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Liver X receptor antagonists with a phthalimide skeleton derived from thalidomide-related glucosidase inhibitors

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Abstract—α-Glucosidase inhibitors with a chlorinated phthalimide or a thiophthalimide skeleton, derived from thalidomide, were found to possess liver X receptor (LXR) antagonistic activity. Novel LXR antagonists with a 2′-substituted phenylphthalimide skeleton were obtained by structural development of glucosidase inhibitors derived from thalidomide.

Liver X receptors (LXRα and LXRβ) are members of the nuclear receptor superfamily (a family of ligand-dependent transcription factors which modulate specific gene expression and thereby influence diverse biological processes, including cell growth, differentiation, and metabolism).[1,2] The physiological ligands of LXRs are considered to be oxysterols, such as 24(*S*),25-epoxycholesterol (**1**, EPC),[1,2] and several synthetic agonists, including GW3965 (**2**), and T0901317 (**3**), have been reported (Fig. 1).[3,4] LXRs function as heterodimers with other nuclear receptors, the retinoid X receptors (RXRα, RXRβ, and RXRγ), to regulate important aspects of cholesterol homeostasis by controlling expression of their target genes, including ATP binding cassette ABCA1 and CYP7A genes.[5,6] LXRs also regulate the expression of several genes involved in glucose metabolism.[7,8] Thus, LXRs have been regarded as members of the metabolic subfamily of nuclear receptors, participating in the regulation of both lipid and sugar metabolism. Mitro et al. reported that LXRs act as glucose sensors, that is, D-glucose and D-glucose-6-phosphate act as ligands for LXRs and activate their transcription activity with EC_{50} values of 308 μM for LXRα and 3141 μM for LXRβ.[9] This finding indicates that LXRs recognize both oxysterols and glucose derivatives as their physiological ligands.

We have been engaged in structural development studies of thalidomide, a drug first launched as a sedative/hypnotic agent, but withdrawn from the market because of its severe teratogenicity, focusing on its potential for the treatment of a range of diseases, including cancers, diabetes, and rheumatoid arthritis.[10–12] We have developed a series of potent α-glucosidase inhibitors, including CP0P (**4**), CP4P (**5**), and PPS-33 (**6**) (Fig. 2).[10–15] CP0P (**4**) is a non-competitive inhibitor of α-glucosidase, whereas CP4P (**5**) is a competitive inhibitor.[13] PPS-33 (**6**) is another competitive α-glucosidase inhibitor, but it also inhibits other enzymes, including maltase and dipeptidylpeptidase type IV.[16] The competitive inhibition of α-glucosidase by CP4P (**5**) and PPS-33 (**6**) indicated that these compounds might be structural mimics of glucose.

On this basis, we considered that CP4P (**5**) and PPS-33 (**6**) might be recognized as glucose mimics by LXRs (*vide supra*) and might act as ligands for LXRs. Here, we describe the discovery of LXR-antagonistic activity of CP4P (**5**), PPS-33 (**6**), and some related derivatives, and the development of novel LXR ligands with a 2′-substituted phenylphthalimide skeleton.

*Effects of CP0P (**4**), CP4P (**5**), and PPS-33 (**6**) on LXRs*. First we investigated the effects of CP0P (**4**), CP4P (**5**), and PPS-33 (**6**) on LXRs using a reporter gene assay method with CMX-GAL4N-hLXR as the recombinant receptor gene, TK-MH100x4-LUC as the reporter gene, and the CMX β-galactosidase gene for

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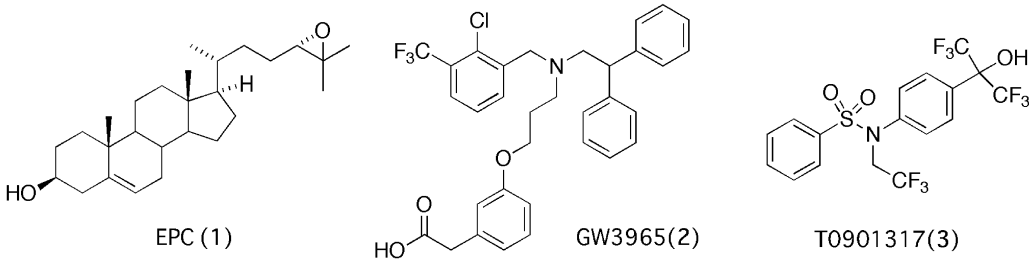


Figure 1. Structures of known LXR agonists, EPC (**1**), GW3965 (**2**), and T0901317 (**3**).[1–4]

