

RT206 Couples Partial PPAR α / γ Agonism with Ligand-Dependent Allosteric Potentiation of FXR Signaling in Liver Cells

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Supplementary Materials

Table S1. PPAR α and PPAR γ transactivation data for compounds selected from the series previously reported by Fracchiolla et al.

Compound	X	PPAR α EC50 (μ M)	PPAR α Efficacy	PPAR γ EC50 (μ M)	PPAR γ Efficacy
S-4	CH ₂ CH ₂	0.15 $\pm$ 0.04	83 $\pm$ 10	0.40 $\pm$ 0.20	33 $\pm$ 5
15	CH ₂ CH ₂ CH ₂	0.72 $\pm$ 0.20	67 $\pm$ 15	0.87 $\pm$ 0.16	30 $\pm$ 6
14	C $\equiv$ C	1.36 $\pm$ 0.06	36 $\pm$ 3	0.540 $\pm$ 0.017	39 $\pm$ 1
12	CH=CH trans	1.75 $\pm$ 0.12	57 $\pm$ 4	0.72 $\pm$ 0.27	50 $\pm$ 1
R-2	CH ₂	2.4 $\pm$ 0.8	82 $\pm$ 2	5.7 $\pm$ 0.4	21 $\pm$ 1
9	CH ₂ NH	3.6 $\pm$ 1.5	78 $\pm$ 4	13 $\pm$ 3	27 $\pm$ 6

Table S2. MM-GBSA binding free energy estimates calculated on docked complexes with PPAR α and PPAR γ .

Compound	X	MM-GBSA ΔG_{bind} (kcal mol ⁻¹) (PPAR α)	MM-GBSA ΔG_{bind} (kcal mol ⁻¹) (PPAR γ)
S-4	CH ₂ CH ₂	-62.17	-50.94
S-15	CH ₂ CH ₂ CH ₂	-65.52	-57.17
R-15	CH ₂ CH ₂ CH ₂	-64.55	-51.16

Table S3. SiteMap-derived properties of the identified pocket within different FXR LBD structures. The threonine residue previously identified by mutagenesis is highlighted in bold.

PDB	Ligand	SiteScore	Size	Dscore	Volume (Å ³)	Residues
6HL1	CDCA	1.097	100	1.009	150.234	Chain A: 259, 260, 261, 263, 264, 265, 293, 294, 297, 298, 301, 327, 330, 331, 334, 379, 386
3DCT	GW4064	1.018	145	0.971	312.473	Chain A: 259, 260, 261, 262, 263, 264, 265, 266, 267, 270, 293, 294, 297, 298, 300, 301, 304, 327, 330, 331, 334, 335, 337, 338, 340, 342, 372, 373, 375, 376, 377, 378, 379, 382, 386
6HL0	APO	0.933	36	0.88	72.03	Chain A: 260, 263, 294, 295, 297, 298, 301, 324, 327, 328, 331, 386 , 390
1OSV	OCA (RAT)	0.994	247	1.001	594.076	Chain A: 202, 256, 257, 258, 259, 260, 261, 262, 263, 264, 267, 268, 270, 287, 291, 294, 295, 298, 301, 324, 328, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 345, 346, 350, 360, 363, 366, 367, 374, 375, 376, 379, 383 , 387

Table S4. Summary of the systems simulated in this study.

System	Starting structure (PDB ID)	RT206 / GG binding site	Ligand placement protocol	Sampling (replicas × length)
<i>Ternary complexes</i>				
FXR / GW4064 / RT206	3DCT	S2 backdoor	Glide SP (grid on SiteMap-identified S2 pocket)	3 × 1 μs
FXR / OCA / RT206	6HL1 ^a	S2 backdoor	Glide SP (grid on SiteMap-identified S2 pocket)	3 × 1 μs
FXR / GW4064 / GG	6HL1 ^b	S2 backdoor	Induced Fit Docking (IFD)	3 × 1 μs
FXR / OCA / GG	6HL1 ^a	S2 backdoor	Induced Fit Docking (IFD)	3 × 1 μs
<i>Binary complexes</i>				
FXR / GW4064	3DCT	n.a.	Co-crystallized pose retained	3 × 1 μs
FXR / OCA	6HL1 ^a	n.a.	Ligand modeled onto the CDCA-bound pose	3 × 1 μs
FXR / RT206 (S1)	6HL1	S1 orthosteric	Glide SP (grid on co-crystallized ligand)	3 × 1 μs
FXR / RT206 (S2)	6HL1	S2 backdoor	Glide SP (grid on SiteMap-identified S2 pocket)	3 × 1 μs
FXR apo	6HL0	n.a.	n.a.	3 × 1 μs

In each simulation, the coactivator peptide co-crystallized in the starting PDB structure was retained and solvated together with the receptor.

^a The OCA model was generated from the CDCA-bound 6HL1 structure by modifying the co-crystallized CDCA coordinates to introduce the 6α-ethyl substituent, followed by local minimization.

