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Diphenylamine-based retinoid antagonists: Regulation of RAR and RXR function depending on the N-substituent

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ABSTRACT

Based upon the structure–activity relationships of diphenylamine derivatives with retinoid synergistic activity (RXR agonists), novel diphenylamine derivatives with a long alkyl chain (**9a** and **9b**) or a benzyl group (**10a**–**f**) as the N-substituent were designed and synthesized. All the synthesized compounds dose-dependently inhibited HL-60 cell differentiation induced by 3.3×10^{-10} M Am80. Among them, compound **10f** showed the most potent inhibitory activity, and the mechanism was shown, by means of transactivation assay for RARs and RXRs, to involve antagonism against RARs. The N-substituent of the diphenylamine skeleton plays an important role in determining the receptor selectivity for RARs or RXRs, as well as the agonist or antagonist nature of the activity.

1. Introduction

Retinoids, that is, natural and synthetic analogues of all-*trans*-retinoic acid (ATRA, **1**), play an important role in cell differentiation, proliferation and embryonic development in vertebrates,[1] and are used as therapeutic agents in the fields of dermatology and oncology.[2] Their biological activities are mediated by binding to and activating two types of specific nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each having three subtypes, α, β, γ.[3] RARs and RXRs are ligand-inducible transcription factors and their endogenous ligands are **1** and 9-*cis*-retinoic acid (**2**), respectively (Fig. 1).[4] Major retinoidal activities are elicited by RAR–RXR heterodimers. RXR is a silent partner of RAR,[5] and the heterodimers can be activated by an RAR agonist such as TTNPB (**3**)[6] and Am80 (**4**, Fig. 2a),[7] but not by an RXR agonist. On the other hand, an RXR agonist, such as LGD1069 (**5**) or HX600 (**6**), acts as a retinoid synergist, since it dose-dependently increases the activity of a low concentration of RAR agonist (Fig. 1).[8,9] The synthetic compounds **3** and **5** should have high stability as a result of transformation of the unstable polyene structures of **1** and **2** into benzene rings. Modifications of retinoid structures with aromatic rings and the introduction of heteroatoms have afforded potent RAR-selective agonists with remarkable chemical stability and good bioavailability, such as Am80 (**4**).[10,11]

Therefore, we have developed various RAR and RXR ligands containing nitrogen atoms (Fig. 2).[11] A heterocyclic RXR agonist HX600 (**6**) was developed by mimicry of the bent structure of **2** with a benzodiazepine structure.[9] We also found potent RXR agonists DA024 (**7a**) and PA024 (**7b**) bearing a nitrogen atom as the linking group between two aromatic rings.[12,13] The diphenylamine skeleton has some advantages for drug discovery, because the linker nitrogen atom can be readily modified with various substituents. Among the synthesized diphenylamine derivatives, DA010 (**8**) showed dual RAR and RXR agonist activities. RAR agonist activity was remarkably diminished by the introduction of an *N*-alkyl substituent such as a cyclopropylmethyl group, which affected the potency of RXR agonist activity, as well as discriminating the two receptors in terms of affinity (Fig. 2b).

Figure 3 summarizes the structure–activity relationship for retinoid synergy of DA010 derivatives bearing an *N*-alkyl group in the presence of 1×10^{-10} M Am80.[12] As the *N*-alkyl group was made

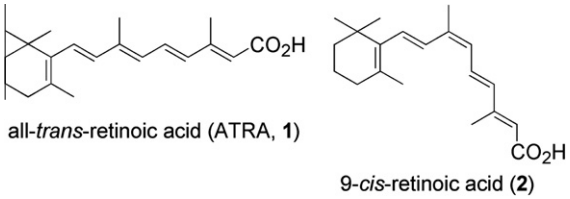

Figure 1. The structures of endogenous RAR and RXR ligands.

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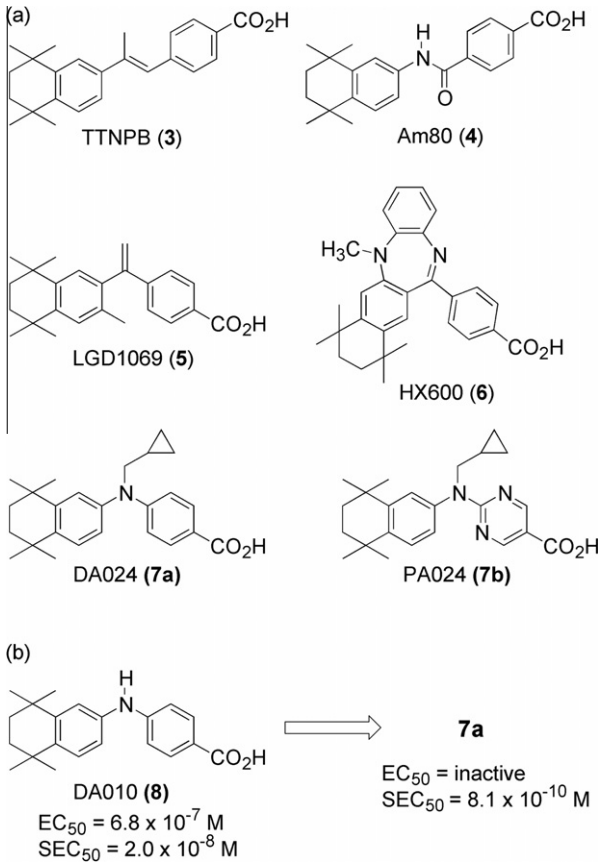


