(Savkur et al., 2003)
			(Faucette et al., 2007)
		SV0	(Jinno et al., 2004)
		SV1	(Arnold et al., 2004)
		CAR1	(Auerbach et al., 2007)
NM_001077480	hCAR-SV23[b]	Transcript variant 2	NCBI Database[a]
		hCAR-4aaINS	(Auerbach et al., 2003)
		SV1	(Jinno et al., 2004)
		SV3	(Arnold et al., 2004)
		CAR2	(Auerbach et al., 2007)
N.A.	hCAR-SV24[b]	hCAR-5aaINS	(Auerbach et al., 2003)
		SV2	(Arnold et al., 2004)
			(Jinno et al., 2004)
		CAR3	(Auerbach et al., 2005)
			(Faucette et al., 2007)
NM_001077482	hCAR-SV25[b]	Transcript variant 1	NCBI Database[a]

hCAR-dblINS	(Auerbach et al., 2003)
hCAR2	(Savkur et al., 2003)
SV3	(Jinno et al., 2004)
SV6	(Arnold et al., 2004)

[a]National Center for Biotechnology Information database; [b](Lamba et al., 2005); N.A., not available.

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TABLE 2

Quantity of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, and flavonol glycosides in five individual lots of *G. biloba* extract

The terpene trilactones were determined by gas chromatography (Indena S.A., Milan, Italy) and the flavonol glycosides were analyzed by liquid chromatography-mass spectrometry (ChromaDex, Inc., Santa Ana, CA).

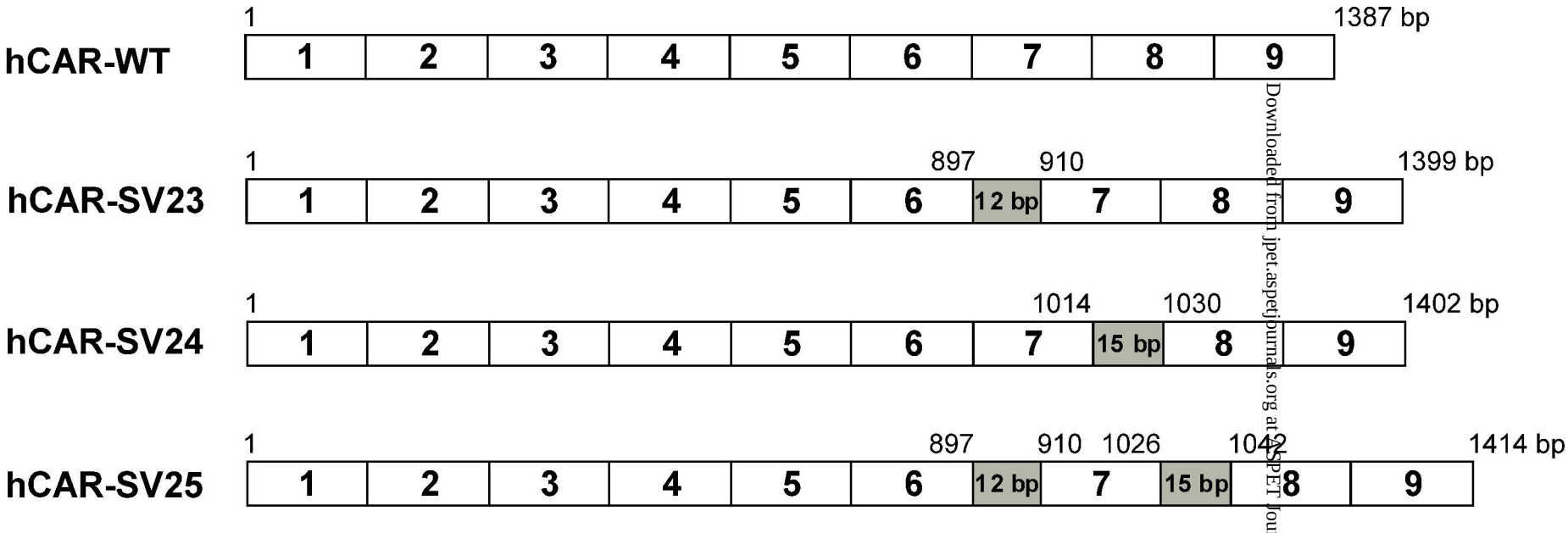
	Quantity in *G. biloba* Extract (% w/w)				
	Lot A	Lot B	Lot C	Lot D	Lot E
Terpene trilactone					
Ginkgolide A	1.1	0.9	1.3	1.5	1.3
Ginkgolide B	0.3	0.3	0.6	0.6	0.6
Ginkgolide C	1.4	1.5	1.4	1.4	1.4
Ginkgolide J	0.6	0.6	0.5	0.6	0.5
Bilobalide	2.8	2.9	3.0	3.0	3.0
Total terpene trilactones	6.2	6.2	6.8	7.1	6.8
Total flavonol glycosides	21.0	24.4	24.4	24.3	24.4

