BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 121–125 (1997)
ARTICLE NO. RC976414

Action Mechanism of Retinoid-Synergistic Dibenzodiazepines

Hiroki Umemiya,* Hiroyuki Kagechika,* Hiroshi Fukasawa,* Emiko Kawachi,* Masayuki Ebisawa,* Yuichi Hashimoto,† Ghislaine Eisenmann,‡ Cathie Erb,‡ Astrid Pornon,‡ Pierre Chambon,‡ Hinrich Gronemeyer,‡ and Koichi Shudo*,[1]

**Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan; †Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan; and ‡Institut de Génétique et de Biologie Moléculaire et Cellulaire, 3 Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, 67404 Illkirch cedex, Strasbourg, France*

Received February 26, 1997

4-[5*H*-2,3-(2,5-Dimethyl-2,5-hexano)-5-methyldibenzo-[*b,e*][1,4]diazepin-11-yl]benzoic acid (HX600), as well as its oxa- (HX620) and thia- (HX630) analogs, enhanced the activity of retinoic acid and a receptor α (RARα)-selective agonist Am80 in HL-60 cell differentiation assays. HX600 synergizes with Am80 by binding to, and transactivating through, the RXR subunit of the RXR-RAR heterodimer. HX600 exhibited RXR pan-agonist activity in transient transfections with a DR1-based reporter gene and synergized with RA-bound RARα and RARβ in inducing transcription from a DR5-based reporter. In addition, all three compounds at high concentrations acted as RAR pan-antagonists in stably transfected RAR "reporter cells." These efficient synergists bind only weakly with RXRs *in vitro,* suggesting that they are RXR-RAR heterodimer-selective activators. These HX retinoids exhibited dual functionality, since they affected signalling through both retinoid receptor families (RARs and RXRs).

The three retinoic acid (RAR) and three retinoid X (RXR) receptors (α, β and γ, and their isoforms), belong to the nuclear receptor (NR) superfamily and act as ligand-inducible transcriptional regulators transducing the pleiotropic effects of retinoic acids on morphogenesis, differentiation and homeostasis during embryonal development and post-natal life. (1-3) RARs are activated by both all-*trans*-retinoic acid (RA) and 9-*cis*-retinoic acid (9cRA), while RXRs bind to and are activated only by 9cRA. Although RARs and RXRs can form homodimers, heterodimerization increases the efficiency of binding to the cognate response elements. RXR acts as a promiscuous heterodimerization partner, since it also forms complexes with various other nuclear receptors, such as the thyroid hormone and vitamin D_3 receptors, as well as certain "orphan" receptors.

RA and several synthetic retinoids are important therapeutic agents in the fields of dermatology and oncology (4,5) and various retinoids, such as receptor isotype-selective retinoid agonists and antagonists, have been developed with the aims of increasing the specificity and efficiency of treatment, and reducing RA side effects. (1,6-8) In addition, retinoids that can synergize with other beneficial treatments (9) may lead to novel therapeutic strategies. Recently, we reported that the dibenzodiazepine derivative HX600 (Fig. 1) exhibits a synergistic activity with RA, or the synthetic retinoid Am80, in the differentiation-induction assay with human HL-60 promyelocytic leukemia cells. (10) In view of the structural similarity between HX600 and other synthetic retinoids, we anticipated that this synergistic effect reflects a direct HX600-retinoid receptor interaction. Several synthetic RXR-selective ligands have recently been reported to synergize with RAR-selective ligands. (11-13) Here we describe the structural and biological properties of HX600 and characterize its interaction with, and transcription regulatory effects via, RARs and RXRs.

MATERIALS AND METHODS

Materials. HX600 was prepared similarly to the retinoid antagonist LE135 (an isomer of HX600). (14) Briefly, 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene was coupled with 2-nitroaniline in the presence of copper iodide, followed by *N*-methylation. After reduction of the nitro group, *N*-methyl-*N*-(1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthyl)phenylenediamine was condensed with terephthalic acid monomethyl ester chloride. The resultant amide compound was cyclized in polyphosphoric acid, followed by ester hydrolysis to generate HX600 (mp 282 °C). 4-[2,3-(2,5-Dimethyl-2,5-hexano)dibenzo[*b, f*][1, 4]oxazepin-11-yl]benzoic acid (HX620, mp

0006-291X/97 $25.00

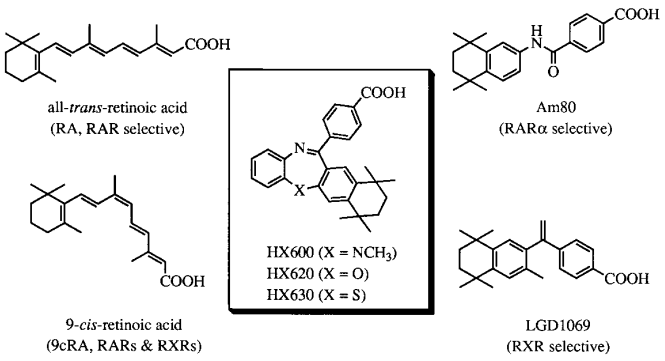

FIG. 1. Structures of retinoids and retinoid synergists.