^b GW4064 coordinates were transferred from PDB 3DCT to 6HL1 by structural superposition of the two LBDs prior to IFD of GG into the S2 pocket.

Table S5. Nucleotide sequences of the primers used in this study.

Target gene		Sequence	T _m (°C)
PPARG	FW	5'-CGTGGCCGCAGATTTGAA-3'	58
	RV	5'-CTTCCATTACGGAGAGATCCAC-3'	
CD36	FW	5'-CTTATACGTACAGAGTTCGTT-3'	60
	RV	5'-AGATGGCACCATTGGGCTGC-3'	
INSIG1	FW	5'-CCTTTGGTGGACATTTGATCGT-3'	60
	RV	5'-GCGTAGCTAGAAAAGCTATGGT-3'	
PPARA	FW	5'-CTATCATTTGCTGTGGAGATCG-3'	60
	RV	5'-AAGATATCGTCCGGGTGGTT-3'	
CPT1A	FW	5'-CCAGACGAAGAACGTGGTCA-3'	58
	RV	5'-ATCTTGCCGTGCTCAGTGAA-3'	
ACOX1	FW	5'-ATGGTGTGTTGTCAGGTCCTTCCT-3'	62
	RV	5'-CTGGGTTTGAAAATCCAAAATCT-3'	
CYP7A1	FW	5'-CACAATGCCCGGGAGAAA-3'	56

	RV	5'-AAAGTCGCTGGAATGGTGTGTTG-3'	
<i>SHP</i>	FW	5'-CTCTTCCTGCTTGGGTGGC-3'	60
	RV	5'-GCACATCGGGGTGAAGAGG-3'	
<i>BSEP</i>	FW	5'-ACATGCTTGCGAGGACCTTTA-3'	60
	RV	5'-GGAGGTTTCGTGCACCAGGTA-3'	
<i>OSTA</i>	FW	5'-TTCCTCTAAAACCAGGTCTCAAGT-3'	62
	RV	5'-GCACAGTCATTAGAAAAGTCTCCA-3'	
<i>OSTB</i>	FW	5'-CAGGAGGTGCTGGAAGAGAT-3'	62
	RV	5'-GACCATGCTTATAATGACCACCA-3'	
<i>NQO1</i>	FW	5'-GAAGAGCACTGATCGTACTGGC-3'	60
	RV	5'-GGGTCCTTCAGTTTACCTGTGAT-3'	
<i>MYC</i>	FW	5'-CACCAGCAGCGACTCTGA-3'	60
	RV	5'-GATCCAGACTCTGACCTTTTGC-3'	
<i>18S</i>	FW	5'-GGGAGCCTGAGAAACGGC-3'	58-60
	RV	5'-GGGTCGGGAGTGGGTAATTT-3'	

Table S6. Antibodies used for Western blotting in this study.

Target protein	Reference	Host	Supplier	Dilution
Anti-PPAR γ	2443	Rabbit	Cell Signaling Technologies	1:1000
Anti-PPAR α	ab227074	Rabbit	Abcam	1:1000
Anti- α -Tubulin	T5168	Mouse	Merck	1:10000
Anti-Rabbit-HRP	7074	-	Cell Signaling Technologies	1:2000
Anti-Mouse-HRP	7076	-	Cell Signaling Technologies	1:2000