Figure 2. (a) The structures of synthetic agonists of RARs and RXRs. (b) The activity alterations caused by introduction of the *N*-cyclopropylmethyl substituent. EC_{50} means the concentration that induced differentiation of 50% of cells in the presence of the compound alone in HL-60 cell differentiation assay. SEC_{50} (synergistic effective concentration) means the concentration that induced differentiation of 50% of cells in the presence of both the compound and an RAR agonist (1×10^{-10} M Am80).[12]

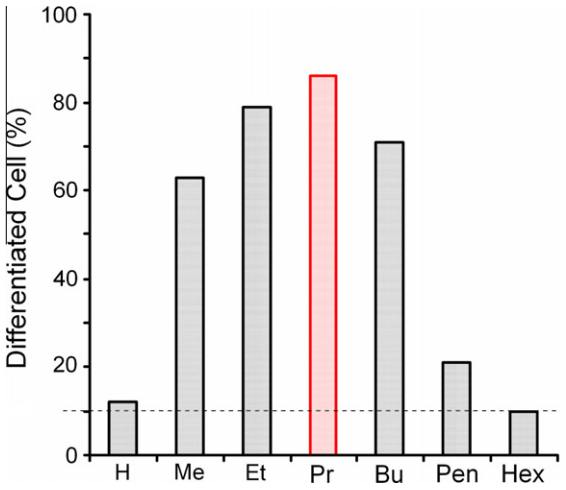


Figure 3. Structure–activity relationship for retinoid synergy of diphenylamine derivatives with various *N*-alkyl substituents. Each column indicates the activity of the test compound at the concentration of 1×10^{-8} M in the presence of 1×10^{-10} M Am80 in HL-60 cell differentiation assay. The dashed line shows the level of differentiated cells (%) induced by 1×10^{-10} M Am 80 alone. Abbreviations are Pr: *n*-propyl, Bu: *n*-butyl, Pen: *n*-pentyl, and Hex: *n*-hexyl group as the N-substituent.

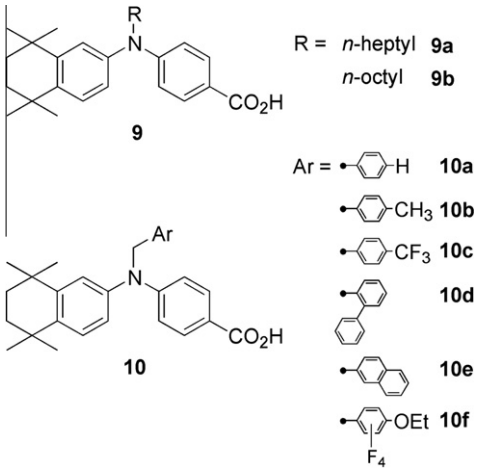


Figure 4. Diphenylamine derivatives **9** and **10** with longer or larger N-substituent, respectively.

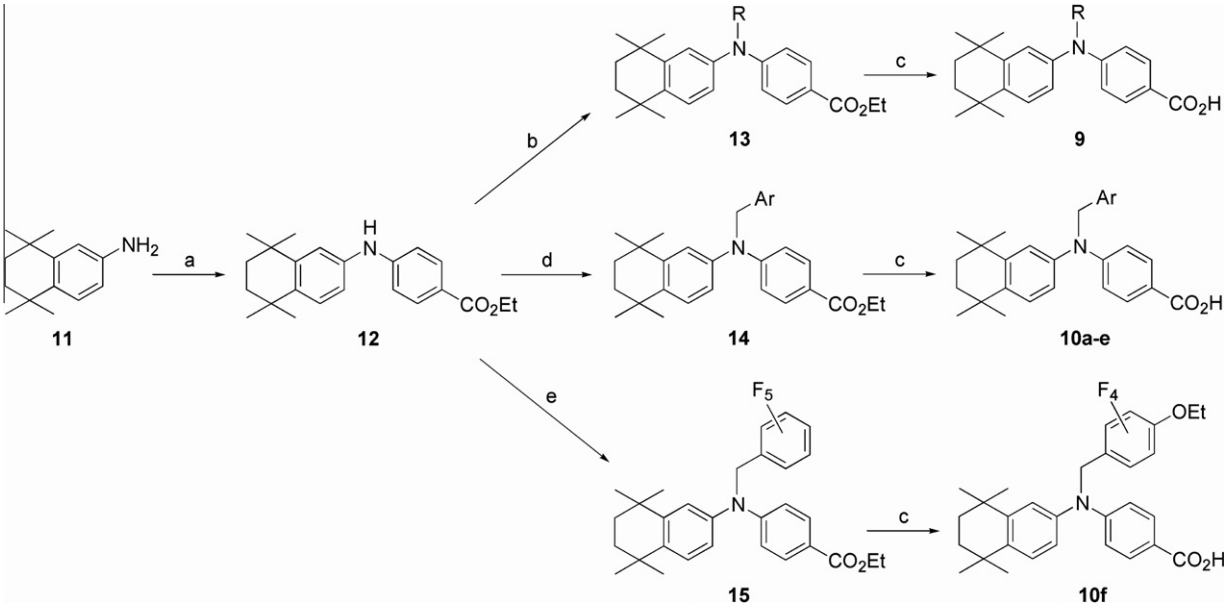
longer, the synergistic activity increased, and the *N*-*n*-propyl compound showed the most potent synergistic activity. Introduction of *N*-alkyl groups longer than *n*-propyl reduced the synergistic activity, and the activity was completely lost in the case of the *N*-*n*-hexyl compound. Based on the structure–activity relationships of RXR ligands, we focused on further modification of the N-substituent on the diphenylamine skeleton. Here, we describe the design, synthesis and biological activities of novel diphenylamine-based retinoid modulators bearing a long or large N-substituent.