TABLE 3

Sequences of primers used for the construction of plasmids

	Primer Sequence (5′ to 3′)
hCAR-WT-LBD (Gln-105 to Ser-348)	5′-GGA-GGA-ATT-CCA-ACT-GAG-TAA-GGA-GCA-AGA-A-3′ (forward)
	5′-GGG-AGG-ATC-CTC-AGC-TGC-AGA-TCT-CCT-GG-3′ (reverse)
hCAR-SV23-LBD (Gln-105 to Ser-352)	5′-GGA-GGA-ATT-CCA-ACT-GAG-TAA-GGA-GCA-AGA-A-3′ (forward)
	5′-GGG-AGG-ATC-CTC-AGC-TGC-AGA-TCT-CCT-GG-3′ (reverse)
hSRC1-RID (Asp-621 to Asn-765)	5′-GGA-GGA-ATT-CGA-TGG-AGA-CAG-TAA-ATA-CTC-TC-3′ (forward)
	5′-GGG-AGG-ATC-CTC-AGT-TTG-GAG-TTG-ATC-TTA-AAT-3′ (reverse)
hSRC2-RID (Lys-583 to Thr-779)	5′-GGA-GGA-ATT-CAA-AGA-CTG-TTT-TGG-ACT-ATA-TG-3′ (forward)
	5′-GGG-AGG-ATC-CTC-ATG-TGT-TAC-TGG-CAG-GAT-CTG-3′ (reverse)
hSRC3-RID (Ser-582 to Asp-782)	5′-GGA-GGG-ATC-CGT-AGT-TCA-ATG-TGT-CAG-TCA-AAT-AG-3′ (forward)
	5′-GGG-AAA-GCT-TTC-AGT-CTT-TCT-CTT-GAC-TTG-AGC-3′ (reverse)

Figure 1



12 bp insert = TAT-CTC-CCA-CAG; 15 bp insert = CTC-CCT-ATC-TTA-CAG

	218	275
hCAR-WT (348 aa)	LCGPLRYTIEDGARV - - - - GFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSP - - - - - DRPGV	
	218 232 237	279
hCAR-SV23 (352 aa)	LCGPLRYTIEDGARV **SPTV** GFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSP - - - - - DRPGV	
	218 270 276	280
hCAR-SV24 (353 aa)	LCGPLRYTIEDGARV - - - - GFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSP **APYLT** DRPGV	
	218 232 237 274 280	284
hCAR-SV25 (357 aa)	LCGPLRYTIEDGARV **SPTV** GFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSP **APYLT** DRPGV	