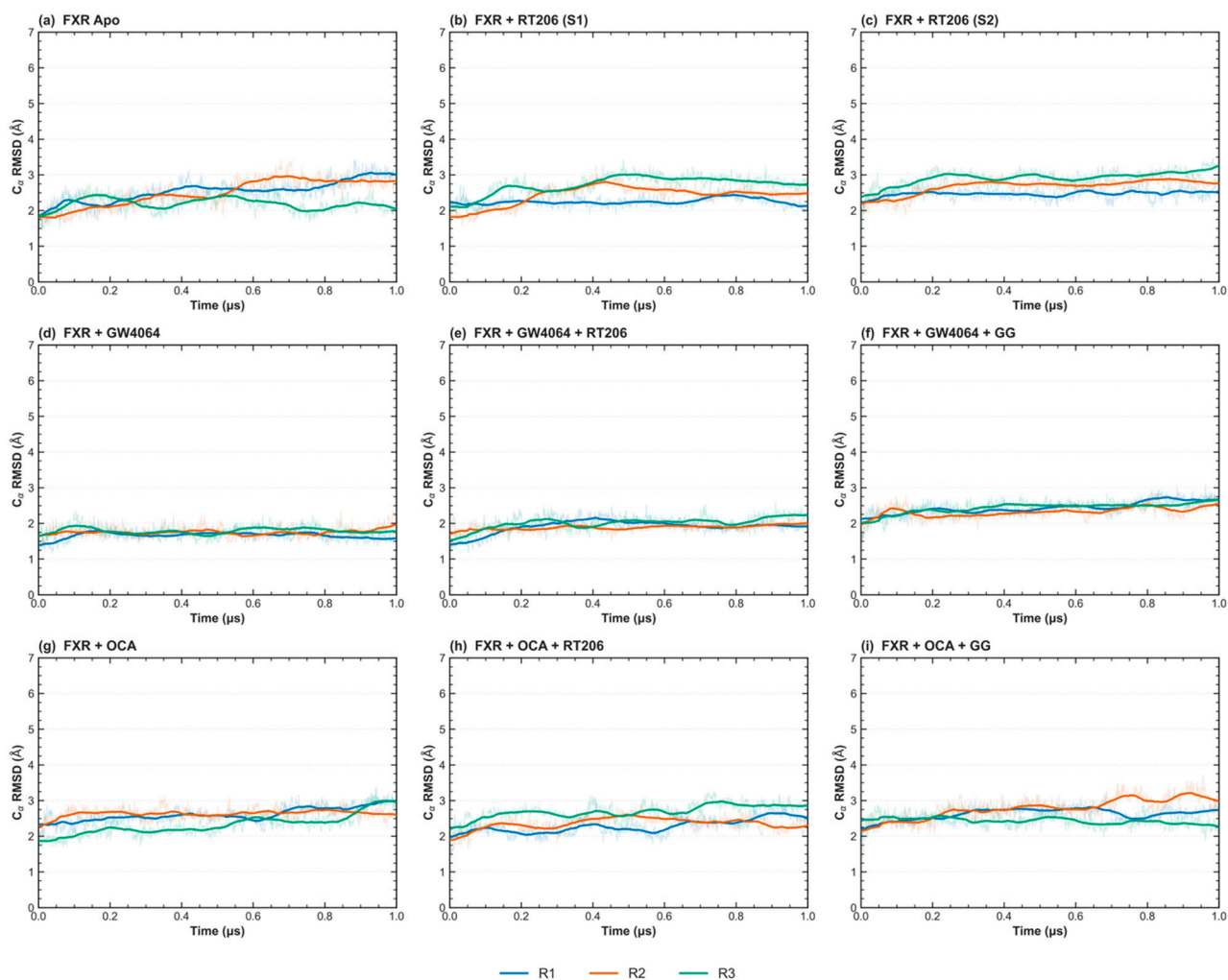


Figure S1. Backbone $C\alpha$ RMSD profiles of the simulated systems along 1 μ s MD trajectories. **A)** apo-FXR; **B)** FXR + RT206 docked in the orthosteric S1 pocket; **C)** FXR + RT206 docked in the S2 backdoor pocket; **D)** FXR + GW4064; **E)** FXR + GW4064 + RT206; **F)** FXR + GW4064 + GG; **G)** FXR + OCA; **H)** FXR + OCA + RT206; **I)** FXR + OCA + GG. For each system, three independent replicas (R1, blue; R2, orange; R3, green) are shown; light traces correspond to the raw frame-wise RMSD values, while solid bold traces represent the moving average (window = 50 ns) and are provided to aid visual comparison across systems.