2. Results

2.1. Chemistry

Our previous studies showed that introduction of a bulky alkyl group of moderate size (3 or 4 carbons) into the diphenylamine skeleton is effective for obtaining potent RXR agonist activity. In order to understand the effects of various N-substituents on the biological activities and also to obtain retinoid antagonists, we designed N-substituted diphenylamine derivatives (**9**) and (**10**) with various *n*-alkyl and benzyl groups as longer and larger N-substituents, respectively (Fig. 4). In terms of *N*-benzyl groups, we chose six substituents with quite different structures and electric properties to understand the steric and electric effects.

The divergent synthesis of the designed molecules **9** and **10** is summarized in Scheme 1. The key intermediate (**12**) with a diphenylamine skeleton was synthesized according to the reported procedure: Pd-catalyzed amination of aniline derivative (**11**) with ethyl 4-iodobenzoate.[13] Long alkyl chains, *n*-heptyl and *n*-octyl, were introduced on the nitrogen atom of **12** by reaction with the corresponding iodoalkane in the presence of NaH as a base. The ester group of N-alkylated compounds (**13**) was hydrolyzed with aqueous 20% KOH solution to give compounds **9**. Compound **12** was treated with various benzyl halides in the presence of NaH as a base to afford *N*-benzylated compounds (**14**), which were hydrolyzed with 20% KOH aqueous solution to afford the corresponding carboxylic acids **10a–e**. An *N*-pentafluorobenzyl derivative (**15**) was synthesized similarly from the reaction of **12** with the corresponding benzyl bromide. During the following hydrolysis of the ester group, nucleophilic substitution reaction on the pentafluorophenyl ring of **15** by an ethoxide anion produced from 20% KOH and EtOH also occurs to afford an *N*-4-ethoxytetrafluorobenzyl derivative **10f** in quantitative yield.[14] The structure of **10f** was identified by several analytical methods. Low-MS spectra showed a



Scheme 1. Divergent synthesis of diphenylamine derivatives **9** and **10**. Reagents and conditions: (a) $Pd_2(dba)_3$, *rac*-BINAP, NaO^{t}Bu, ethyl 4-iodobenzoate, toluene, 48% yield; (b) NaH, *n*-$C_7H_{15}I$ or *n*-$C_8H_{15}I$, DMF, 96%-quantitative yields; (c) 20% KOH aq EtOH, 90%-quantitative yields; (d) NaH, benzyl halides, DMF, 93%-quantitative yields; (e) NaH, pentafluorobenzyl bromide, DMF, 99% yield.

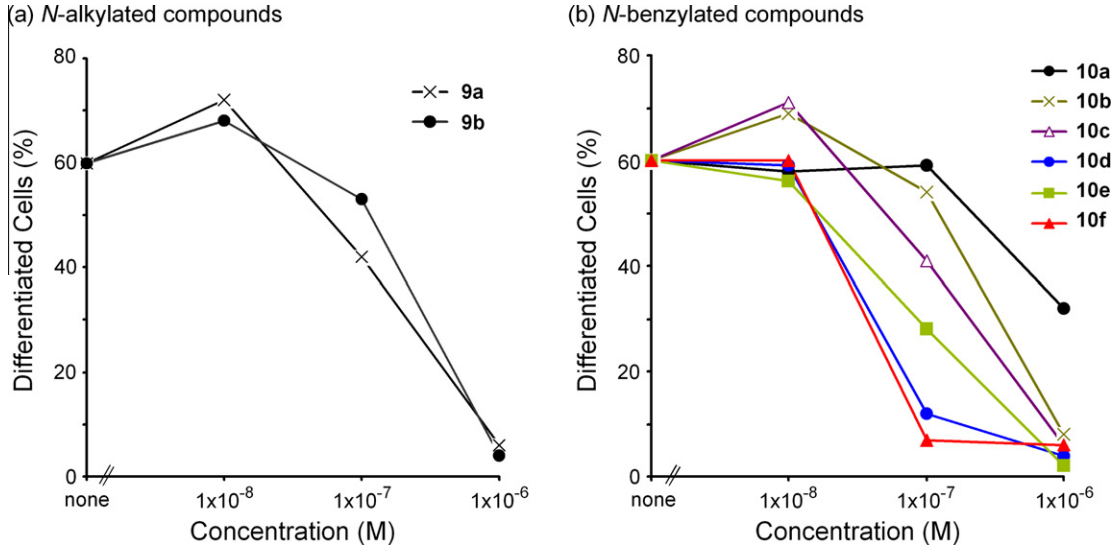


Figure 5. Inhibitory activity of (a) N-alkylated and (b) N-benzylated compounds toward HL-60 cell differentiation induced by 3.3×10^{-10} M Am80.

molecular ion peak at *m*/*z* 529 in high-resolution MS analysis, with the chemical composition $C_{30}H_{31}F_4NO_3$. ^{1}H NMR spectra indicated the presence of an ethoxy group and ^{19}F spectra showed two kinds of peaks of AA′XX′ split type. In the ^{13}C NMR spectra, we observed four kinds of split aromatic carbon peaks caused by coupling with fluorine atoms. Therefore, we identified the structure of compound **10f** as 4-[*N*-(4-ethoxy-2,3,5,6-tetrafluoro)benzyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid. The purity of all synthesized compounds was confirmed by elemental analyses.