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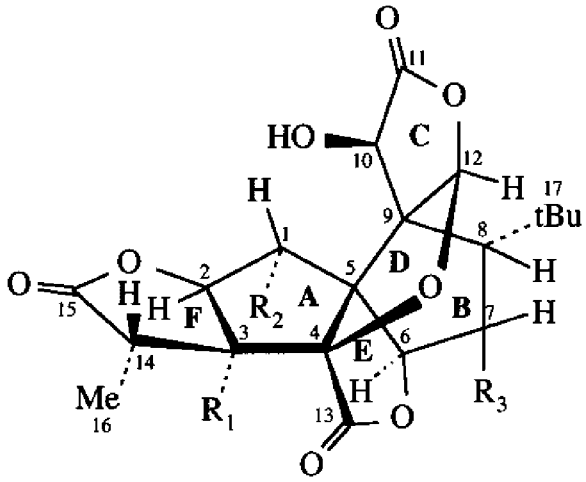
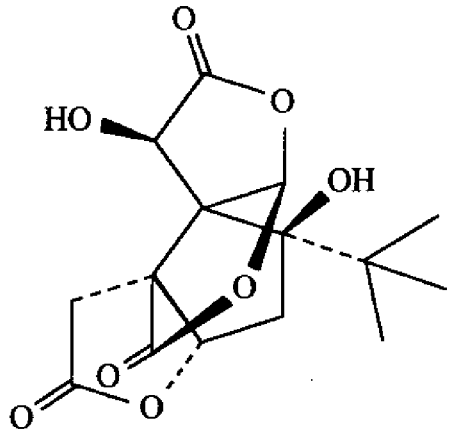


Ginkgolides



Bilobalide

	R1	R2	R3
Ginkgolide A	**OH**	**H**	**H**
Ginkgolide B	**OH**	**OH**	**H**
Ginkgolide C	**OH**	**OH**	**OH**
Ginkgolide J	**OH**	**H**	**OH**

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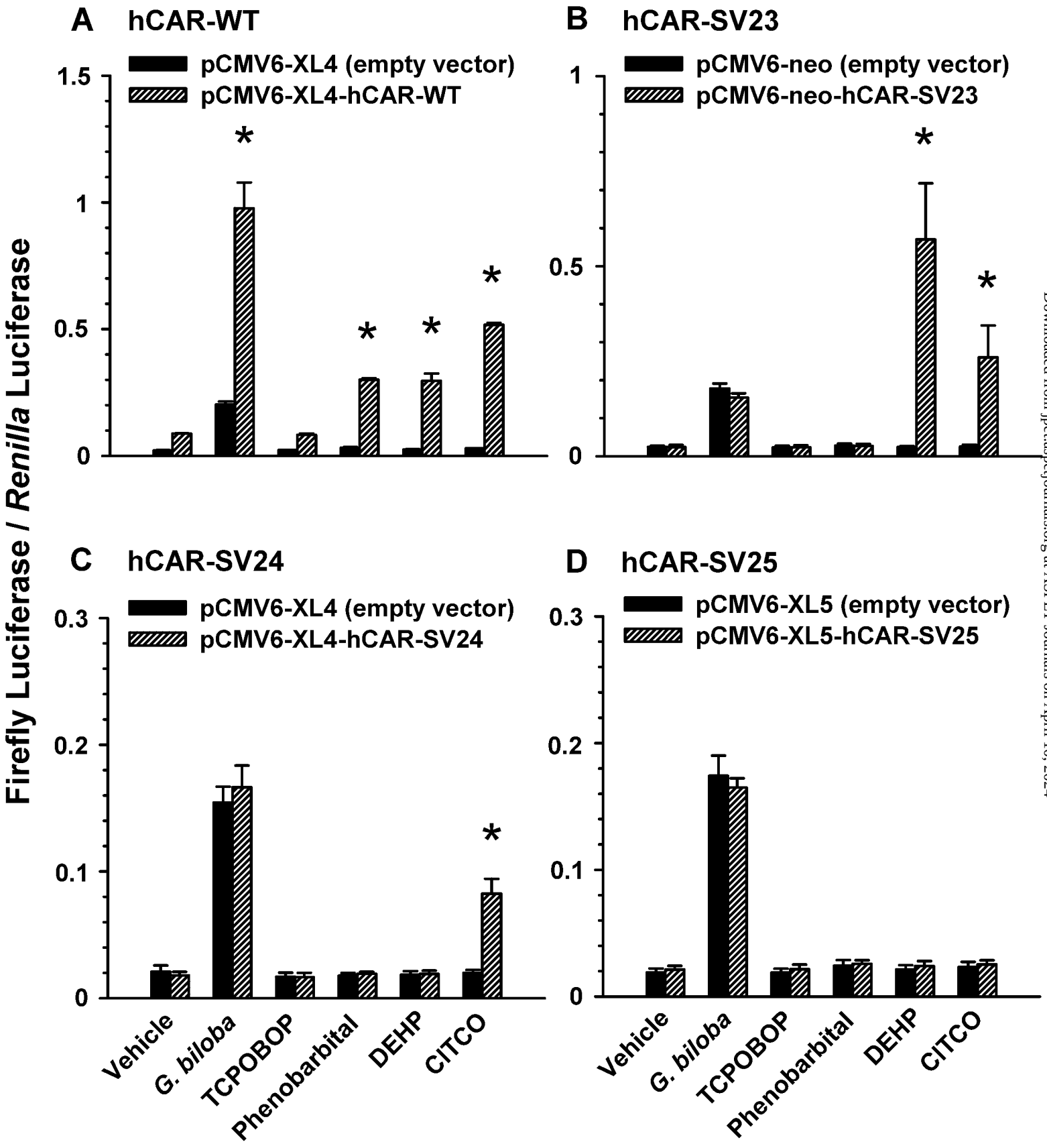
Figure 3



