

HETEROCYCLES, Vol. 83, No. 9, 2011, pp. 2137 - 2147. © The Japan Institute of Heterocyclic Chemistry
Received, 20th June, 2011, Accepted, 8th July, 2011, Published online, 20th July, 2011
DOI: 10.3987/COM-11-12287

ESTROGEN RECEPTOR α/β LIGANDS DERIVED FROM THALIDOMIDE

Tomomi Noguchi-Yachide,* Kazuyuki Sugita, and Yuichi Hashimoto

Institute of Molecular & Cellular Biosciences, The University of Tokyo, 1-1-1
Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

E-mail : noguchi@iam.u-tokyo.ac.jp

Abstract – We have developed a series of thalidomide-derived phthalimide-type estrogen receptor (ER) modulators with characteristic subtype (ER α /ER β) selectivity, and investigated their structure-activity relationship for ER agonistic and antagonistic activities.

INTRODUCTION

Estrogen receptors (ER α and ER β) are members of the nuclear receptor superfamily (a family of ligand-dependent transcription factors that modulate expression of specific genes and thereby influence diverse biological processes, including cell growth, differentiation and metabolism).^{1,2} The major endogenous ligands of ERs are estrogens, such as estrone (E1), 17 β -estradiol (E2) and estriol (E3), and several synthetic ER modulators, including tamoxifen and raloxifene (**6**) (**Figure 1**), have been reported.¹⁻³ Estrogens play a central role in female reproductive function and proliferation of uterine and vaginal epithelium cells through binding to the ERs, which function as ER α or ER β homodimers or ER α/β heterodimer.^{1,2,4} ERs exist not only in normal cells, including uterine and ovarian cells, but also in a number of cancer cells, such as breast cancer cells and uterine cancer cells, and cancer growth is supported through the ERs.^{1,5,6} Furthermore, estrogens regulate expression of their target genes and have important roles in a variety of physiological and pathological states in both males and females, including glucose homeostasis, insulin resistance, regulation of the circulating levels of cholesterol and lipids, and regulation of bone density, in addition to their role in the female reproductive system.⁷⁻⁹

We have been engaged in structural development studies of thalidomide (**1**) (**Figure 1**). Thalidomide (**1**) is a sedative/hypnotic agent, which was withdrawn from the market because of its serious teratogenicity, but has subsequently shown potential for the treatment of a range of diseases, including cancers, diabetes, and rheumatoid arthritis.¹⁰⁻¹² Tumor necrosis factor alpha (TNF- α) production-inhibitory activity was

initially considered to be one of the key mechanisms of thalidomide's actions, but thalidomide (**1**) has also been discovered to have various other biological activities, including anti-inflammatory, anti-angiogenic, and cyclooxygenase (COX)-inhibitory activities.¹⁰⁻¹⁴ However, these activities are thought to be linked only partially to the pharmacological effects elicited by thalidomide (**1**). Although the precise molecular mechanisms of its actions remain unclear, current research suggests that thalidomide (**1**) acts on many target molecules and is thus a multi-target agent. Thalidomide (**1**) is easily metabolized *in vivo*, and various metabolites of thalidomide (**1**), such as compounds **2-4**, have been reported.¹⁵⁻²² Not only thalidomide itself, but also its metabolites, play an important role in the pharmacological effects of thalidomide (**1**).

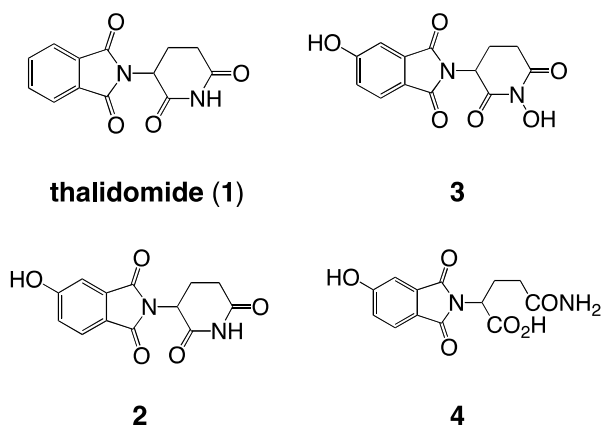


Figure 1. Structures of thalidomide and its metabolites

The multi-target nature of thalidomide (**1**) suggested that superior lead compounds for various target molecules could be created by structural development of thalidomide (**1**). The structural resemblance between synthetic ER ligands, including diethylstilbestrol (**10**) and raloxifene (**11**) (**Figure 2**), and thalidomide metabolites **2** and **3** (**Figure 1**) led us to speculate that (i) the anti-carcinogenic activity of thalidomide (**1**) might be related to functions of ERs, and/or (ii) novel ER ligands might be created by structural development of thalidomide (**1**). Therefore, we performed structure-activity relationship studies of thalidomide derivatives for ER α and ER β agonistic and antagonistic activities.