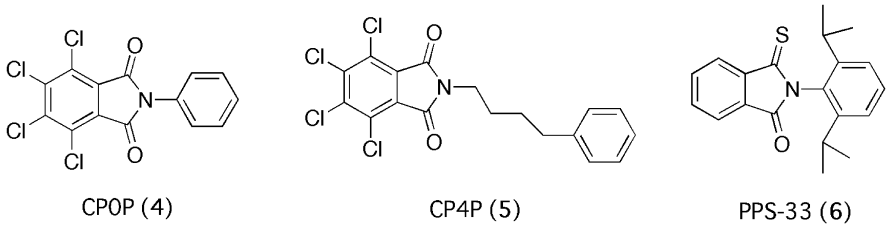
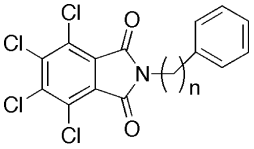
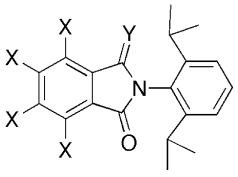
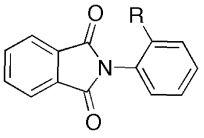


Figure 2. Structures of α-glucosidase inhibitors (**4**–**6**) derived from thalidomide.[10–12]

normalization, as previously reported.[17–19] Briefly, human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfections were performed by the calcium phosphate coprecipitation method. Test compounds with or without 0.1 μM T0901317 (**3**) were added 8 h after the transfection, and luciferase and β-galactosidase activities were assayed using a luminometer and microplate reader. The experiment was repeated three times, and the normalized average values are presented in this paper. None of the compounds tested show agonistic activity under the experimental conditions used (data not shown). α-Glucosidase-inhibitory activity was evaluated as reported previously.[13–15] Briefly, α-glucosidase (Wako Pure Chemical Industries Ltd., 25 mU/mL) was incubated with various concentrations of test compounds in 10 mM phosphate buffer (pH 7.2) at 37 °C for 10 min. The substrate (*p*-nitrophenyl-α-D-glucopyranoside) was added at the final concentration of 0.16 mM and the mixture was incubated at 37 °C for 30 min. It was basified by adding 0.5 M Na_2CO_3, and the amount of released *p*-nitrophenol (Abs. 400 nm) was measured. The IC_{50} values are shown in Table 1.

As shown in Figure 3, a non-competitive α-glucosidase inhibitor, CP0P (**4**, IC_{50} value of 2.6 μM toward α-glucosidase, Table 1), did not show LXR-antagonistic activity, while the competitive inhibitors CP4P (**5**, IC_{50} value of 2.0 μM toward α-glucosidase, Table 1) and PPS-33 (**6**, IC_{50} value of 8.0 μM toward α-glucosidase, Table 1) showed dose-dependent antagonistic activity toward both LXRα and LXRβ. CP4P (**5**) seems to be an almost non-selective (very slightly LXRα-selective) LXR antagonist with calculated IC_{50} values of 88 μM for LXRα and 101 μM for LXRβ. On the other hand, PPS-33 (**6**) seems to be an LXRα-selective antagonist with IC_{50} values of 3.1 μM for LXRα and 18 μM for LXRβ. LXR-antagonistic activity is not correlated with α-glucosidase inhibitory activity, because the non-competitive α-glucosidase inhibitor CP0P (**4**) did not show LXR-antagonistic activity. The finding that the competitive α-glucosidase inhibitors CP4P (**5**) and

Table 1. α-Glucosidase-inhibitory activity of CPnP (**4**, **5**, and **7**–**11**), PPS-33 (**6**), CPP-33 (**14**), PP60 (**15**), and PP2P (**16**)

Compound	IC_{50} (μM)
	
n = 0: CP0P (**4**)	2.6
n = 1: CP1P (**7**)	10.9
n = 2: CP2P (**8**)	6.0
n = 3: CP3P (**9**)	4.5
n = 4: CP4P (**5**)	2.0
n = 5: CP5P (**10**)	3.5
n = 10: CP10P(**11**)	6.8
	