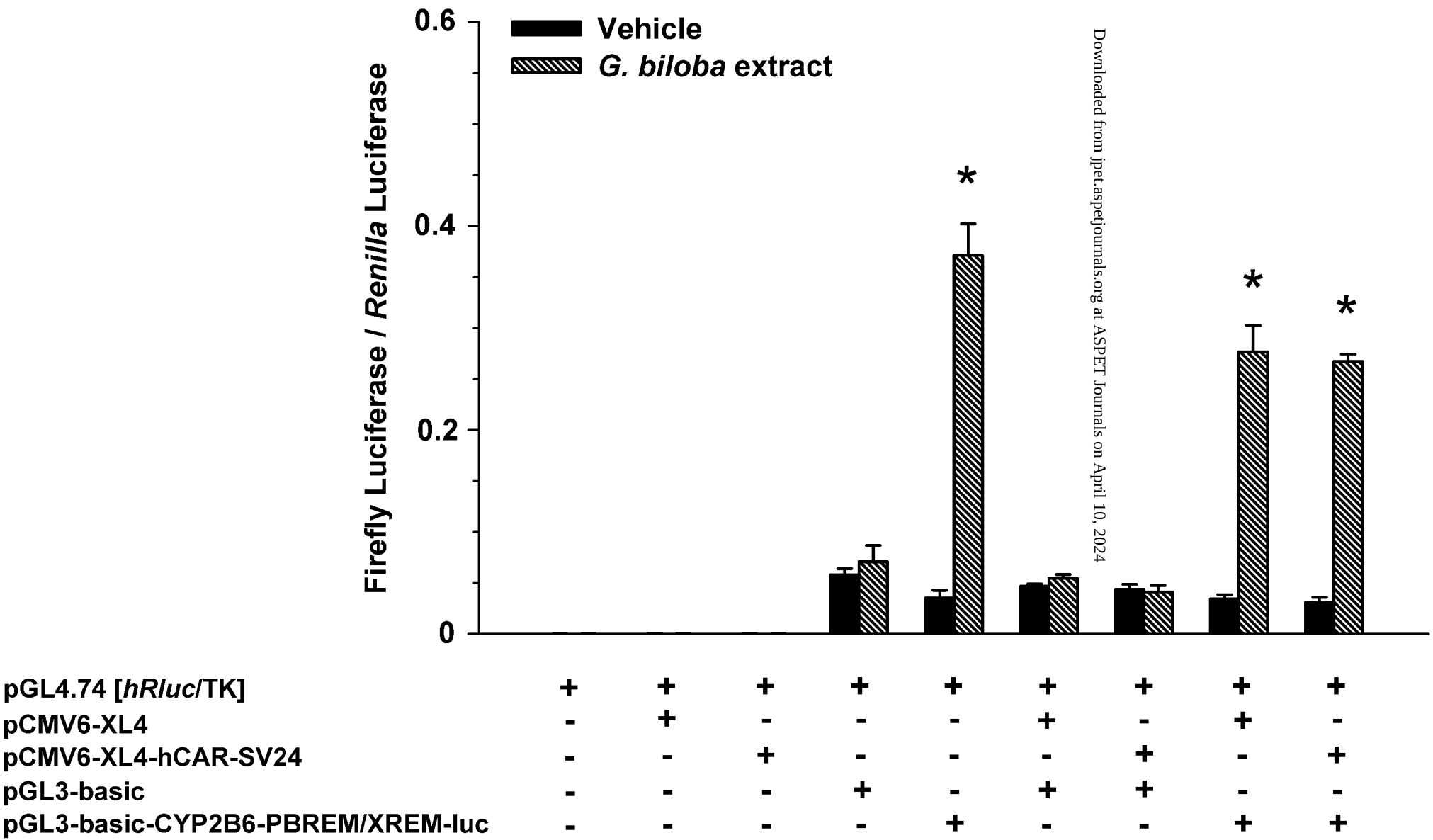


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Figure 5