RESULTS

We investigated the effects of our compounds on ERs using a reporter gene assay method with CMX-GAL4N-hER as the recombinant receptor gene, TK-MH100x4-LUC as the reporter gene, and the CMX β -galactosidase gene for normalization, as previously reported.²³⁻²⁵ As shown in **Table 1**, agonistic activity of test compounds was expressed as EC₅₀ values, which were estimated from the sigmoidal

dose-response curves using R software. The results of ER-antagonistic activity assay in the presence of 0.3 nM E2 are shown in **Table 2**. Antagonistic activity of test compounds was expressed as IC₅₀ values, which were estimated from the sigmoidal dose-response curves using R software. “N.A.” means no activity at 30 μM in **Tables 1** and **2** and “>30” means that the compound does not achieve 50% inhibition at 30 μM in **Table 2**.

First, we evaluated the ER α and ER β agonistic and antagonistic activities of thalidomide (**1**), its major metabolites (**2-4**) (**Figure 1**), and some simple phthalimide derivatives, **5-9** (**Figure 2**). The derivatives **5**, **6**, **8** and **9** were designed to possess a halogen atom or a hydroxyl group at the position corresponding roughly in the molecular shape to one of the phenolic hydroxyl groups of diethylstilbestrol (**10**) (a synthetic nonsteroidal estrogen, **Figure 2**) or raloxifene (**11**) [a selective estrogen modulator (SERM),^{1,2} **Figure 2**]. However, compounds **1-9** showed neither agonistic nor antagonistic activity towards ER α and ER β . These results, together with our previous finding that the *N*-phenylphthalimide skeleton is a superior template for various biologically active compounds,¹⁰⁻¹⁴ prompted us to convert the glutalimide group of thalidomide to a substituted or non-substituted *N*-phenyl group.

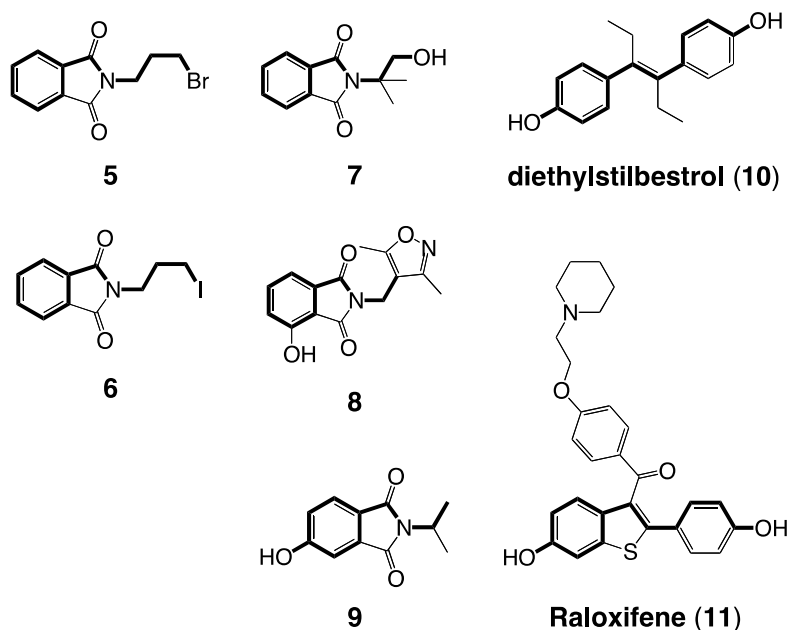


Figure 2. Thalidomide derivatives lacking the *N*-phenyl group in relation to diethylstilbestrol (**10**) and raloxifen (**11**)

Structure-activity relationship of *N*-phenylphthalimide derivatives: Agonistic activity

PP-00 (**12**), which has no substituents in the *N*-phenyl group, did not show agonistic activity toward either ER α or ER β . Introduction of a hydroxyl group into the phthaloyl group at the 5-position, *i.e.*,

5HPP-00 (**21**), resulted in the appearance of moderate agonistic activity toward both ER α and ER β with EC₅₀ values of 1.30 μ M and 2.74 μ M, respectively. 4HPP-00 (**17**), which is a regio-isomer of **21**, did not show agonistic activities, suggesting the importance of the *meta*-hydroxyl group for the activity. We then investigated the effects of a hydroxyl group introduced into the *N*-phenyl group of PP-00 (**12**). PP-H (**13**), PP-0H (**15**) and PP-00H (**16**), which possess a hydroxyl group at the 2'-, 3'- or 4'-position, respectively, did not show agonistic activity toward either ER α or ER β . Among the hydroxylated *N*-phenylphthalimide derivatives so far mentioned (**13**, **15**, **16**, **17**, **21**), only 5HPP-00 (**21**) showed ER agonistic activity. Next, we converted the hydroxyl group of 5HPP-00 (**21**) to an amino or a nitro group, *i.e.*, 5APP-00 (**25**) and 5NPP-00 (**26**), respectively. Both 5APP-00 (**25**) and 5NPP-00 (**26**) showed agonistic activity toward ER α and ER β . The corresponding regio-isomers, *i.e.*, 4APP-00 (**19**) and 4NPP-00 (**20**), respectively, were inactive, as was the case for the hydroxyl compounds 5HPP-00 (**21**: active)/4HPP-00 (**17**: inactive).