2.2. Cell differentiation assay using HL-60

The biological activity of the synthesized compounds **9** and **10** was examined in terms of the ability to induce differentiation of human promyelocytic leukemia HL-60 cells.[11] Differentiated cells were identified by nitro blue tetrazolium (NBT) reduction assay.[15] Compounds **9** and **10** did not show differentiation-inducing activity alone, which means these compounds did not act as RAR agonists. Next, their inhibitory activity toward cell differentiation induced by the RAR agonist Am80 was examined. In the presence of 3.3×10^{-10} M Am80, which induced differentiation of about 60% of cells, compounds **9** and **10**, except for **10a**, dose-dependently inhibited cell differentiation, and the number of differentiated cells decreased to less than 10% at 1×10^{-6} M test compound (Fig. 5). The inhibitory activity of **9a** with a *n*-heptyl group is similar to that of **9b** with a *n*-octyl group. Compound **10a** with an *N*-benzyl group showed weak inhibitory activity, compared to those of **9a** and **9b**. Compounds **10b** and **10c** with an *N*-4-methylbenzyl and *N*-4-trifluoromethylbenzyl group, respectively, showed more potent inhibitory activity than **10a**. Since the inhibitory activities of **10b** and **10c** were similar, the

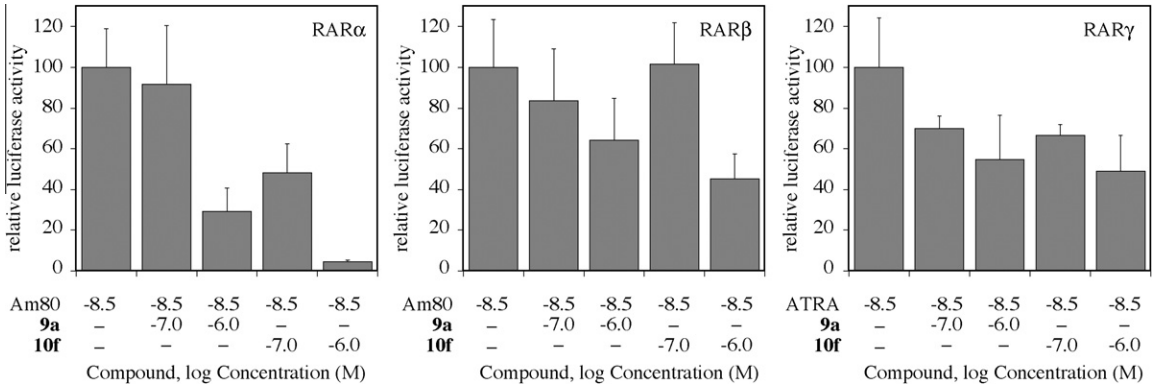


Figure 6. Transactivation-inhibitory activity of compounds **9a** and **10f**. Transactivation assays were carried out using COS-1 cells transfected with hRAR (α, β, or γ) and $(TREpal)_3$-TKLUC. Receptor transactivation was induced with 3×10^{-9} M Am80 for RARα and β, or 3×10^{-9} M ATRA for RARγ. The vertical scale is the transactivation relative to that with agonist only, taken as 100.

electronic properties of the aromatic ring appear not to be significant for the inhibitory activity. Compounds with a bulkier substituent, such as biphenyl structure (compound **10d**) or a naphthyl group (compound **10e**), showed higher activity, and inhibited the Am80-induced cell differentiation to about 10% and 30% at 1×10^{-7} M test compound, respectively. Compound **10f** was the most potent inhibitor among the test compounds, and inhibited the retinoidal effect of Am80 to below the 10% level at 1×10^{-7} M. The cell differentiation–inhibitory activity was affected by the bulkiness of the *N*-benzyl substituent.

2.3. Transactivation assay of 10f for RARs and RXRs

In order to confirm the inhibitory mechanism of the diphenylamine derivatives, transient transactivation assay for RARs and RXRs was examined using two selected compounds, **9a** with an *N*-alkyl group, and **10f** with an *N*-benzyl group that was the most active compound in HL-60 cell differentiation assay.[16] Unexpectedly, compounds **9a** and **10f** did not inhibit the transactivation of RXRs activated with 1×10^{-8} M RXR agonist PA024 (**7b**) (data not shown). On the other hand, the transactivation induced by 3×10^{-9} M Am80 (RARα and β) or ATRA (RARγ) with all subtypes of RARs was dose-dependently inhibited by **9a** or **10f** (Fig. 6). The result indicated that the introduction of a bulky *N*-alkyl group into the diphenylamine skeleton of an RXR agonist afforded an RAR antagonist.

3. Discussion

Various synthetic ligands for RARs and RXRs have been developed. Typical synthetic RAR agonists consist of two aromatic rings with hydrophobic substituents or a carboxyl group linked by two or three atoms, and have a rather planar extended structure, while typical RXR agonists have a folded structure with two aromatic moieties linked by one atom. DA010 (**8**) acted as dual, but weak agonist of RARs and RXRs. The introduction of an *N*-alkyl group with 1–5 carbon atoms (R_1) or a substituent at the ortho position to the nitrogen atom on the hydrophobic aromatic ring (R_2) increased the RXR agonist potency with loss of RAR agonist activity (Fig. 7a). Some aza derivatives, such as PA024 (**7b**), are more potent RXR agonists. Introduction of an *N*-acyl group, such as an acetyl group, into DA010 (**8**) was not effective for obtaining RXR agonist activity (our unpublished results). Several RXR antagonists have been developed based on the structures of DA024 (**7a**) and PA024 (**7b**) as lead compounds. Thus, PA452 (**16**), with a long substituent on the aromatic ring, and compound **17** showed RXR-selective antagonist activity (Fig. 7b).[17,18] The present study suggests that the diphenylamine derivatives **9a** and **10f** binds to the RAR site of RXR–RAR heterodimers and inhibits the cell differentiation-inducing activity of the RAR agonist. Although compounds **9a** and **10f** have a similar skeleton to compounds PA452 (**16**) and **17**, its receptor selectivity was completely different (Fig. 7b). We speculate that the difference arises from a difference in the conformations of the N-substituents of these compounds. Sulfonamide generally exists in a gauche-like conformation, and the torsion angles of C(Ar)–N–S–C(Ar) are large. In the case of compounds **9a** and **10f**, the N-substituent (*n*-alkyl or benzyl, respectively) would have little effect on the extended conformation of the diphenylamine structure, and so compounds **9a** and **10f** bind preferentially to RARs over RXRs. As summarized in Figure 7a, the diphenylamine skeleton is suitable for binding to both receptors, RARs and RXRs, and the N-substituent determines the receptor selectivity and the biological activity of the compounds. Similar findings were reported for diphenylamine