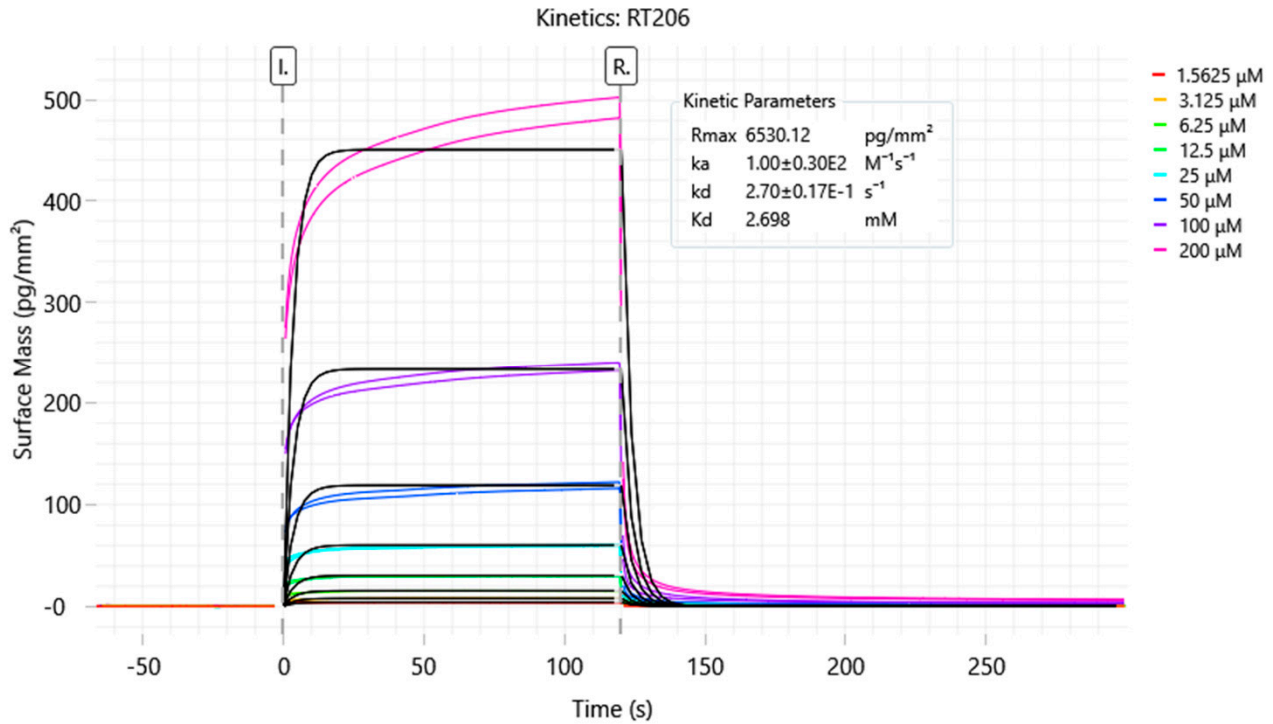


Figure S2. Kinetic binding analysis of RT206 to immobilized FXR-LBD by grating-coupled interferometry.

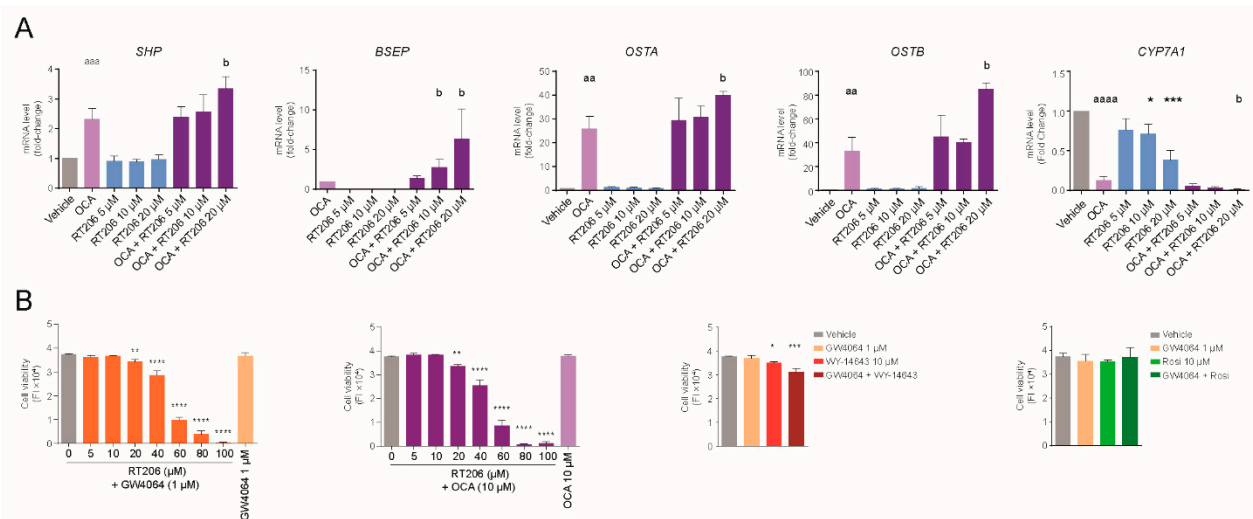


Figure S3. A) Expression of *SHP* (n=4), *BSEP* (n=5), *OSTA* (n=3), *OSTB* (n=3), and *CYP7A1* (n=3) genes in HepG2 cells treated for 24 h with the vehicle (DMSO) or OCA (10 μM), the indicated doses of RT206, alone or in combination. **B)** Viability (n=3) of HepG2 cells treated for 24 h with the indicated concentrations of RT206 alone or in combination with GW4064, OCA, WY-14643, or rosiglitazone. All histograms report normalization of mRNA levels to 18S in RT-qPCR analysis. Only for *BSEP* expression, the fold-change is referred to OCA treatment, as the basal level is very low or undetermined. Statistical significance was considered when a $p < 0.05$, aa $p < 0.01$, aaa $p < 0.001$, or aaaa $p < 0.0001$ (t-test vs Vehicle); b $p < 0.05$ (t-test with Bonferroni correction vs OCA); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or **** $p < 0.0001$ (ANOVA with Dunnett's post-test vs Vehicle).