Then, we investigated the effects of substituents introduced at the 4'-position of the *N*-phenyl group. The agonistic activity of compounds with a 4'-substituent increased in the order of: 5HPP-0CF (**23**) < 5HPP-0I (**24**) < 5HPP-0F (**22**) < 5HPP-00 (**21**), for both ER α and ER β . 4HPP-0N0CF (**18**), which possesses a nitro group at the 4'-position, did not show agonistic activity. Thus, introduction of a substituent at the 4'-position seems to reduce the activity.

Generally speaking, molecular planarity is one of critical factors affecting the activity of nuclear receptor ligands. Therefore, introduction of two alkyl groups at the 2'- and 6'-positions, which will change the torsion angle between the two aromatic groups of *N*-phenylphthalimide derivatives, was investigated. Among the prepared *N*-2',6'-dialkylphenylphthalimide derivatives [PP-00 (**12**), PP-11 (**27**), PP-22 (**31**), PP-33 (**35**)], only PP-11 (**27**) showed agonistic activity toward both ER α and ER β with EC₅₀ values of 2.77 μ M and 12.1 μ M, respectively. For 5HPP-00 derivatives, the agonistic activity increased in the order of: 5HPP-22 (**33**) ~ 5HPP-33 (**36**) << 5HPP-00 (**12**) < 5HPP-11 (**28**), for both ER α and ER β . The 2',6'-dimethylphenyl analog 5HPP-11 (**28**) showed the most potent activity among this series of the compounds, as was the case in the PP-series. 5HPP-11 (**28**) showed the most potent agonistic activities among our thalidomide-derived test compounds, with EC₅₀ values of 0.0577 μ M and 0.768 μ M for ER α and ER β , respectively.

We next investigated the effects of 2',6'-dialkyl substitution of derivatives possessing a substituent other than a hydroxyl group at the 5-position, *i.e.*, the 5APP series (5-amino derivatives) and 5NPP series (5-nitro derivatives). In the 5APP series, the ER α / β -agonistic activity increased in the order of: 5APP-33 (**40**) << 5APP-00 (**25**) << 5APP-11 (**29**) < 5APP-22 (**33**). In the 5NPP series, the activity increased in the order of: 5NPP-00 (**26**) < 5NPP-33 (**41**) << 5NPP-11 (**30**) < 5NPP-22 (**34**), for both ER α and ER β . The 2',6'-diethyl derivatives [5APP-22 (**33**) and 5NPP-22 (**34**)] showed the most potent ER α / β -agonistic activities among the 5-amino- or 5-nitro-substituted derivatives, while the 2',6'-dimethyl derivative was

the most potent among the 5-hydroxy-substituted derivatives. 5APP-22 (**33**) and 5NPP-22 (**34**) showed ER α / β -agonistic activity with EC₅₀ values of 0.0820 μ M and 0.705 μ M for ER α , respectively, and 0.397 μ M and 3.20 μ M for ER β , respectively. The results suggest that a 5-substituted *N*-2',6'-dimethylphenyl- or 5-substituted *N*-2',6'-diethylphenylphthalimide skeleton is a structural requirement for potent ER α / β -agonistic activity, with a tendency for ER α -selectivity (**Fig. 3**). Interestingly, 5NPP-33 (**41**) was found to be a selective ER α agonist and its EC₅₀ value was estimated to be 3.95 μ M for ER α under our experimental conditions. A regio-isomer of 5APP-11, *i.e.*, 5APP-0101 (**51**), with 3',5'-dimethyl substituents, possesses quite weak (if any) ER α agonistic activity and moderate ER β agonistic activity (EC₅₀ is 3.20 μ M for ER β), so that it is a selective ER β agonist. 5NPP-33 (**41**) possesses ER α agonistic activity, while its 1-decarbonylated analogue, 5NIDO-33 (**49**), is inactive. The 1,3-decarbonylated analogues (**43-49**, **52-55**) tested in our experiments were all inactive. These results suggest an important role of the 1,3-dicarbonyl moiety on the phthalimide group in ER α / β -agonistic activity.

Structure-activity relationship of *N*-phenylphthalimide derivatives: Antagonistic activity

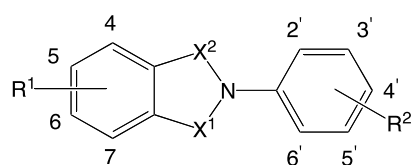
As shown in Table 2, PP-00 (**12**), *N*-hydroxyphenyl phthalimide derivatives [PP-H (**13**), FPP-H (**14**), PP-0H (**15**) and PP-00H (**16**)] and 4-substituted-phenyl phthalimide derivatives [4HPP-00 (**17**), 4HPP-H0N0CF (**18**), 4APP-00 (**19**) and 4NPP-00 (**20**)] showed no or very weak ER α / β -antagonistic activity. Among the 5-substituted phenylphthalimide derivatives [5HPP-00 (**21**), 5HPP-0F (**22**), 5HPP-0CF (**23**), 5HPP-0I (**24**), 5APP-00 (**25**) and 5NPP-00 (**26**)], only 5HPP-0F (**22**) showed moderately selective ER α antagonistic activity.