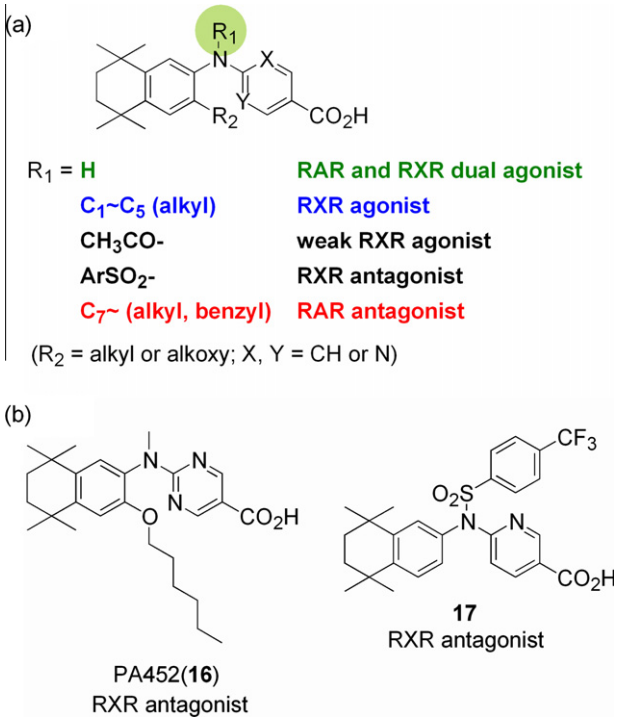


Figure 7. (a) The structure–activity relationships of N-substituents of diphenylamine derivatives. (b) The structures of the RXR antagonists PA452 (**16**) and N-sulfonylated compound **17**.

derivatives showing agonist or antagonist activity for other nuclear receptors, such as thyroid hormone receptors, estrogen receptor and androgen receptor.[19–21] Thus, the diphenylamine skeleton seems to be a key basic structure to develop specific nuclear receptor modulators.

4. Conclusion

In conclusion, novel diphenylamine derivatives **9a**, **9b**, and **10a–f** with a long alkyl chain or a benzyl group as an N-substituent, inhibited the differentiation of HL-60 cells induced by RAR agonists. The inhibitory mechanism of **9a** and **10f** was shown, by means of transactivation assays for RARs and RXRs, to be antagonism of the RAR agonists at the RAR site of RAR–RXR heterodimers. Interestingly, diphenylamine derivatives with a shorter alkyl group (C_1–C_5) as the N-substituent acted as RXR agonists, while the derivatives with a benzyl group acted as RAR antagonists, not RXR ligands. The N-substituent of diphenylamine derivatives plays an important role in determining the receptor selectivity between RARs and RXRs, as well as the agonist or antagonist nature of the activity.

5. Experimental

5.1. General considerations

Melting points were determined with a Yanaco micro melting point apparatus and were not corrected. ^{1}H NMR, ^{13}C NMR, and ^{19}F spectra were recorded with JEOL JNM-GX-400 and JNM-EX-270 spectrometers. Chemical shifts for ^{1}H NMR spectra were referenced to tetramethylsilane (0.0 ppm) as an internal standard. Chemical shifts for ^{13}C NMR spectrum were referenced to residual ^{13}C present in the deuterated solvent. Chemical shift values for ^{19}F spectra were referenced relative to external trifluoroacetic acid (CF_3CO_2H, 0.0 ppm, with negative values upfield). The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet) and m (multiplet). Elemental analyses were carried out in the Microanalytical Laboratory, Faculty of Pharmaceutical Sciences, The University of Tokyo, and were within ±0.3% of the theoretical values. Column chromatography was carried out using Merck silica gel 60 (0.063–0.200 μm) and TLC was performed on Merck silica gel F_{254}. Reagents were purchased from Wako Pure Chemical Industries, Ltd, Sigma–Aldrich Co., and Tokyo Chemical Industry, Ltd (TCI). All solvents were commercial products of reagent grade, and were used without further purification.

5.2. Synthesis

5.2.1. General procedure of N-alkylation or N-benzylation of diphenylamine derivatives

A suspension of NaH in dry DMF was added to a solution of **11** in dry DMF under an Ar atmosphere, and the mixture was stirred for 20 min. The corresponding iodoalkane or benzyl halide was added to the mixture, and the whole was stirred at room temperature, then poured into water, and extracted with ether. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified with flash column chromatography on silica gel (AcOEt/*n*-hexane = 1:10) to afford the corresponding N-substituted compound.