289 °C) and 4-[2,3-(2,5-dimethyl-2,5-hexano)dibenzo[*b,f*][1, 4]thiazepin-11-yl]benzoic acid (HX630, mp 299 °C) were synthesized similarly. LGD1069 was prepared as described. (15)

Differentiation-inducing assay. The human promyelocytic leukemia cell line HL-60 was provided by Prof. F. Takaku (Faculty of Medicine, University of Tokyo) in 1980, and has been maintained since then. Differentiation-induction assays with retinoids were carried out as reported previously. (14) The percentages of differentiated cells were determined both morphologically and by NBT reduction assay.

Competitive binding assay. The binding activities for RARs and RXRs were measured by the nitrocellulose filter binding assay method as described previously. (14) Recombinant receptors were prepared as fusion proteins with the maltose-binding protein (MBP) for RARα and β (ligand-binding domain), or with glutathione-*S*-transferase (GST) for RXRs. Recombinant RARγ was prepared as described. (16) Competitive binding assays were carried out using 8 nM [^{3}H]Am80 (65 Ci/mmol, Amersham) for RARα, 20 nM [^{3}H]Am80 for RAR β, 4 nM [^{3}H]9cRA (48 Ci/mmol, Amersham, used at 8 Ci/mmol by dilution with cold 9cRA) for RARγ, or 10 nM [^{3}H]9cRA for RXRs. The Ki values of compounds were calculated according to the following equation: $Ki = IC_{50}/(1+[L]/Kd)$, where IC_{50} is the concentration of competing compounds required to decrease specific binding of the labeled compound by 50%, and [L] and Kd are the concentration and the dissociation constant of the labeled compound, respectively. (17)

Transactivation assay. Ligand-regulated transactivation was assayed by transient transfections and by using the stably transfected "reporter cells" described previously. (12) Transient transactivation assays were carried out using HeLa cells transfected with mRAR(α, β or γ) and DR5-tk-CAT or mRXR(α, β or γ) and DR1-tk-CAT, using RA or 9cRA as the activating ligand. Reporter cell assays were carried out using HeLa cells stably transfected with GAL-RARα, β or γ chimeras and (17mer) × 5-globin-luciferase reporters.

RESULTS

Synergism between the RAR Agonist Am80 and HX600 in the HL-60 Differentiation Assay

Retinoids are known to induce the differentiation of HL-60 human promyelocytic leukemia cells to mature granulocytes and the potencies of retinoids in this assay correlate well with other retinoid activities. Alone, HX600 (Fig. 1) did not affect the proliferation or differentiation of HL-60 cells (maximal concentration tested 1×10^{-6} M). However, HX600 strongly enhanced the differentiation-inducing activity of Am80 (Fig. 2), since the percentage of cells differentiated by 3×10^{-10} M Am80 (appox. 15%) was increased to 41% and 72% in the presence of 1×10^{-8} and 1×10^{-7} M HX600, respectively. (10) A similar synergy with Am80 was seen with the oxa- (HX620) and thia- (HX630) analogs of HX600 (Figs. 1 and 2). For comparison, we studied the synergistic activity of LGD1069 (Fig. 1), a typical RXR-selective retinoid, (15) in combination with Am80. The order of the synergistic potency was LGD1069 ≈ HX630 > HX620 (Fig. 2). The concentration causing 50% of the maximal synergistic effect (EC_{50}) at 3×10^{-10} M Am80 was about $10^{-9} - 10^{-8}$ M for both LGD1069 and HX630, possibly reflecting the higher affinity of HX630 to RXR, compared with HX600 and HX620.