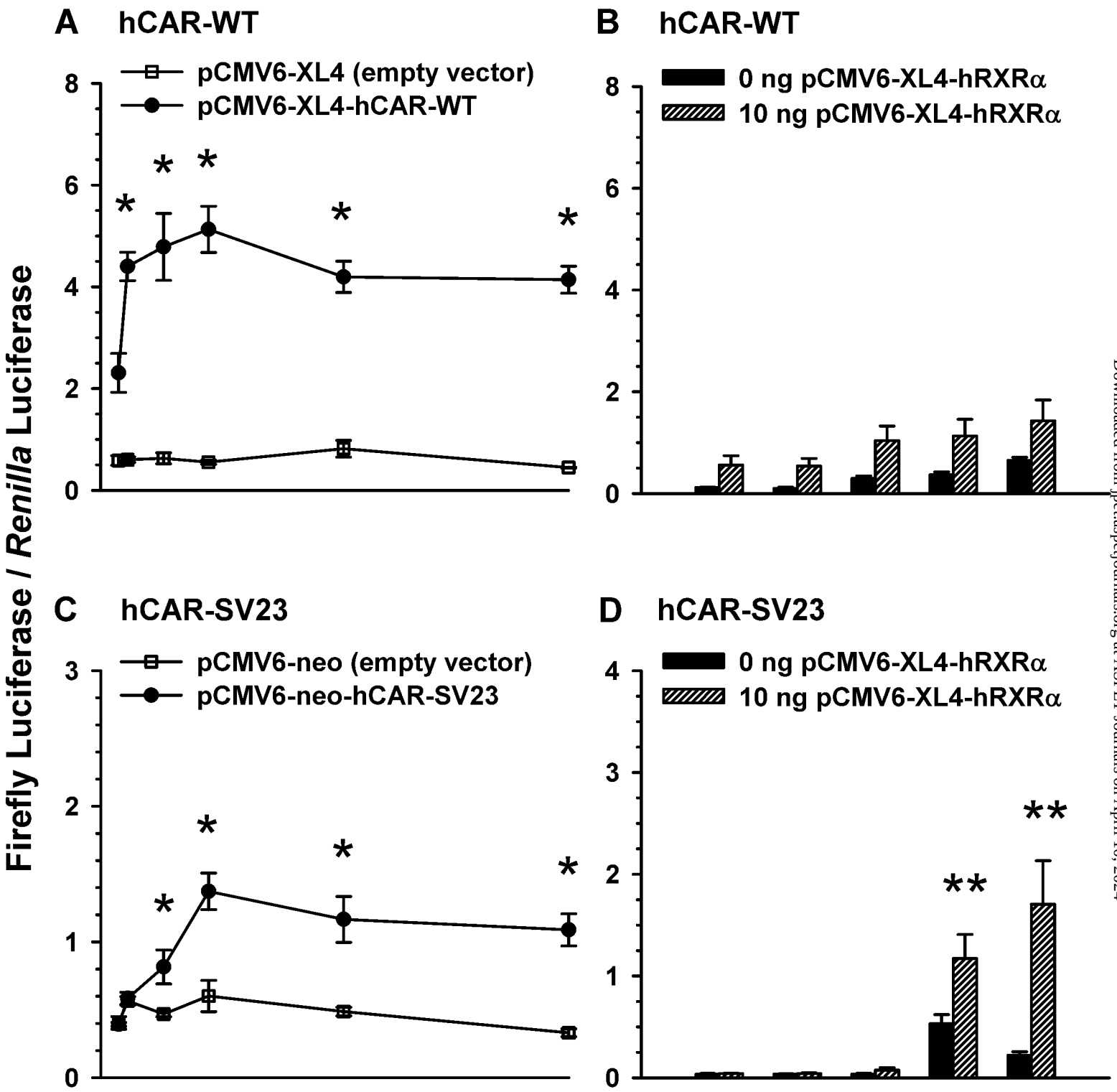

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Figure 5