5.2.2. Ethyl 4-[*N*-*n*-heptyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

Quantitative yield; colorless powder (*n*-hexane); mp 65.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* = 7.3 Hz, 3H), 1.24 (s, 6H), 1.27 (br m 8H), 1.30 (s, 6H), 1.35 (t, *J* = 10.0 Hz, 3H), 1.64 (br m, 2H), 1.70 (s, 4H), 3.66 (t, *J* = 7.7 Hz, 2H), 4.31 (q, *J* = 7.0 Hz, 2H), 6.64 (d, *J* = 8.8 Hz, 2H), 6.92 (dd, *J* = 2.6 Hz, 8.1 Hz, 1H), 7.10 (d, *J* = 2.2 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.82 (d, *J* = 8.8 Hz, 2H). Anal. Calcd for $C_{30}H_{43}NO_2$: C, 80.13; H, 9.64; N, 3.12. Found: C, 80.17; H, 9.46; N, 3.11.

5.2.3. Ethyl 4-[*N*-*n*-octyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

96% Yield; colorless powder (*n*-hexane); mp 70 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, *J* = 7.0 Hz, 3H), 1.24 (s, 6H), 1.26 (br m 10H), 1.30 (s, 6H), 1.35 (t, *J* = 7.3 Hz, 3H), 1.68 (br m, 2H), 1.70 (s, 4H), 3.65 (t, *J* = 7.7 Hz, 2H), 4.31 (q, *J* = 7.3 Hz, 2H), 6.64 (d, *J* = 8.8 Hz, 2H), 6.91 (dd, *J* = 2.2 Hz, 8.4 Hz, 1H), 7.09 (d, *J* = 2.2 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.82 (dd, *J* = 2.2 Hz, 9.2 Hz, 2H).

5.2.4. Ethyl 4-[*N*-benzyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

Quantitative yield; colorless needles (*n*-hexane); mp 128.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.21 (s, 6H), 1.28 (s, 6H), 1.33 (t, *J* = 7.0 Hz, 3H), 1.68 (s, 4H), 4.31 (q, *J* = 7.0 Hz, 2H), 4.99 (s, 2H), 6.74 (dd, *J* = 1.8 Hz, 8.8 Hz, 2H), 7.03 (dd, *J* = 2.6 Hz, 8.4 Hz, 1H), 7.20 (d, *J* = 2.6 Hz, 1H), 7.22–7.25 (m, 1H), 7.29 (d, *J* = 8.4 Hz, 1H), 7.31 (d, *J* = 4.4 Hz, 4H), 7.80 (dd, *J* = 1.8 Hz, 9.2 Hz, 2H).

5.2.5. Ethyl 4-[*N*-(4-methylbenzyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

Quantitative yield; colorless powder (*n*-hexane); mp 107–108 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.21 (s, 6H), 1.28 (s, 6H), 1.33 (t, *J* = 7.0 Hz, 3H), 1.68 (s, 4H), 2.32 (s, 3H), 4.29 (q, *J* = 7.0 Hz, 2H), 4.60 (s, 2H), 6.74 (dd, *J* = 1.8 Hz, 9.2 Hz, 2H), 7.02 (dd, *J* = 2.2 Hz, 8.4 Hz, 1H), 7.11 (d, *J* = 7.7 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 1H), 7.20 (d, *J* = 2.2 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 4H), 7.79 (d, *J* = 9.2 Hz, 2H).

5.2.6. Ethyl 4-[*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)-*N*-(4-trifluoromethylbenzyl)amino]benzoate

93% Yield; colorless prisms (*n*-hexane); mp 97 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.29 (s, 6H), 1.33 (t, *J* = 7.3 Hz, 3H), 1.69 (s, 4H), 4.30 (q, *J* = 7.3 Hz, 2H), 5.04 (s, 2H), 6.71 (d, *J* = 9.2 Hz, 2H), 7.01 (dd, *J* = 2.6 Hz, 8.4 Hz, 1H), 7.19 (d, *J* = 2.2 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.43 (d, *J* = 8.1 Hz, 2H), 7.57 (d, *J* = 8.1 Hz, 2H), 7.82 (d, *J* = 9.2 Hz, 2H).

5.2.7. Ethyl 4-[*N*-(biphen-2-ylmethyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

Quantitative yield; colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.19 (s, 6H), 1.28 (s, 6H), 1.33 (t, *J* = 7.3 Hz, 3H), 1.68 (s, 4H), 4.29 (q, *J* = 7.3 Hz, 2H), 4.86 (s, 2H), 6.65 (dd, *J* = 1.8 Hz, 8.8 Hz, 2H), 6.98 (dd, *J* = 2.6 Hz, 8.4 Hz, 1H), 7.15 (d, *J* = 2.6 Hz, 1H), 7.23–7.32 (m, 6H), 7.35–7.45 (m, 3H), 7.48–7.52 (m, 1H), 7.77 (dd, *J* = 2.2 Hz, 9.2 Hz, 2H).

5.2.8. Ethyl 4-[*N*-(naphthalene-2-ylmethyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

99% Yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.28 (s, 6H), 1.32 (t, *J* = 7.3 Hz, 3H), 1.68 (s, 4H), 4.29 (q, *J* = 7.3 Hz, 2H), 5.15 (s, 2H), 6.80 (dd, *J* = 2.2 Hz, 9.2 Hz, 2H), 7.09 (dd, *J* = 2.2 Hz, 8.4 Hz, 1H), 7.28 (d, *J* = 2.6 Hz, 1H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.42–7.47 (m, 3H), 7.73–7.82 (m, 4H), 7.81 (dd, *J* = 2.2 Hz, 9.2 Hz, 2H).