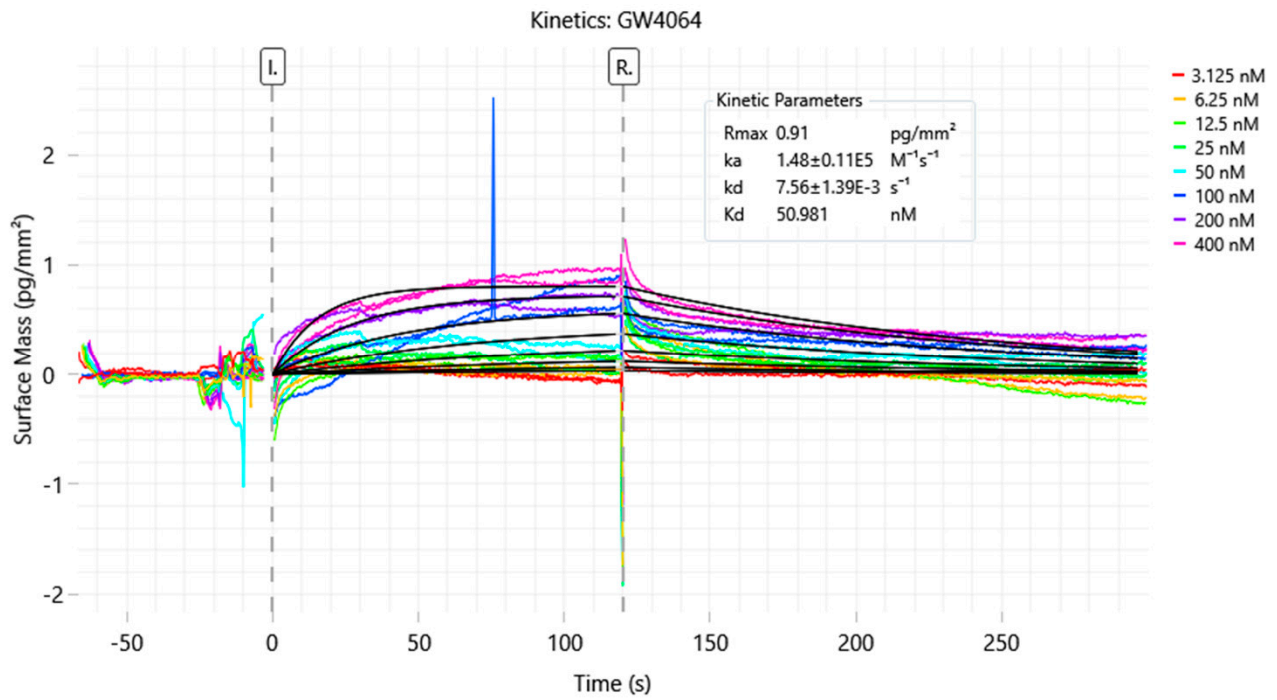
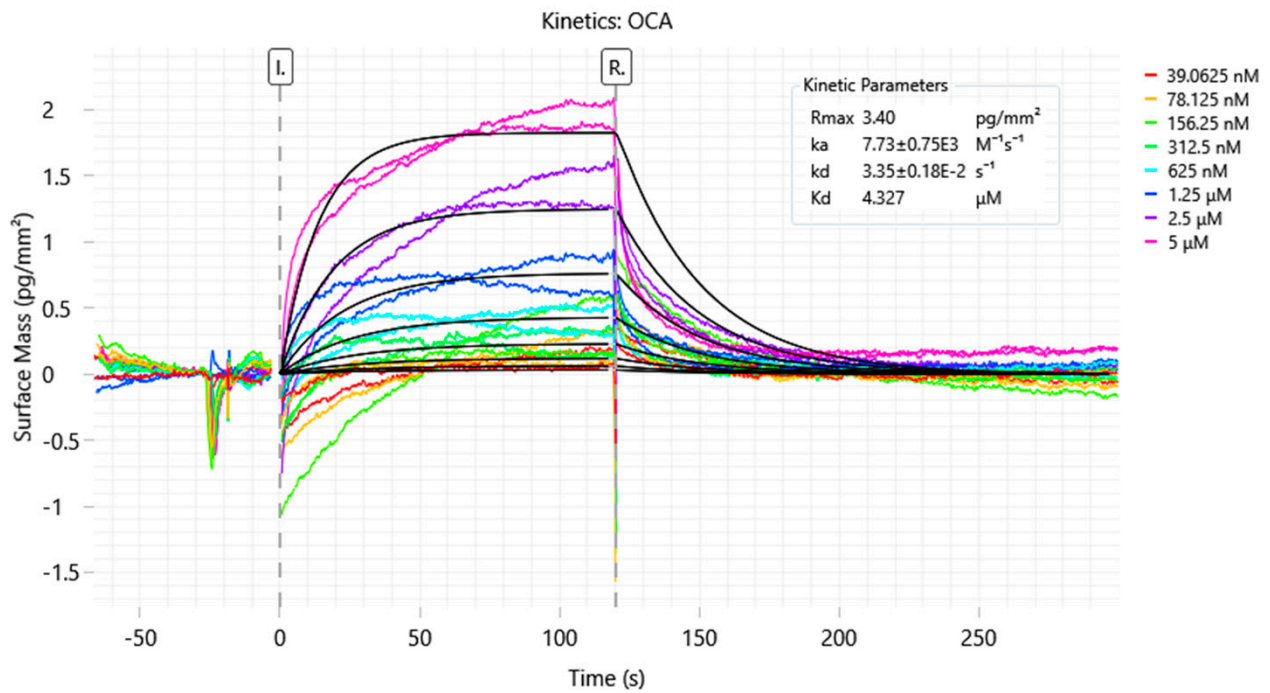
A**B**

Figure S4. A) GW4064 GCI kinetic analysis towards immobilized FXR; **B)** OCA GCI kinetic analysis towards immobilized FXR.