X = H, Y = S: PPS-33 (**6**)	8.0
X = CI, Y = O: CPP-33 (**14**)	2.4
	
R = $(CH_2)_5CH_3$: PP60 (**15**)	24.7
R = $(CH_2)_2Ph$: PP2P (**16**)	16.2

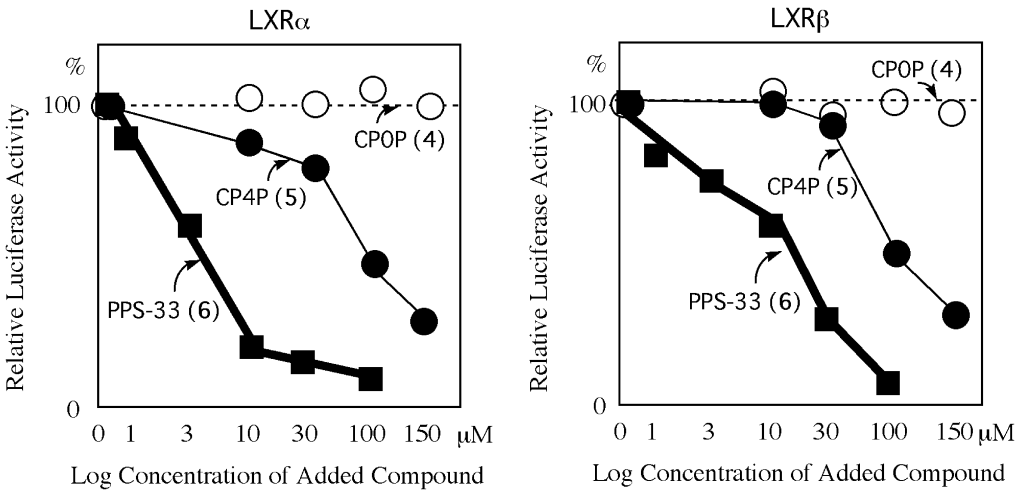
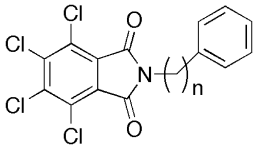
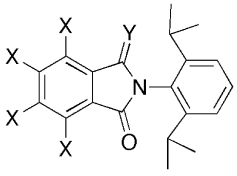
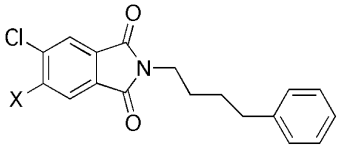


Figure 3. LXR-antagonistic activities of α-glucosidase inhibitors (**4**–**6**) measured by means of reporter gene assay. Various concentrations of the test compounds were added in the presence of 0.1 μM T0901317 (**3**). The relative luciferase activity (vertical scale) induced with 0.1 μM T0901317 (**3**) alone was defined as 100%.

PPS-33 (**6**) show LXR antagonistic activity suggests that they are indeed recognized by LXRs as glucose mimics, as we had expected (*vide supra*).

*Effects of chlorinated phthalimide analogs (***4***,* **5***, and* **7***,* **14***) on LXRs*. Next, we investigated the effects of several chlorinated phthalimide derivatives on LXRs. The derivatives [CP1P (**7**),[13] CP2P (**8**),[13] CP3P (**9**),[13] CP5P (**10**),[13] CP10P (**11**),[14] 5CP4P (**12**),[20] 56CP4P (**13**)[14], and CPP-33 (**14**)[14]] were prepared by usual organic synthetic methods, that is, condensation of appropriate amines with chlorinated phthalic anhydride, as previously reported.[13–15] None of these compounds showed LXR-agonistic activity (data not shown). The results of LXR-antagonistic activity assay in the presence of 0.1 μM T0901317 (**3**) are presented in Table 2. Compounds **7**–**11** are derivatives with various lengths of methylene spacer linking a tetrachlorophthalimide moiety and a phenyl moiety. CP0P (**4**) is inactive toward both LXRα and LXRβ, as mentioned above. However, insertion of a spacer with one to four methylene units between the tetrachlorophthalimide moiety and the phenyl moiety resulted in the appearance of antagonistic activity, that is, CP1P (**7**), CP2P (**8**), CP3P (**9**), and CP4P (**5**) at 100 μM showed 30–60% inhibition of T0901317 (**3**)-induced transcriptional activation of both LXRα and LXRβ. Although no clear structure–activity relationship concerning the methylene spacer length was observed, the compounds with an odd number of methylene units [CP1P (**7**) and CP3P (**9**)] seemed to be more potent than those with an even number of methylene units [CP2P (**8**) and CP4P (**5**)] (though the difference is not large). However, CP5P (**10**), a derivative with a five methylene unit spacer, showed weaker antagonistic activity toward LXRs than those of CP4P (**5**). The result suggests that insertion of an over-long spacer causes disappearance of the activity. A derivative with a ten methylene unit spacer, CP10P (**11**), was inactive towards both LXRα and LXRβ.