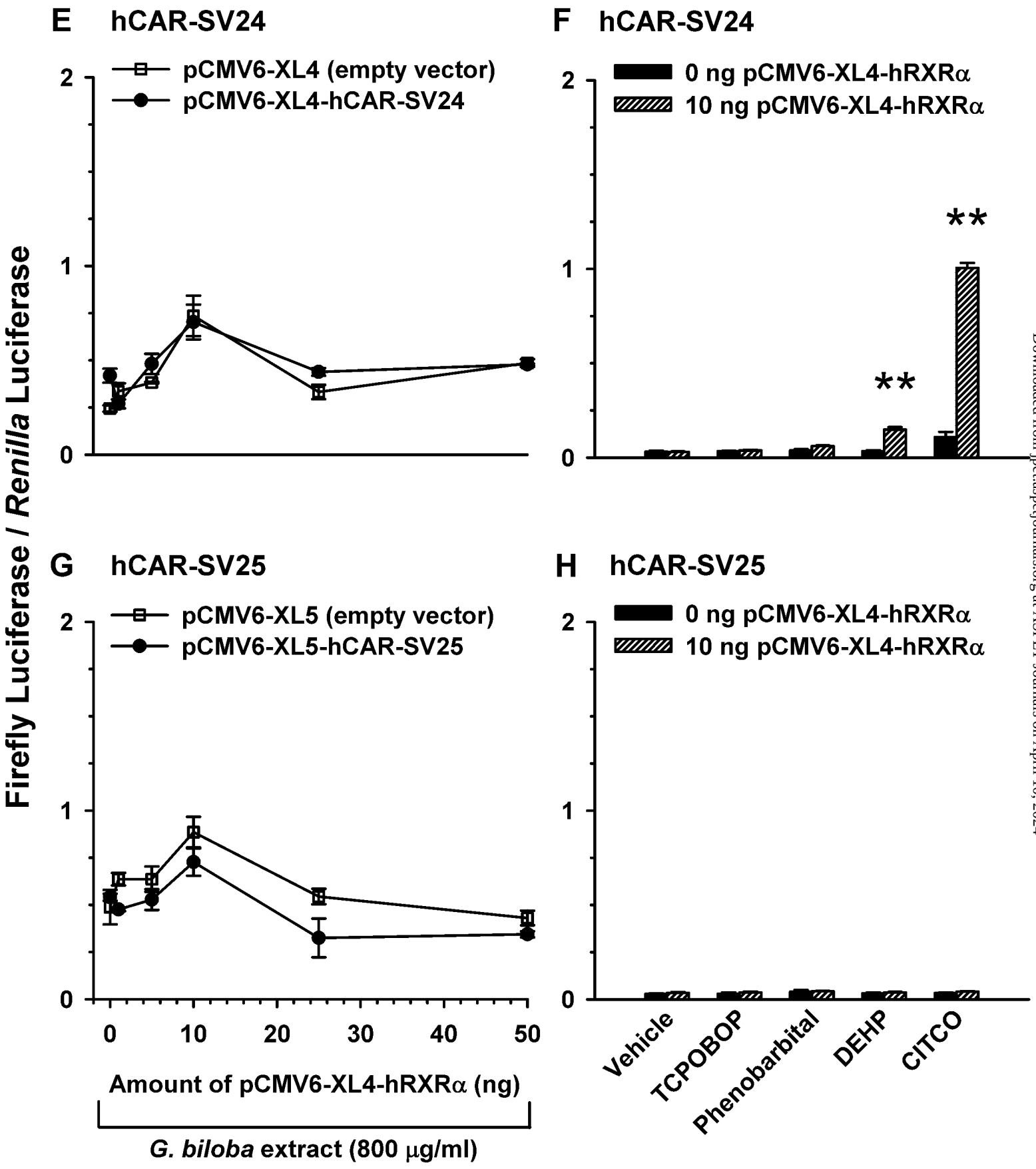

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Figure 6