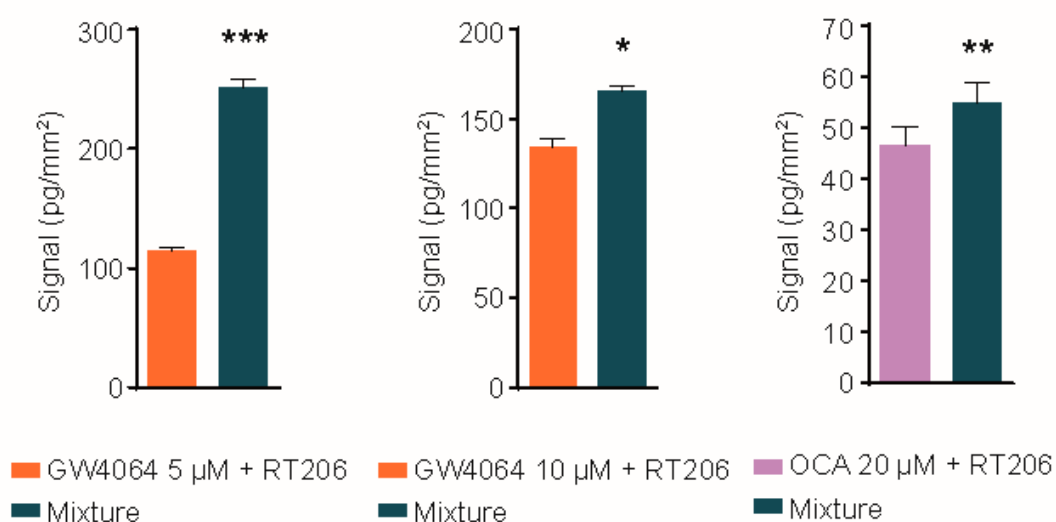


Figure S5. GCI signals recorded upon injection of 100 µM RT206 and 5 or 10 µM GW4064 either individually (orange bar) or in combination (blue bar) onto immobilized FXR. The same experiment was conducted by injecting 100 µM RT206 and 20 µM OCA either individually (violet bar) or in combination (blue bar). Statistical significance was considered when *p < 0.05, **p < 0.01, and ***p < 0.001 (paired t-test).

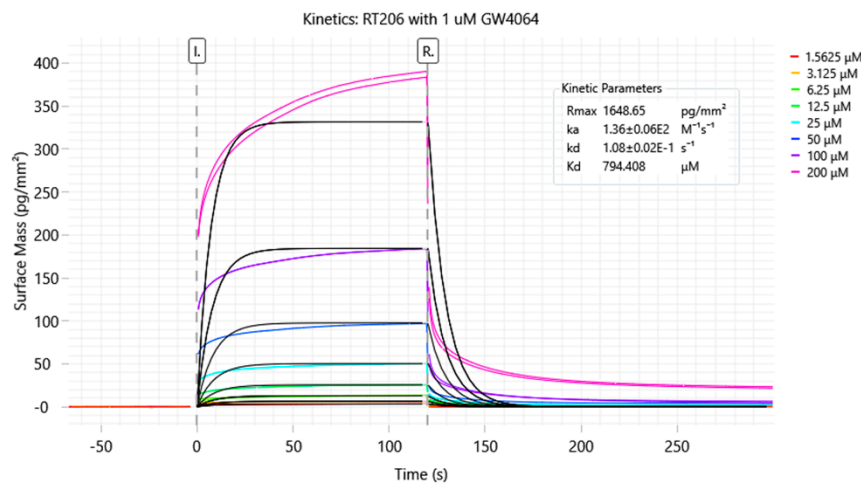
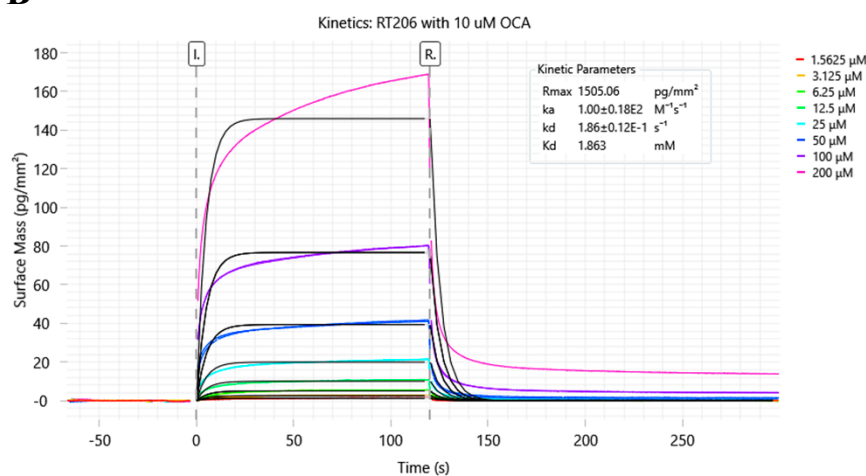
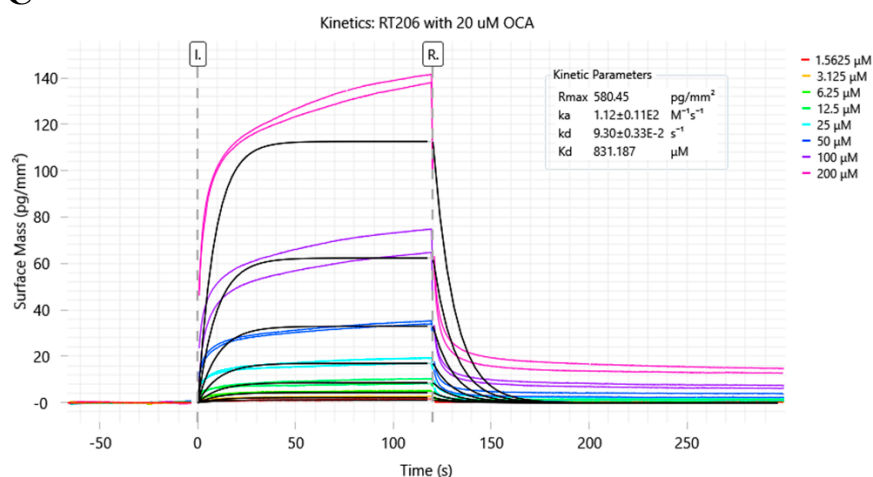
A**B****C**