Among the *N*-2',6'-dialkylphenylphthalimide derivatives [PP-00 (**12**), PP-11 (**27**), PP-22 (**31**), PP-33 (**35**)], only PP-33 (**35**) showed ER β antagonistic activities, with an IC₅₀ value of 13.0 μ M under our experimental conditions. All the *N*-3',5'-dialkylphenylphthalimide analogues investigated in our experiments [4APP-0101 (**50**), 5APP-0101 (**51**), 5AIDO-0101 (**52**), 6AIDO-0101 (**53**), 7AIDO-0101 (**54**) and 5AIDOL-0101 (**55**)] were inactive. These results suggest that 2',6'-disubstitution of the *N*-phenyl group is a structural requirement for both ER α - and ER β -antagonistic activity, as was the case for ER α / β -agonistic activity.

The ER α antagonistic activity of 5-hydroxy-2',6'-dialkylphenylphthalimide derivatives [5HPP-11 (**28**), 5HPP-22 (**32**) and 5HPP-33 (**36**)] increased in the order of 5HPP-11 (dimethyl: **28**) < 5HPP-22 (diethyl: **32**) < 5HPP-33 (di-isopropyl: **36**). In the case of 5-amino-2', 6'-dialkylphenylphthalimide derivatives [5APP-11 (**29**), 5APP-22 (**33**) and 5APP-33 (**40**)], the order of antagonistic potency toward ER β showed the same tendency as in the case of the 5HPP series of compounds, *i.e.*, 5APP-11 (dimethyl: **29**) < 5APP-22 (diethyl: **33**) < 5APP-33 (di-isopropyl: **40**). Interestingly, the order was just reversed in the case

of antagonistic activity toward ER α , *i.e.*, 5APP-33 (di-isopropyl: **40**) < 5APP-22 (diethyl: **33**) < 5APP-11 (dimethyl: **29**). These results suggest that (i) introduction of two small alkyl groups (that is, methyl groups) at the 2'- and 6'-positions of the *N*-phenyl group is favorable for ER α -antagonistic activity, while (ii) introduction of larger alkyl groups (isopropyl groups) at the corresponding positions is favorable for ER β -antagonistic activity (**Figure 3**). Among 5-nitro-2',6'-dialkylphenylphthalimide derivatives [5NPP-11 (**30**), 5NPP-22 (**34**) and 5NPP-33 (**41**)], only 5NPP-33 (**41**) showed ER α / β -antagonistic activity, though it was weak. Possibly the introduction of an electron-donating group at the 5-position is critical for potent ER α / β -antagonistic activity (**Figure 3**). Overall, 5HPP-33 (**36**) is the most potent ER α / β dual antagonist among our test compounds, and its IC₅₀ values are 0.772 μ M for ER α and 0.630 μ M for ER β . Further, 5HPP-11 (**28**) and 5APP-33 (**40**) were ER α -selective and ER β -selective antagonists, respectively, as shown in Table 2. The IC₅₀ values are 0.915 μ M for 5HPP-11 (**28**) toward ER α (at least more than 33-fold selectivity over ER β) and 1.53 μ M for 5APP-33 (**40**) toward ER β .

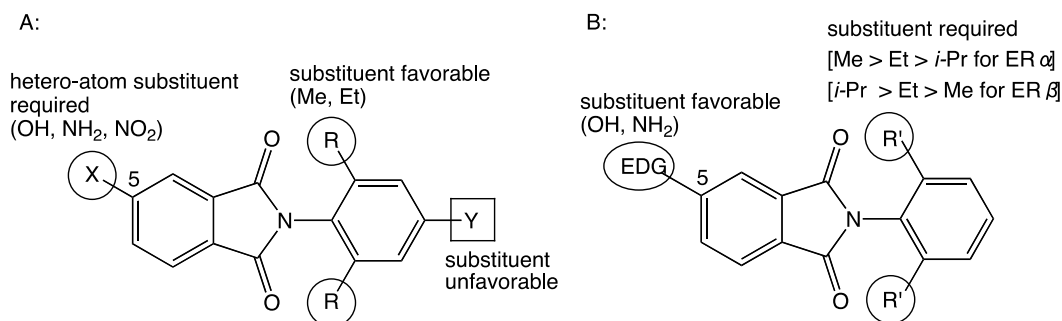
Table 1. Agonistic activity of phenylphthalimide derivatives