Table 2. LXR-antagonistic activities of CPnP (**4**, **5**, and **7**–**11**), PPS-33 (**6**), 5CP4P (**12**), 56CP4P (**13**), and CPP-33 (**14**) measured by means of reporter gene assay

Compound	% Inhibition	
	LXRα	LXRβ
		
n = 0: CP0P (**4**)	Inactive	Inactive
n = 1: CP1P(**7**)	60	51
n = 2: CP2P(**8**)	38	38
n = 3: CP3P(**9**)	59	52
n = 4: CP4P (**5**)	53	49
n = 5: CP5P(**10**)	42	3-10
n = 10: CP10P(**11**)	Inactive	Inactive
		
X = H, Y = S: PPS-33 (**6**)	94	92
X = CI, Y = O: CPP-33 (**14**)	61	49
		
X = H: 5CP4P (**12**)	66	45
X = CI: 56CP4P (**13**)	50	35

The test compounds (100 μM) were added in the presence of 0.1 μM T0901317 (**3**). Inhibition (%) of the relative luciferase activity induced by 0.1 μM T0901317 (**3**) alone is presented.

5CP4P (**12**) and 56CP4P (**13**) are dechlorinated derivatives of CP4P (**5**). 5CP4P (**12**) and 56CP4P (**13**) showed similar antagonistic activity toward both LXRα and LXRβ, with the former being slightly more potent. CPP-33 (**14**) is a tetrachloro/carbonyl derivative of PPS-33 (**6**). Although CPP-33 (**4**) shows more potent α-glucosidase-inhibitory activity (IC_{50}: 2.4 μM) than PPS-33 (**6**) (Table 1), its LXR-antagonistic activity was weaker than that of PPS-33 (**6**) (Table 2). Thus, potency of α-glucosidase-inhibitory activity again does not correlate with potency of LXR-antagonistic activity in this case.

*Structural development of novel LXR antagonists (***15** *and* **16***) based on a phthalimide skeleton*. The above results indicate that a phenylphthalimide skeleton can be regarded as a novel scaffold for LXR antagonists. The reported crystal structures of the ligand binding domains of LXRs suggest that the ligand-binding pocket is larger than that of retinoic acid receptors (RARs) and smaller than that of peroxisome proliferator-activated receptors (PPARs).[21,22] The amino acid sequences of LXRs and the results of molecular evolutional analysis suggest that LXRs are evolutionally close to PPARs, which have been established to possess a Y-shaped ligand-binding pocket of approximately 1300–1400 $Å^3$.[23] Based on these findings, we designed Y-shaped, branched, 2′-substituted phenylpthalimide derivatives, PP60 (**15**), and PP2P (**16**) (Fig. 4).

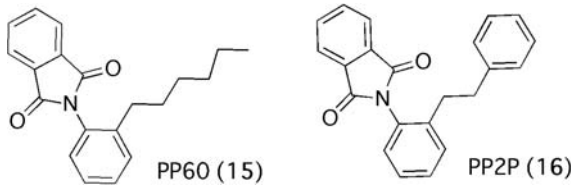


Figure 4. Structures of the designed branched, Y-shaped compounds, PP60 (**15**), and PP2P (**16**).

PP60 (**15**)[24] and PP2P (**16**)[25] were synthesized by usual organic synthetic methods. Briefly, pentyl [for PP60 (**15**)] or benzyl [for PP2P (**16**)] triphenyl phosphonium bromide was treated with butyl lithium (1 eq.) and reacted with 2-nitrobenzaldehyde. The resultant *ortho*-substituted nitrobenzene was reduced with H_2 gas over Pd/C, followed by condensation with phthalic anhydride. The α-glucosidase-inhibitory activity and the effects on LXRs of PP60 (**15**) and PP2P (**16**) were evaluated as mentioned above, and the results are presented in Table 1 and Figure 5, respectively.

Both PP60 (**15**) and PP2P (**16**) showed moderate α-glucosidase-inhibitory activity with IC_{50} values of 24.7 and 16.2 μM (less potent than other compounds in Table 1), respectively (Table 1), and more potent antagonistic activity toward both LXRα and LXRβ than CP4P (**5**) (Figs. 3 and 5), with no agonistic activity on LXRs (data not shown). As shown in Figure 5, both PP60 (**15**) and PP2P (**16**) seem to be LXRα-selective antagonists, that is, PP60 (**15**) antagonized transcriptional activation of LXRs by 0.1 μM T0901317 (**3**) with IC_{50} values of 13 μM for LXRα and 65 μM for LXRβ. The IC_{50} values of PP2P (**16**) determined under the same experimental conditions were 9.8 μM for LXRα and 44 μM for LXRβ.