Figure S6. RT206 kinetic analysis of binding to immobilized FXR in presence of a fixed concentration of **A)** GW4064 1 µM or **B)** OCA 10 µM or **C)** OCA 20 µM.

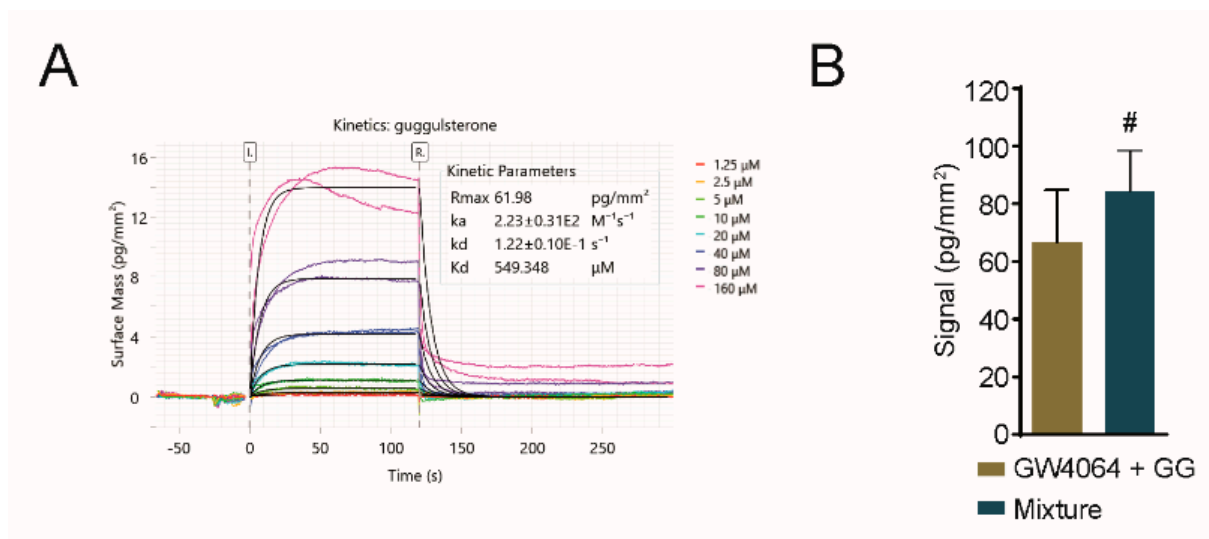


Figure S7. A) GG kinetic analysis towards immobilized FXR; **B)** GCI signals recorded upon injection of 10 μM GW4064 and 100 μM GG either individually (brown bar) or in combination (blue bar) onto immobilized FXR. Statistical significance was considered when # $p < 0.05$ (paired t-test).