Two important observations were made in these cell differentiation assays: (i) at a concentration of 1×10^{-6} M the three azepine derivatives alone were completely inactive, whereas LGD1069 showed a significant differentiation-inducing activity; (18) (ii) the high concentration of 1×10^{-6} M HX600, weakly but reproducibly, decreased the synergistic efficiency of differentiation by 3×10^{-10} M Am80. A similar, but more marked, decrease of synergism of high-dose HX600 was observed when RA was used as the retinoid, as reported previously. (10) These results suggest that HX600, unlike LGD1069, may possibly act as an RAR antagonist at this high concentration.

Binding Affinities to Retinoid Nuclear Receptors RARs and RXRs

Our previous studies showed that, as in the case of aromatic retinoid agonists, (6) the bulky alkyl group (2,5-dimethyl-2,5-hexano group) of HX600 is important

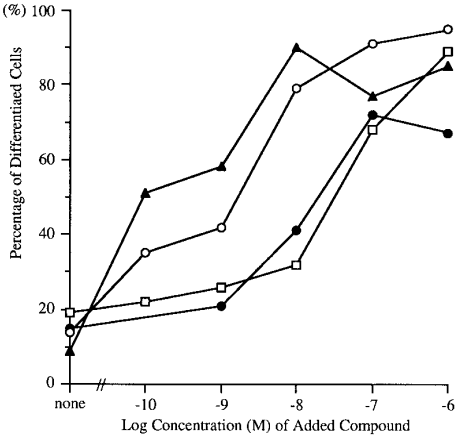

FIG. 2. Synergistic effects on HL-60 cell differentiation induced by 3×10^{-10} M Am80. The added compound was HX600 (●), HX620 (□), HX630 (○), or LDG1069 (▲). The vertical scale is the percentage of differentiated cells evaluated from NBT reduction assay, and the horizontal scale is the molar concentration of the added compound.

TABLE 1

Receptor Binding Affinities of Retinoids and Retinoid Synergists

	Ki values, $\times 10^{-9}$ M					
	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
RA	3.3	4.1	0.04	330	54	120
9cRA	No data	No data	1.0	12	3.8	11
Am80	6.5	30	Not bind[a]	Not bind	Not bind	Not bind
HX600	Not bind	680	Not bind	1900	640	1000
HX620	980	320	Not bind	3800	1200	2100
HX630	900	320	Not bind	900	400	620
LGD1069	180	50	130	16	5.9	8.3

[a] 'Not bind' means that 1000 fold excess of test compound did not affect the binding of the labeled compound to the receptors.

for the synergistic activity. (10) Therefore, HX600 action was expected to be mediated by retinoid receptors. Table 1 shows the competitive binding ability of the retinoids used in this study, as determined with recombinant RARs and RXRs. In our experiments, LGD1069 is a potent ligand for all three RXRs with Ki values (5-16 nM) similar to that of 9cRA. The three HX compounds bound very weakly to all RXRs, their Ki values being between 400 and 3800 nM. Thus, the RXR-binding affinities of these azepine derivatives are much lower than would be expected from their maximal synergistic activities in the HL-60 assay (1 - 10 nM). HX compounds also bound weakly to RARs, with RARβ exhibiting the highest affinity.

Transactivation Properties of HX600

In transient transactivation assays HX600 acted as a triple RXR agonist (a pan-RXR agonist, Fig. 3a). No RAR agonist activity of HX600 could be detected (Fig. 3b). However, when HX600 (1×10^{-6} M) was added together with 1×10^{-8} M RA, a moderate synergistic effect was observed in the cases of RARα and β. Since HX600 did not activate RARs, this synergism is most likely due to the interaction of HX600 with the endogenous RXRs.

The effect of HX600 on RAR-dependent transactivation was also studied by using the reporter cell system (Fig. 4). (8) At low concentration (1×10^{-8} M), HX600 further augmented the activity seen with 1×10^{-9} M RA, but at higher concentration HX600 acted in this system rather as an antagonist of RA-induced transactivation. The difference from the above-described absence of RAR-antagonistic activity in the transient transactivation experiments was not due to the use of chimeric Gal-RARs (which act as stably expressed activators in these reporter cells), since in transient transfections with these same chimeras no RAR-antagonistic potential of HX600 could be observed (data not shown). Thus, it is likely that the RAR-antagonistic