5.2.9. Ethyl 4-[*N*-pentafluorobenzyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate

99% Yield; colorless powder (*n*-hexane); mp 116–120 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.17 (s, 6H), 1.26 (s, 6H), 1.35

(t, J = 7.3 Hz, 3H), 1.67 (s, 4H), 4.32 (q, J = 7.0 Hz, 2H), 4.95 (s, 2H), 6.75 (dd, J = 2.2 Hz, 9.2 Hz, 2H), 6.85 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 6.98 (d, J = 2.2 Hz, 1H), 7.26 (d, J = 8.4 Hz, 1H), 7.87 (dd, J = 1.8 Hz, 8.8 Hz, 2H).

5.2.10. General procedure of hydrolysis of ethyl ester

A mixture of the ester compound **12**, **13** or **14** in EtOH and 20% aqueous solution of KOH was stirred at room temperature. The reaction mixture was poured into 1 M hydrochloric acid, and the solution was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated. The residue was recrystallized from appropriate solvents to afford the corresponding carboxylic acid derivatives.

5.2.11. 4-[*N*-*n*-Heptyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (9a)

99% Yield; colorless powder (CH_2Cl_2–*n*-hexane); mp 168 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, J = 6.6 Hz, 3H), 1.25 (s, 6H), 1.25–1.31 (br m 8H), 1.31 (s, 6H), 1.70 (br m, 2H), 1.70 (s, 4H), 3.66 (t, J = 7.7 Hz, 2H), 6.63 (dd, J = 8.8 Hz, 2H), 6.93 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.10 (d, J = 1.8 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.86 (d, J = 9.2 Hz, 2H). Anal. Calcd for $C_{28}H_{39}NO_2$: C, 79.76; H, 9.32; N, 3.32. Found: C, 79.80; H, 9.62; N, 3.06.

5.2.12. 4-[*N*-*n*-Octyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (9b)

98% Yield; colorless cotton (CH_2Cl_2–*n*-hexane); mp 160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.87 (t, J = 6.6 Hz, 3H), 1.25 (s, 6H), 1.26 (br m 10H), 1.31 (s, 6H), 1.70 (br m, 2H), 1.70 (s, 4H), 3.66 (t, J = 8.1 Hz, 2H), 6.63 (d, J = 9.2 Hz, 2H), 6.92 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.10 (d, J = 2.2 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.87 (dd, J = 2.2 Hz, 9.2 Hz, 2H); Anal. Calcd for $C_{29}H_{41}NO_2$: C, 79.95; H, 9.49; N, 3.22. Found: C, 79.92; H, 9.54; N, 3.18.

5.2.13. 4-[*N*-Benzyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (10a)

Quantitative yield; colorless powder (CH_2Cl_2–*n*-hexane); mp 272–273 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 5.00 (s, 2H), 6.73 (dd, J = 1.8 Hz, 9.2 Hz, 2H), 7.04 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 7.23–7.25 (m, 1H), 7.30 (d, J = 7.3 Hz, 1H), 7.32 (d, J = 4.8 Hz, 4H), 7.84 (dd, J = 2.2 Hz, 9.2 Hz, 2H); Anal. Calcd for $C_{28}H_{31}NO_2$: C, 81.32; H, 7.56; N, 3.39. Found: C, 81.05; H, 7.49; N, 3.57.

5.2.14. 4-[*N*-(4-Methylbezyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (10b)

Quantitative yield; colorless powder (CH_2Cl_2–*n*-hexane); mp 246–248 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.29 (s, 6H), 1.68 (s, 4H), 2.32 (s, 3H), 4.96 (s, 2H), 6.73 (dd, J = 2.9 Hz, 9.2 Hz, 2H), 7.03 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.12 (d, J = 8.1 Hz, 2H), 7.20 (d, J = 6.6 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 7.30 (d, J = 8.4 Hz, 4H), 7.83 (d, J = 2.2 Hz, 9.2 Hz, 2H); Anal. Calcd for $C_{29}H_{33}NO_2$: C, 81.46; H, 7.78; N, 3.28. Found: C, 81.28; H, 7.82; N, 3.44.

5.2.15. 4-[*N*-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)-*N*-(4-trifluoromethylbenzyl)amino]benzoic acid (10c)

90% Yield; colorless powder (*n*-hexane); mp 209–210 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.29 (s, 6H), 1.69 (s, 4H), 5.04 (s, 2H), 6.70 (d, J = 8.8 Hz, 2H), 7.02 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.19 (d, J = 2.2 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.44 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 8.1 Hz, 2H), 7.86 (d, J = 2.2 Hz, 9.2 Hz, 2H); Anal. Calcd for $C_{29}H_{30}NO_2F_3$: C, 72.33; H, 6.28; N, 2.91. Found: C, 72.15; H, 6.41; N, 2.94.

5.2.16. 4-[*N*-(Biphen-2-ylmethyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (10d)

97% Yield; colorless prisms (CH_2Cl_2–*n*-hexane); mp 237–239 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.19 (s, 6H), 1.28 (s, 6H), 1.68 (s, 4H), 4.87 (s, 2H), 6.64 (d, J = 9.2 Hz, 2H), 6.98 (dd, J = 2.2 Hz, 8.8 Hz, 1H), 7.15 (d, J = 2.6 Hz, 1H), 7.23–7.31 (m, 6H), 7.35–7.44 (m, 4H), 7.48–7.79 (m, 1H), 7.80 (d, J = 8.8 Hz, 2H); Anal. Calcd for $C_{34}H_{35}NO_2$: C, 83.40; H, 7.21; N, 2.86. Found: C, 83.11; H, 7.50; N, 2.75.