Compound	X ¹ , X ²	R ¹	R ²	EC ₅₀ (μ M)		Compound	X ¹	X ²	R ¹	R ²	EC ₅₀ (μ M)	
				ER α	ER β						ER α	ER β
PP-00 (12)	- CO -	H	H	N.A.	N.A.	PP-33 (35)			H		N.A.	N.A.
PP-H (13)		H	2'-OH	N.A.	N.A.	5HPP-33 (36)			5-OH		N.A.	N.A.
FPP-H (14)		4,5,6,7-tetraF	2'-OH	N.A.	N.A.	5OMePP-33 (37)			5-OMe		N.A.	N.A.
PP-0H (15)	- CO -	H	3'-OH	N.A.	N.A.	5OMe4,7DHPP-33 (38)	- CO -	- CO -	5-OMe-4,7-(OH) ₂	2',6'- <i>i</i> -Pr	N.A.	N.A.
PP-00H (16)		H	4'-OH	N.A.	N.A.	4,7DHPP-33 (39)			4,7-(OH) ₂		N.A.	N.A.
4HPP-00 (17)		4-OH	H	N.A.	N.A.	5APP-33 (40)			5-NH ₂		N.A.	N.A.
4HPP-0NO ₂ CF (18)		4-OH	4'-NO ₂ -5'-CF ₃	N.A.	N.A.	5NPP-33 (41)			5-NO ₂		3.95	N.A.
4APP-00 (19)	- CO -	4-NH ₂	H	N.A.	N.A.	FPPSS-33 (42)	- CS -	- CS -	4,5,6,7-tetraF	2',6'- <i>i</i> -Pr	N.A.	N.A.
4NPP-00 (20)		4-NO ₂	H	N.A.	N.A.	5HIDO-33 (43)		- CO -	5-OH		N.A.	N.A.
5HPP-00 (21)		5-OH	H	1.30	2.74	6HIDO-33 (44)		- CO -	6-OH		N.A.	N.A.
5HPP-0F (22)		5-OH	4'-F	N.A.	N.A.	5HIDOS-33 (45)		- CS -	5-OH		N.A.	N.A.
5HPP-0CF (23)	- CO -	5-OH	4'-CF ₃	3.84	2.90	6HIDOS-33 (46)	- CH ₂ -	- CS -	6-OH	2',6'- <i>i</i> -Pr	N.A.	N.A.
5HPP-0I (24)		5-OH	4'- <i>i</i> -Pr	4.12	11.9	5AIDO-33 (47)		- CO -	5-NH ₂		N.A.	N.A.
5APP-00 (25)		5-NH ₂	H	18.9	12.9	6AIDO-33 (48)		- CO -	6-NH ₂		N.A.	N.A.
5NPP-00 (26)		5-NO ₂	H	N.A.	N.A.	5NIDO-33 (49)		- CO -	5-NO ₂		N.A.	N.A.
PP-11 (27)		H		2.77	12.1	4APP-0101 (50)	- CO -	- CO -	4-NH ₂		N.A.	N.A.
5HPP-11 (28)		5-OH		0.0577	0.768	5APP-0101 (51)	- CO -	- CO -	5-NH ₂		>30	3.20
5APP-11 (29)	- CO -	5-NH ₂	2',6'-Me	0.139	1.60	5AIDO-0101 (52)	- CH ₂ -	- CO -	5-NH ₂	3',5'- <i>i</i> -Pr	N.A.	N.A.
5NPP-11 (30)		5-NO ₂		1.50	8.32	6AIDO-0101 (53)	- CH ₂ -	- CO -	6-NH ₂		N.A.	N.A.
PP-22 (31)		H		N.A.	N.A.	7AIDO-0101 (454)	- CH ₂ -	- CO -	7-NH ₂		N.A.	N.A.
5HPP-22 (32)		5-OH		N.A.	N.A.	5AIDOL-0101(55)	- CH ₂ -	- CH ₂ -	5-NH ₂		N.A.	N.A.
5APP-22 (33)	- CO -	5-NH ₂	2',6'-Et	0.0820	0.397							
5NPP-22 (34)		5-NO ₂		0.705	3.20							