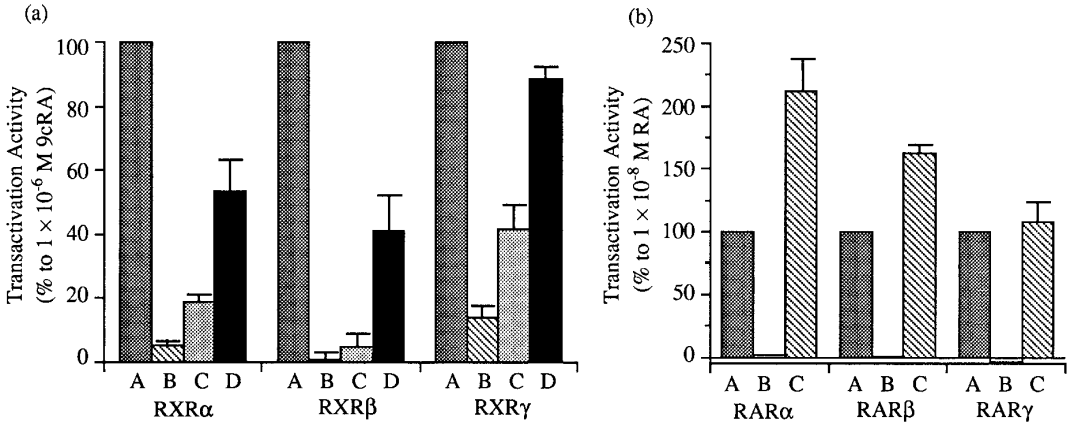

FIG. 3. (a) CAT assays in Hela cells transiently transfected with RXR/DR1-tk-CAT. The vertical scale is the transactivation relative to that with 1×10^{-6} M 9cRA (100%). Added compounds were 1×10^{-6} M 9cRA (A), 1×10^{-8} M HX600 (B), 1×10^{-7} M HX600 (C), or 1×10^{-6} M HX600 (D). (b) CAT assays in Hela cells transiently transfected with RAR/DR5-tk-CAT. The vertical scale is the transactivation relative to that with 1×10^{-8} M RA (100%). Added compounds were 1×10^{-8} M RA (A), 1×10^{-6} M HX600 (B), or 1×10^{-8} M RA plus 1×10^{-6} M HX600 (C).

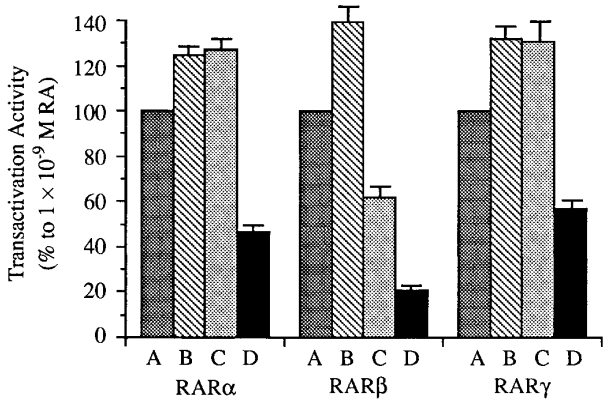

FIG. 4. Reporter cell assays in Hela cells stably transfected with GAL-RAR chimera and (17mer) × 5-globin-luciferase reporter. The vertical scale is 1×10^{-9} M RA-induced luciferase activity (% with respect to induction by RA only, A). Molar concentrations of added HX600 were 1×10^{-8} M (B), 1×10^{-7} M (C), and 1×10^{-6} M (D).

activity of HX600 can be observed only under conditions where activator and/or reporter are limiting, i.e. in assay systems with endogenous or stably transfected receptors and target genes. Note that this RAR antagonistic potential of HX600 may limit its RXR agonist activity-based synergism with Am80 or RA in the HL-60 cell assay only at high ligand concentrations.

DISCUSSION

The biological actions of retinoids, such as those affecting cell differentiation and proliferation, result from the modification of the transcriptional activity of specific gene networks and are mediated through their cognate nuclear receptors RARs and RXRs. In keeping with the observation of RAR-RXR heterodimer formation *in vitro,* recent studies have supported the concept that complex biological functions are also controlled *in vivo* by such heterodimers. (2) This notion is further supported by the observation that RXR ligands which exhibited very weak "retinoidal" activities on their own, significantly increased the potency of RAR-selective ligands. (11-13)