5.2.17. 4-[*N*-(Naphthalen-2-ylmethyl)-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (10e)

Quantitative yield; colorless powder (CH_2Cl_2–*n*-hexane); mp 233 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (s, 6H), 1.28 (s, 6H), 1.68 (s, 4H), 5.15 (s, 2H), 6.79 (dd, J = 2.2 Hz, 9.2 Hz, 2H), 7.09 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 7.27 (d, J = 2.6 Hz, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.44 (m, 3H), 7.78 (m, 4H), 7.84 (dd, J = 2.2 Hz, 9.2 Hz, 2H); Anal. Calcd for $C_{32}H_{33}NO_2$: C, 82.90; H, 7.18; N, 3.02. Found: C, 82.66; H, 7.48; N, 2.73.

5.2.18. 4-[*N*-(4-Ethoxy-2,3,5,6-tetrafluoro)benzyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic acid (10f)

Quantitative yield; colorless powder (CH_2Cl_2–*n*-hexane); mp 227–229 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.16 (s, 6H), 1.25 (s, 6H), 1.36 (t, J = 7.3 Hz, 3H), 1.66 (s, 4H), 4.22 (q, J = 7.0 Hz, 2H), 4.92 (s, 2H), 6.75 (dd, J = 2.2 Hz, 9.2 Hz, 2H), 6.87 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 6.98 (d, J = 2.2 Hz, 1H), 7.26 (d, J = 8.4 Hz, 1H), 7.90 (dd, J = 1.8 Hz, 8.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 31.2, 31.4, 33.7, 33.9, 34.4, 42.8, 70.8, 113.1, 119.3, 124.9, 125.4, 127.9, 130.8, 136.0 (t), 138.6 (d), 141.0, 142.2 (d), 142.8, 143.2 (t), 145.8, 151.7, 167.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ (ppm) −31.82 (AA'XX'), −17.37 (AA'XX'); MS (EI) m/z 529 (M^+), 514 (100%); HRMS Calcd for $C_{30}H_{31}NO_3F_4$: 529.2240, Found: 529.2239; Anal. Calcd for $C_{30}H_{31}NO_3F_4$: C, 68.04; H, 5.90; N, 2.65. Found: C, 67.78; H, 5.89; N, 2.61.

5.3. HL-60 cell differentiation-inducing assay

The human promyelocytic leukemia cell line HL-60 was provided by Prof. F. Takaku (Faculty of Medicine, The University of Tokyo) in 1980 and has been maintained in continuous suspension culture. The cells are cultured in plastic flasks in RPMI1640 medium, supplemented with 5% fetal bovine serum (FBS, not delipidized), and antibiotics (penicillin G and streptomycin) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Test compounds were dissolved in ethanol at 2 mM and added to the cells, which were seeded at about 8×10^4 cells/mL; the final ethanol concentration was kept below 0.5%. Control cells were given only the same volume of ethanol. Am80, as a positive control, was always assayed at the same time. The cells were incubated for four days and stained with Wright–Giemsa in order to check for morphological change. The percentages of differentiated cells were determined by NBT reduction assay. Cells were incubated for 20 min at 37 °C in RPMI1640 medium (5% FBS) and an equal volume of phosphate-buffered saline (PBS) containing NBT (0.2%) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 200 ng/mL). The percentage of cells containing blue-black formazan was determined using a minimum of 200 cells. The evaluation of the differentiation from NBT reduction assay was always consistent with the morphological result. Synergistic activity with Am80 was examined in the presence of a suitable concentration of test compound according to the method described above. In this experiment, the independent effects of Am80 and the test compound were always assayed, and the percentages of differentiated cells were determined by NBT reduction assay. The assays of test compounds were performed at least twice, and the average of the two experiments was taken.

5.4. Transactivation assay for RARs and RXRs

Transient transactivation assays were carried out using COS-1 cells transfected with hRAR (α, β, or γ) and $(TREpal)_3$-TKLUC or mRXR (α, β, or γ) and $(DR1)_5$-pGL-TK. The COS-1 cells were obtained from the Japanese Cancer Resources Bank (JCRB) and were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 5% FBS (5% FBS/DMEM). The reporter plasmid, $(TREpal)_3$-TKLUC contains three copies of the thyroid hormone-responsive palindromic element AGGTCA–TGACCT. The reporter plasmid, $(DR1)_5$-pGL-TK, was constructed by introducing five copies of RXRE, GGTTCAGAGTTCA and the herpes simplex virus thymidine kinase promoter into the NheI–Hind III sites of the pGL3-Basic luciferase reporter vector (Promega). For reporter gene assay, COS-1 cells were seeded in 24-well tissue culture plates at 8×10^4 cells per well with 5% FBS/DMEM. The cells were cultured at 37 °C in 5% CO_2 overnight and allowed to attach to the plates. Then, the medium was exchanged for 0.4 mL of serum-free DMEM and transfection was performed by use of the transfection reagent LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. For each well, cells were transfected with 100 ng of receptor-expression plasmid, 200 ng of $(TREpal)_3$-TKLUC or $(DR1)_5$-pGL-TK, 100 ng of the reference plasmid pCMVβ (Clontech), and carrier plasmid pUC18 to adjust the total DNA amount to 800 ng. DMEM (0.1 mL) containing transfection reagent-DNA complex was added to each well. After 4 h of culture at 37 °C in 5% CO_2, 0.5 mL of DMEM with 10% charcoal dextran-treated FBS (Hyclone) and test compounds were added to cells. Each test compound was added as an EtOH solution (final 0.5% EtOH). After an additional 40 h of incubation, the cells were harvested, and a luciferase assay was performed with the Luciferase Assay System (Tokyo Ink Mfg. Co. Ltd). The luciferase activities were normalized to β-galactosidase activities. Assays were done in triplicate under each condition.

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