Table 2. Antagonistic activity of phenylphthalimide derivatives

Compound	χ^1, χ^2	R ¹	R ²	IC ₅₀ (μ M)		Compound	χ^1	χ^2	R ¹	R ²	IC ₅₀ (μ M)	
				ER α	ER β						ER α	ER β
PP-00 (12)	- CO -	H	H	>30	>30	PP-33 (35)			H		>30	13.0
PP-H (13)		H	2'-OH	N.A.	N.A.	5HPP-33 (36)			5-OH		0.772	0.630
FPP-H (14)		4,5,6,7-tetraF	2'-OH	N.A.	N.A.	5OMePP-33 (37)			5-OMe		>30	>30
PP-0H (15)	- CO -	H	3'-OH	N.A.	N.A.	5OMe4,7DHPP-33 (38)	- CO -	- CO -	5-OMe-4,7-(OH) ₂	2',6'- <i>i</i> -Pr	N.A.	N.A.
PP-00H (16)		H	4'-OH	N.A.	N.A.	4,7DHPP-33 (39)			4,7-(OH) ₂		N.A.	N.A.
4HPP-00 (17)		4-OH	H	N.A.	N.A.	5APP-33 (40)			5-NH ₂		>30	1.53
4HPP-0N0CF (18)		4-OH	4'-NO ₂ -5'-CF ₃	>30	>30	5NPP-33 (41)			5-NO ₂		6.09	6.98
4APP-00 (19)	- CO -	4-NH ₂	H	>30	N.A.	FPPSS-33 (42)	- CS -	- CS -	4,5,6,7-tetraF	2',6'- <i>i</i> -Pr	N.A.	N.A.
4NPP-00 (20)		4-NO ₂	H	N.A.	N.A.	5HIDO-33 (43)		- CO -	5-OH		>30	2.02
5HPP-00 (21)		5-OH	H	>30	>30	6HIDO-33 (44)		- CO -	6-OH		6.12	5.90
5HPP-0F (22)		5-OH	4'-F	2.65	>30	5HIDOS-33 (45)		- CS -	5-OH		5.86	7.72
5HPP-0CF (23)		5-OH	4'-CF ₃	N.A.	N.A.	6HIDOS-33 (46)	- CH ₂ -	- CS -	6-OH	2',6'- <i>i</i> -Pr	3.09	0.917
5HPP-0I (24)	- CO -	5-OH	4'- <i>i</i> -Pr	>30	>30	5AIDO-33 (47)		- CO -	5-NH ₂		N.A.	N.A.
5APP-00 (25)		5-NH ₂	H	N.A.	N.A.	6AIDO-33 (48)		- CO -	6-NH ₂		N.A.	N.A.
5NPP-00 (26)		5-NO ₂	H	N.A.	N.A.	5NIDO-33 (49)		- CO -	5-NO ₂		N.A.	N.A.
PP-11 (27)		H		>30	>30	4APP-0101 (50)	- CO -	- CO -	4-NH ₂		N.A.	N.A.
5HPP-11 (28)		5-OH		0.915	>30	5APP-0101 (51)	- CO -	- CO -	5-NH ₂		N.A.	N.A.
5APP-11 (29)	- CO -	5-NH ₂	2',6'-Me	1.67	>30	5AIDO-0101 (52)	- CH ₂ -	- CO -	5-NH ₂	3',5'- <i>i</i> -Pr	N.A.	N.A.
5NPP-11 (30)		5-NO ₂		>30	>30	6AIDO-0101 (53)	- CH ₂ -	- CO -	6-NH ₂		N.A.	N.A.
PP-22 (31)		H		>30	>30	7AIDO-0101 (454)	- CH ₂ -	- CO -	7-NH ₂		N.A.	N.A.
5HPP-22 (32)		5-OH		1.39	0.889	5AIDOL-0101(55)	- CH ₂ -	- CH ₂ -	5-NH ₂		N.A.	N.A.
5APP-22 (33)	- CO -	5-NH ₂	2',6'-Et	1.72	4.41							
5NPP-22 (34)		5-NO ₂		>30	>30							

**Figure 3.** Structural requirements for ER-modulating activity (A: for agonistic activity, B: for antagonistic activity) EDG: electron-donating group

CONCLUSION

Although thalidomide and its metabolites do not show ER-modulating activity, ER α /ER β agonists and antagonists were structurally developed based on the thalidomide-derived phthalimide skeleton. Their structure-activity relationship was examined. We discovered compounds with ER α - and ER β -selective activity, including 5NPP-33 (**41**) (an ER α selective agonist), 5APP-0101 (**51**) (an ER β selective agonist), and 5APP-33 (**40**) (an ER β selective antagonist). In addition, we discovered compounds with potent ER α /ER β dual agonist/antagonist activity, such as 5HPP-11 (**28**), 5APP-22 (**33**) and 5HPP-33 (**36**).

EXPERIMENTAL

Synthesis

The synthesis of compounds (**5-31**, **33**, **35-37**, **40-55**) has been reported in references 27-31.

General

2-(2,6-Diethylphenyl)-substituted isoindoline-1,3-dione (**32**, **34**)

A mixture of substituted phthalic anhydride and 2,6-diethylaniline was heated at 180 °C for 2 h. The reaction mixture was cooled to 0 °C, and n-hexane was added. The precipitated solid was collected by filtration, washed with cold n-hexane, and dried under reduced pressure. The structure were confirmed by NMR and mass spectroscopy, and gave appropriate analysis data.