Although the LXR-antagonistic activities elicited by PP60 (**15**) and PP2P (**16**) are less potent than those of PPS-33 (**6**), PP60 (**15**), and PP2P (**16**) are thought to be superior lead compounds for the development of LXR ligands. One reason for this is that they are less cytotoxic [PP60 (**15**) and PP2P (**16**) at 100 μM inhibited HEK293 cells to the extent of 57% and 48%, respectively] than PPS-33 (**6**, 73% inhibition of cell growth of HEK293 cells at 100 μM). In addition, they seem to be more selective [possessing no inhibitory activity toward dipeptidylpeptidase type IV, maltase, or other enzymes toward which PPS-33 (**6**) shows inhibitory activity, as well as having less potent α-glucosidase-inhibitory activity] than PPS-33 (**6**).

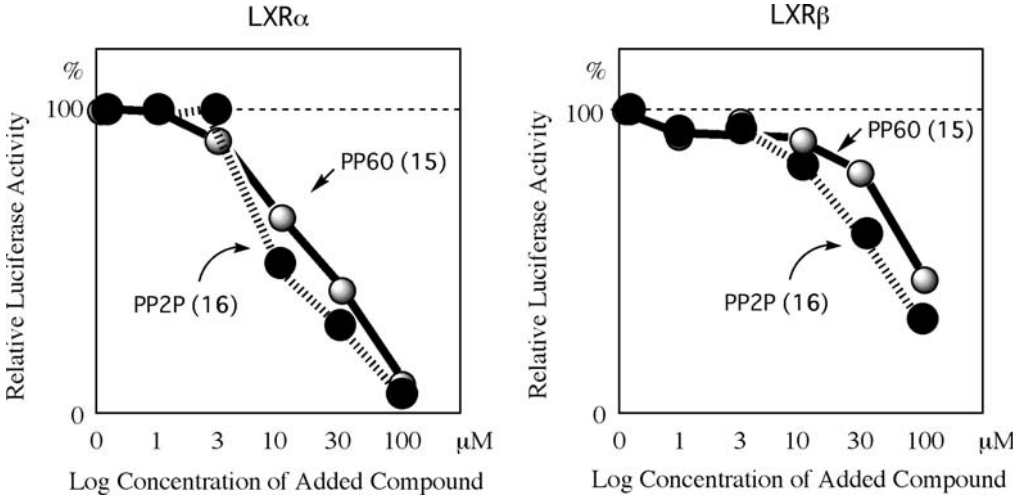


Figure 5. LXR-antagonistic activities of PP60 (**15**) and PP2P (**16**) measured by means of reporter gene assay. Various concentrations of the test compounds were added in the presence of 0.1 μM T0901317 (**3**). The relative luciferase activity induced with 0.1 μM T0901317 (**3**) alone was defined as 100%.

In conclusion, we discovered LXR antagonists [CP4P (**5**), PPS-33 (**6**), and their derivatives] among our α-glucosidase competitive inhibitors derived from thalidomide. Based on a consideration of our findings and reported information, novel LXR antagonists with a 2′-substituted phenylphthalimide skeleton, PP60 (**15**), and PP2P (**16**), were designed and synthesized. Further investigation of the structure–activity relationships, development of superior LXR ligands, and further applications of phenylphthalimide-related structures as glucose mimics are in progress.

Acknowledgments

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24. PP60 (**15**): Mp. 65.3–66.3 °C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.95–7.98 (2H, m), 7.78–7.83 (2H, m), 7.41 (1H, dt, J = 1.22, 7.63 Hz), 7.39 (1H, dd, J = 7.33, 2.44 Hz), 7.33 (1H, dt, J = 2.44, 7.33 Hz), 7.17 (1H, dd, J = 7.63, 1.22 Hz), 2.48 (2H, t, J = 7.94 Hz), 1.49–1.54 (2H, m), 1.23–1.27 (6H, m), 0.77 (3 H, t, J = 6.78 Hz). Anal. Calcd for $C_{20}H_{21}NO_2$: C, 78.15; H, 6.89; N, 4.56. Found: C, 77.95; H, 6.98; N, 4.65.
25. PP2P (**16**): Mp. 90.8–91.2 °C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.94–7.98 (2H, m), 7.78–7.82 (2H, m), 7.40 (1H, dt, J = 1.52, 7.48 Hz), 7.33–7.37 (2H, m), 7.16–7.22 (3H, m), 7.10–7.15 (1H, m), 7.04–7.08 (2H, m), 2.78–2.89 (4 H, m). Anal. Calcd for $C_{22}H_{17}NO_2$: C, 80.71; H, 5.23; N, 4.28. Found: C, 80.85; H, 5.36; N, 4.20.