We show here that HX600 and related compounds (HX620 and HX630) synergize with Am80 to induce the differentiation of HL-60 cells. This phenomenon was also seen when RA or another synthetic retinoid (Am555S or Ch55), was used as the RAR ligand (ref. 10, and data not shown). Interestingly, the GAL-RAR reporter cell assays (Fig. 4) show that HX600 acts as a RAR pan-antagonist at high concentration, while there is a weak, but reproducible, synergy between 1×10^{-9} M RA and HX600 at low concentrations (around 1×10^{-8} M). These observations suggested to us that HX600 may exhibit a dual activity. Indeed, transient transactivation assays showed that HX600 is, in addition to its RAR activity, an RXR homodimer agonist with the cognate DR1-tk-CAT reporter gene. It is therefore likely that at low concentrations HX600 is bound to the RXR subunit of the RXR-RAR heterodimer and can synergize with RA-bound RAR. At higher concentrations HX600 can compete with RA for binding to the RAR subunit and exert the antagonistic activity seen in the reporter cell assays. It is interesting to note that, while HX600 showed no agonist activity in transient transfections, it also did not antagonize RA-induced activity in this system as it did in the reporter cells assays. As discussed above, the absence of an antagonistic effect of HX600 in the transient transfection system is most likely due to the high concentration of receptors expressed in transfected cells. It may thus represent a non-physiological situation, whereas the stably transfected reporter cells are more likely to mimic the physiological concentration of retinoid receptors. It is also worth noting that HX600 can act as an RXR agonist despite its very weak binding affinity, as measured *in vitro.* Note in this context that the ligand binding affinity was determined with recombinant receptors and that *in vitro* and *in vivo* binding affinities may differ for a given ligand, as has recently been suggested for a mutant glucocorticoid receptor. (19) Similar differences between the ligand affinity measured *in vitro* and the ligand efficiency observed *in vivo* may account for the observation that HX630 binds 50 - 100 times more weakly than LGD1069 to the three RXRs, although both RXR ligands exhibit a similar synergistic activity, which is nearly as potent as that of LGD1069 in HL-60 assays. *HX compounds may interact efficiently with RXRs of RXR-RAR heterodimers in vivo.*

Together, our data and those of others raised the fascinating possibility of modulating the activity of a given RXR homo- or heterodimer in multiple ways by choosing the appropriate synthetic retinoid: (i) RXR ligands may synergize with RAR agonists or antagonists and generate some cell type specificity, (11-13) (ii) as shown here, RXR ligands may in a concentration-dependent manner, positively and negatively alter RAR ligand-induced transactivation, and (iii) RXR ligands may distinct effects on homo- and heterodimers. (20) In view of the possibility of dissociating the functions of retinoid receptors for transactivation and transrepression (8,21) and the existence of RAR subtype-specific synthetic retinoids (6-8) an exploitation of these exciting tools may not only facilitate the analysis of the molecular mechanisms involved in retinoid-induced cell differentiation and modulation of cell proliferation and apoptosis, but also pave the way to novel concepts in the treatment of a variety of diseases in which retinoid receptors are known to play a major role.

REFERENCES

1. Sporn, M. B., Roberts, A. B., and Goodman, D. S. (Eds) (1994) The Retinoids, 2nd ed., Raven Press, New York.

2. Kastner, P., Mark, M., and Chambon, P. (1995) *Cell* **83,** 589–869; Mangelsdorf, D. J., and Evans, R. M. (1995) *Cell* **83,** 841–850; Chambon, P. (1996) *FASEB J.* **10,** 940–954.

3. Gronemeyer, H., and Laudet, V. (1995) *Protein Profile* **2,** 1173–1236.

4. Cunliffe, W. J., and Miller, A. J. (Eds) (1984) Retinoid Therapy, MTP Press Limited, Lancaster; Bollag, W., and Holdener, E. E. (1992) *Ann. Oncol.* **3,** 513–526; Hill, D. L., and Grubbs, C. J. (1992) *Ann. Rev. Nutr.* **12,** 161–181.

5. Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhoa, L., Gu, L. J., and Wang, Z. Y. (1988) *Blood* **72,** 567–572; Castaigne, S., Chomienne, C., Daniel, M. T., Ballerini, P., Berger, R., Fenaux, P., and Degos, L. (1990) *Blood* **76,** 1704–1709.

6. Shudo, K., and Kagechika, H. (1993) *Adv. Drug Res.* **24,** 81–119.

7. Recent Selected Papers: Lehmann, J. M., Dawson, M. I., Hobbs, P. D., Husmann, M., and Pfahl, M. (1991) *Cancer Res.* **51,** 4804–4809; Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I., and Pfahl, M. (1992) *Science* **258,** 1944–1946; Martin, B., Bernardon, J.-M., Cavey, M.-T., Bernard, B., Carlavan, I., Charpentier, B., Pilgrim, W. R., Shroot, B., and Reichert, U. (1992) *Skin Pharmacol.* **5,** 57–65; Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W., and Klaus, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7129–7133.