2-(2,6-Diethylphenyl)-5-hydroxyisoindoline-1,3-dione (5HPP-22, **32**):

Mp 174.0-178.0 °C. FAB-MS m/z 296 (M+H)⁺; ¹H-NMR (500MHz, CDCl₃): δ 7.83 (d, J = 7.9 Hz, 1H), 7.37 (t, J = 7.9 Hz, 1H), 7.36 (d, J = 1.8 Hz, 1H), 7.23 (d, J = 7.9 Hz, 2H), 7.15 (dd, J = 7.9, 1.8 Hz, 1H), 6.17 (s, 1H), 2.46 (q, J = 7.3 Hz, 2H), 1.14 (t, J = 7.3 Hz, 3H), 3.38. Anal. Calcd for C₁₈H₁₇NO₃: C, 73.20; H, 5.80; N, 4.74. Found: C, 73.13; H, 5.91; N, 4.66.

2-(2,6-Diethylphenyl)-5-nitroisoindoline-1,3-dione (5NPP-22, **34**):

Mp 145.5-147.0 °C. FAB-MS m/z 325 (M+H)⁺; ¹H-NMR (500MHz, CDCl₃): δ 8.80 (d, J = 1.8 Hz, 1H), 8.70 (dd, J = 8.5, 1.8 Hz, 1H), 7.17 (d, J = 8.5 Hz, 1H), 7.43 (t, J = 7.9 Hz, 1H), 7.27 (d, J = 7.9 Hz, 2H), 2.44 (q, J = 7.3 Hz, 2H), 1.14 (t, J = 7.3 Hz, 3H). HRMS (FAB) Calcd for C₁₈H₁₇N₂O₄: 325.1188. Found: 325.1226 (M+H)⁺.

2-(2,6-Diisopropylphenyl)-4,7-dihydroxy-5-substituted isoindoline-1,3-dione (**38**, **39**)³²

To a prepared THF solution of (TMP)₂Cu(CN)Li₂ was added 5-substituted PP-33 at -78 °C under Ar. The reaction mixture was stirred for 3 hat 0 °C, then ^tBuOOLi was added at -78 °C and the mixture was stirred at -78 °C for 16 h. It was then poured into saturated aqueous NH₄Cl and NaHS₂O₃, and extracted with AcOEt. The AcOEt layer was dried over Na₂SO₄, and the solvent was removed by evaporation in vacuo. Purification by silica gel flash chromatography gave 5OMe₄,7DHPP-33 (**38**) and 4,7DHPP-33 (**39**), which were recrystallized from AcOEt/n-hexane to give yellow solids. Each structure were confirmed by NMR and mass spectroscopy, and gave appropriate analysis data (TMP: 2,2,6,6-tetramethylpiperidino).

2-(2,6-Diisopropylphenyl)-4,7-dihydroxy-5-methoxyisoindoline-1,3-dione (5OMe₄,7DHPP-33, **38**):

Mp 136.5-138.0 °C. FAB-MS m/z 339 (M⁺); ¹H-NMR (400MHz, CDCl₃): δ 7.47 (d, J = 8.0 Hz, 1H), 7.28

(m, 4H), 2.73 (sept, $J = 6.8$ Hz, 2H), 1.18 (d, $J = 6.8$ Hz, 12H). HRMS (FAB) Calcd for $C_{20}H_{21}NO_4$: 339.1471. Found: 339.1450.

2-(2,6-Diisopropylphenyl)-4,7-dihydroxyisoindoline-1,3-dione (4,7DHPP-33, **39**):

Mp 162.0-163.5 °C. FAB-MS m/z 369 (M^+); 1H -NMR (400MHz, $CDCl_3$): δ 7.45 (t, $J = 7.8$ Hz, 1H), 7.28 (d, $J = 8.1$ Hz, 2H), 6.68 (s, 1H), 3.92 (s, 3H), 2.74 (sept, $J = 6.6$ Hz, 2H), 1.17 (d, $J = 6.6$ Hz, 12H). HRMS (FAB) Calcd for $C_{21}H_{23}NO_5$: 369.1576. Found: 369.1551; Anal. Calcd for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.69. Found: C, 67.33; H, 6.77; N, 3.35.

Reporter Gene Assay²³⁻²⁵

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (phenol red free) containing 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfections of CMX-GAL4N-hER α , CMX-GAL4N-hER β and CMX- β -GAL into HEK 293 cells were performed by the calcium phosphate coprecipitation method. Test compounds, with or without 17 β -estradiol, were added 7 hours after the transfection. After overnight incubation, luciferin was added and luminescence was measured on an Wallac ARVOTM-SX (Perkin Elmer) microplate reader. Then, β -galactosidase was added and the absorbance was measured on the microplate reader with emission detection at 405 nm. Reporter gene assay was performed in triplicate (n=3).

ACKNOWLEDGEMENTS

The work described in this paper was partially supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Society for the Promotion of Science.

REFERENCES

1. V. C. Jordan, *J. Med. Chem.*, 2003, **46**, 883.
2. V. C. Jordan, *J. Med. Chem.*, 2003, **46**, 1081.
3. G. G. J. M. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, and J. Gustafsson, *Endocrinology*, 1997, **138**, 863.
4. X. Li, J. Huang, P. Yi, R. A. Bambara, R. Hilf, and M. Mutan, *Mol. Cell. Biol.*, 1999, **19**, 1919.
5. S. Green, P. Walter, V. Kumar, A. Krust, J. Bornert, P. Argos, and P. Chambon, *Nature*, 1986, **320**, 134.
6. G. G. J. M. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, and J. Gustafsson, *Pro. Nat. Acad. Sci. USA*, 1996, **93**, 5925.