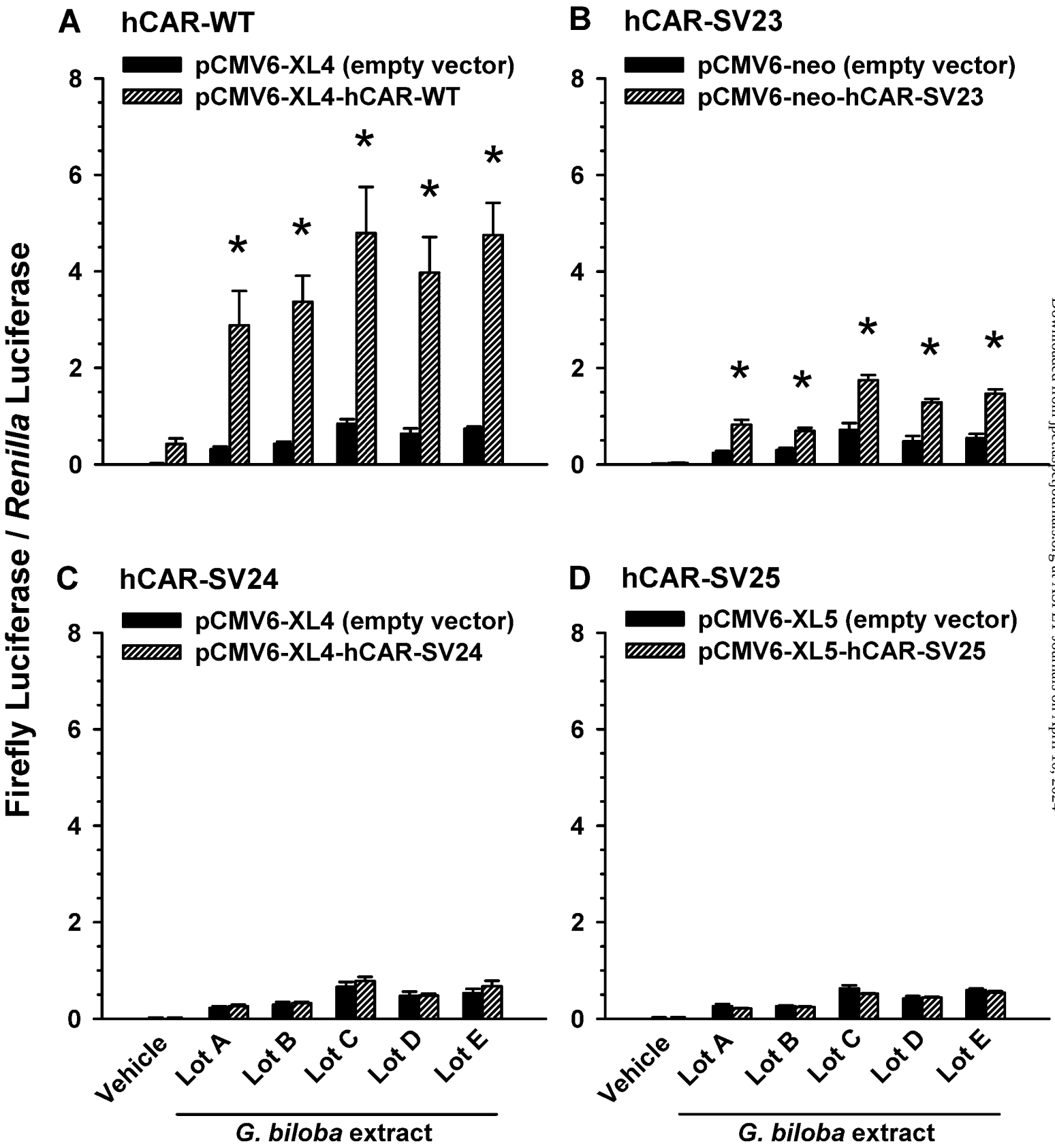

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Figure 7