8. Chen, J.-Y., Penco, S., Ostrowski, J., Balaguer, P., Pons, M., Starrett, J. E., Reczek, P., Chambon, P., and Gronemeyer, H. (1995) *EMBO J.* **14,** 1187–1197.

9. Olsson, I. L., and Breitman, T. R. (1982) *Cancer Res.* **42,** 3924–3927; Peck, R., and Bollag, W. (1991) *Eur. J. Cancer* **27,** 53–57; Bollag, W. (1991) *Br. J. Haematol.* **79,** Suppl. 87–91; Takeuchi, K., Ishihara, H., Tsuneoka, K., and Shikita, M. (1991) *Biochem. Biophys. Res. Commun.* **178,** 263–268; Nakajima, O., Hashimoto, Y., and Iwasaki, S. (1993) *FEBS Lett.* **330,** 81–84; Safonova, I., Reichert, U., Shroot, B., Ailhaud, G., and Grimaldi, P. (1994) *Biochem. Biophys. Res. Commun.* **204,** 498–504; Sidell, N., Wada, R., Han, G., Chang, B., Shack, S., Moore, T., and Samid, D. (1995) *Int. J. Cancer* **60,** 507–514.

10. Umemiya, H., Kawachi, E., Kagechika, H., Fukasawa, H., Hashimoto, Y., and Shudo, K. (1995) *Chem. Pharm. Bull.* **43,** 1827–1829.

11. Roy, B., Taneja, R., and Chambon, P. (1995) *Mol. Cell. Biol.* **15,** 6481–6487; Lotan, R., Dawson, M. I., Zou, C.-C., Jong, L., Lotan, D., and Zou, C-P. (1995) *Cancer Res.* **55,** 232–236.; Apfel, C. M., Kamber, M., Klaus, M., Mohr, P., Keidel, S., and LeMotte, P. K. (1995) *J. Biol. Chem.* **270,** 30765–30772.

12. Chen, J. Y., Clifford, J., Zusi, C., Starrett, J., Tortolani, D., Ostrowski, J., Reczek, P. R., Chambon, P., and Gronemeyer, H. (1996), *Nature* **382,** 819–822.

13. Taneja, R., Roy, B., Plassat, J. L., Zusi, C., Ostrowski, J., Reczek, P. R., Chambon, P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6197–6202.

14. Eyrolles, L., Kagechika, H., Kawachi, E., Fukasawa, H., Iijima, T., Matsushima, Y., Hashimoto, Y., and Shudo, K. (1994) *J. Med. Chem.* **37,** 1508–1517.

15. Boehm, M. F., Zhang, L., Badea, B. A., White, S. K., Mais, D. E., Berger, E., Suto, C. M., Goldman, M. E., and Heyman, R. A. (1994) *J. Med. Chem.* **37,** 2930–2941.

16. Fukasawa, H., Iijimia, T., Kagechika, H., Hashimoto, Y., and Shudo, K. (1993) *Biol. Pharm. Bull.* **16,** 343–348.

17. Cheng, Y. C., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108.

18. LGD1069 was reported to be inactive in HL-60 assay. Nadzan, A. M., Boehm, M. F., Zhang, L., Badea, B. A., Zhi, L., White, S. K., Mais, D. E., Berger, E., Suto, C. M., McClurg, M. R., Davies, P. J. A., and Heyman, R. A. (1995) *Eur. J. Med. Chem.* **30,** 1s–688s.

19. Roux, S., Térouanne, B., Balaguer, P., Jausons-Loffreda, N., Pons, M., Chambon, P., Gronemeyer, H., and Nicolas, J.-C. (1996) *Mol. Endocrinol.* **10,** 1214–1226.

20. Lala, D. S., Mukherjee, R., Schulman, I. G., Canan-Koch, S. S., Dardashti, L. J., Nadzan, A. M., Croston, G. E., Evans, R. M. , and Heyman, R. A. (1996) *Nature* **383,** 450–453; Lee, M.-O., Dawson, M. I., Picard, N., Hobbs, P. D., and Pfahl, M. (1996) *J. Biol. Chem.* **271,** 11897–11903.

21. Fanjui, A., Dawson, M. I., Hobbs, P. D., Jong, L., Cameron, J. F., Harlev, E., Graupner, G., Lu, X.-P., and Pfahl, M. (1994) *Nature* **372,** 107–111; Nagpal, S., Athanikar, J., and Chandraratna, R. A. S. (1995) *J. Biol. Chem.* **270,** 923–927.