7. P. Chambon, [Mol. Endocrinol.](#), 2005, **19**, 1418.
8. S. Nilsson, S. Makela, E. Treuter, M. Tujague, J. Thamsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, and J. Gustafsson, *Physiol. Rev.*, 2001, **81**, 1535.
9. R. P. A. Barros, U. F. Machado, and J. Gustafsson, [Trends. Mol. Med.](#), 2006, **12**, 425.
10. Y. Hashimoto, *Curr. Med. Chem.*, 1998, **5**, 163.
11. Y. Hashimoto, [Bioorg. Med. Chem.](#), 2002, **10**, 461.
12. Y. Hashimoto, A. Tanatani, K. Nagasawa, and H. Miyachi, [Drugs Future](#), 2004, **29**, 383.
13. J. B. Bartlett, K. Dredge, and A. G. Dalglish, [Nat. Rev. Cancer](#), 2004, **4**, 314.
14. J. B. Bartlett, A. Tozer, D. Stirling, and J. B. Zeldis, [British J. Cancer](#), 2005, **93**, 613.
15. J. Lu, N. Hesby, B. D. Palmer, M. Tingle, B. C. Baguley, P. Ketstell, and L.-M. Ching, [J. Pharm. Exp. Ther.](#), 2004, **310**, 571.
16. J. Lu, B. D. Palmer, P. Kestell, P. Browett, B. C. Baguley, G. Muller, and L.-M. Ching, *Clin. Cancer Res.*, 2003, **9**, 1680.
17. M. G. Marks, J. Shi, M. O. Fry, Z. Xiao, M. Trzyna, V. Pokala, M. A. Ihnat, and P.-K. Li, [Biol. Pharm. Bull.](#), 2002, **25**, 597.
18. S. Robin, J. Zhu, H. Galons, C. Pham-Huy, J. R. Vlaude, A. Tomas, and B. Viossat, [Tetrahedron: Assymetry](#), 1995, **6**, 1249.
19. U. Teubert, K. Zwingenberger, S. Wnendt, and K. Eger, [Arch. Pharm.](#), 1998, **331**, 7.
20. F. A. Luzzio, D. Y. Duveau, E. R. Lepper, and W. D. Figg, [J. Org. Chem.](#), 2005, **70**, 10117.
21. M. Doi, Y. Ijiri, M. Akagi, M. Uenishi, and H. Urata, *Anal. Sci.*, 2003, **19**, x51.
22. M. Meyring, J. Muehlbacher, K. Messer, N. Kastner-Pustet, G. Bringmann, A. Mannschreck, and G. Blaschke, [Anal. Chem.](#), 2002, **74**, 3726.
23. M. Makishima, T. T. Lu, W. Xie, G. K. Whitfield, H. Domoto, R. M. Evans, M. R. Haussler, and D. J. Mangelsdorf, [Science](#), 2002, **296**, 1313.
24. M. Makishima, A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan, [Science](#), 1999, **284**, 1362.
25. J. Kasuga, M. Makishima, Y. Hashimoto, and H. Miyachi, [Bioorg. Med. Chem. Lett.](#), 2006, **16**, 554.
26. A. M. Brzozowski, A. C. W. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engström, L. Öhman, G. L. Greene, J. Gustafsson, and M. Carlquist, [Nature](#), 1997, **389**, 753.
27. Y. Shibata, K. Sasaki, Y. Hashimoto, and S. Iwasaki, [Biochem. Biophys. Res. Commun.](#), 1994, **205**, 1992.
28. Y. Shibata, K. Sasaki, Y. Hashimoto, and S. Iwasaki, *Chem. Pharm. Bull.*, 1996, **44**, 156.
29. M. Tetsuhashi, M. Ishikawa, M. Hashimoto, Y. Hashimoto, and H. Aoyama, [Bioorg. Med. Chem.](#), 2010, **18**, 5323.

30. R. Shimazawa, H. Takayama, Y. Fujimoto, M. Komoda, K. Dodo, R. Yamasaki, R. Shirai, Y. Koiso, K. Miyata, F. Kato, M. Kato, H. Miyachi, and Y. Hashimoto, [*J. Enz. Inhib. Med. Chem.*, 1999, **14**, 259.](#)
31. M. Suizu, Y. Muroya, H. Kakuta, H. Kagechika, A. Tanatani, K. Nagasawa, and Y. Hashimoto, [*Chem. Pharm. Bull.*, 2003, **51**, 1098.](#)
32. S. Usui, Y. Hashimoto, J. V. Morey, A. E. H. Wheatley, and M. Uchiyama, [*J. Am. Chem. Soc.*, 2007, **129**, 15102.](#)