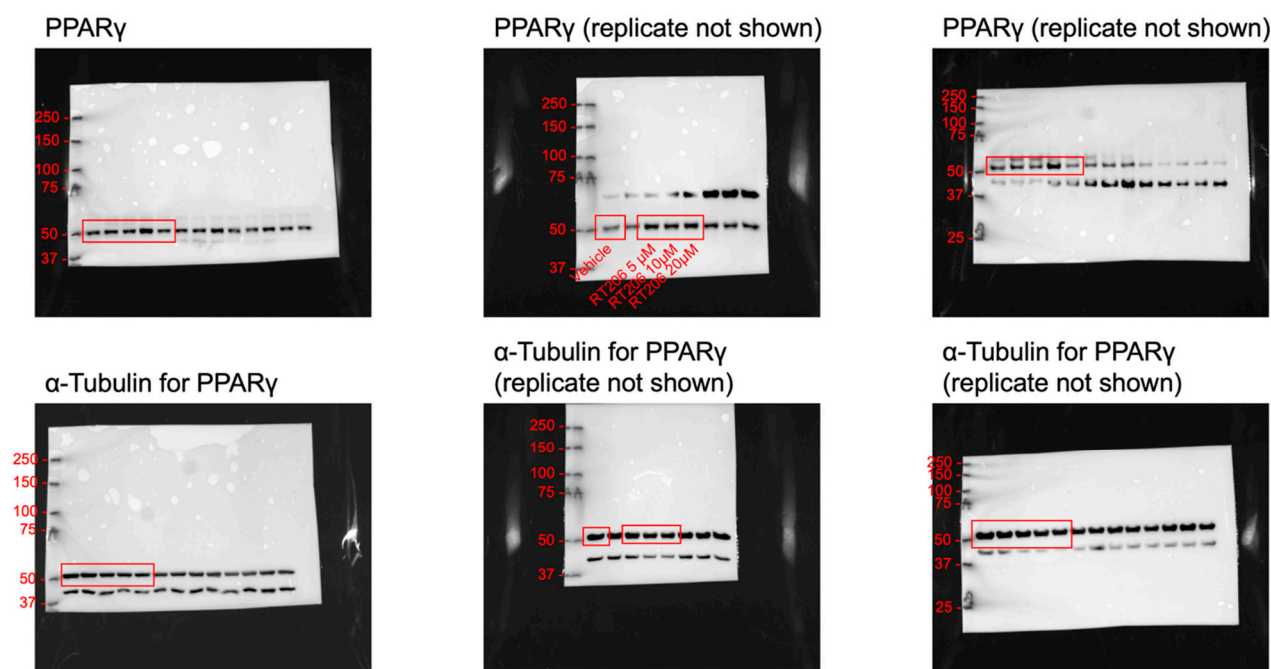


Figure S8. Original Western blots corresponding to Figure 1G. Uncropped Western blot images showing detection of PPAR γ in extracts of HepG2 cells treated as indicated in Figure 1G legend. The PPAR γ bands, circled in red in the upper panels, are from three independent experiments. Band density was normalized to α -Tubulin bands (outlined in red in the lower panels) from the same blots, using ImageLab software, version 6.1.0.

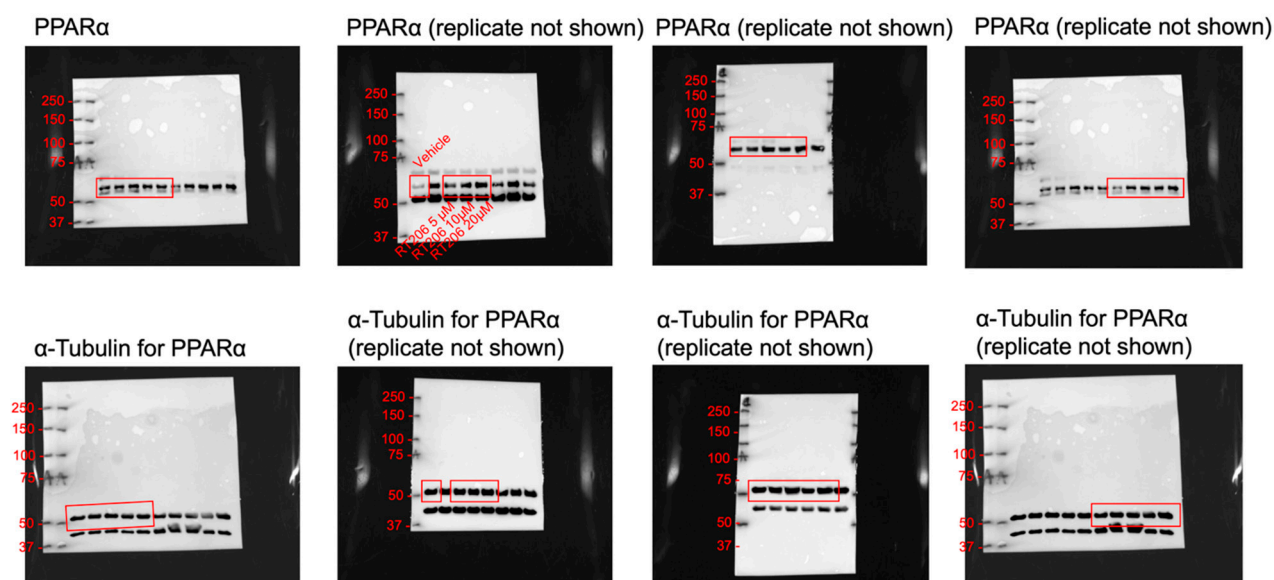


Figure S9. Original Western blots corresponding to Figure 1I. Uncropped Western blot images showing detection of PPAR α in extracts of HepG2 cells treated as indicated in Figure 1I legend. The PPAR α bands, circled in red in the upper panels, are from four independent experiments. Band density was normalized to α -Tubulin bands (outlined in red in the lower panels) from the same blots, using ImageLab software, version 6.1.0.