minimal CMV promoter and the 3' region of the CMV promoter (201 bp and 265 bp)) amplified using the following PCR primers:

5'-ggagatctggccccgcccagcgtcttgtc-3' and

5'-ggaagcttgcggcacgctgttgacgctgttaagcgggtcgctgcaggg-3' (3' region of the TK promoter);

5'-ccagatcttagttattaatagtaatcaattacggggtc-3' and 5'-ccaagcttgatctgacggttcactaaaccagc-3' (CMV promoter);

5'-ccagatcttaggcgtgtacggtgggagg-3' and 5'-ccaagcttaggctggatcggtcccggtg-3' (minimal CMV promoter);

5'-ccagatcttgggagtttgttttggcacc-3' and reverse primer of CMV promoter (CMV 201);

5'-ccagatcttcaatgggcgtggatagcgg-3' and reverse primer of CMV promoter (CMV265).

Double-stranded oligonucleotides containing hormone response elements (RXRE, RARE and FXRE; shown in Fig.1B) were ligated into the upstream region of these promoters using *Mlu*I and *Bgl*II sites. The sequences of the constructed plasmids were confirmed by sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Cotransfection and reporter assay

A monkey kidney cell line, COS-7, was kept in DMEM with 10% FBS. Transfections were performed using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The ratio of the reporter plasmid, receptor expression plasmids (for example, the RARα and RXRα expression plasmids for assay of RARα ligands) and the internal control plasmid was 4:1:1:1. The culture medium was replaced with DMEM without phenol red (Gibco BRL, Gaithersburg, MD) supplemented with 10% charcoal-treated FBS (Hyclone, Logan, UT) when the transfections were performed. At 15 h after transfections, the cells were treated with trypsin/ penicillin reagent and divided among wells of a black,

96-well plate with 100 μl of the culture medium. At 6 h after division among wells, the cells were treated with chemicals. After 40-h incubation, the medium was eliminated by decantation, the cells were washed twice with PBS, and the wells were filled with 200 μl PBS. Fluorescence was detected using a microplate reader (ARVO; Perkin Elmer, Fremont, CA, USA). The fluorescence of EYFP was detected with an excitation filter of 485 nm and an emission filter of 545 nm, and that of ECFP was detected with filters of 420 nm and 486 nm (Perkin Elmer), respectively. The auto-fluorescence in COS-7 cells was subtracted from each of the detected fluorescences, and the EYFP/ECFP ratio was calculated using the resulting values.

Results

Reporter assay system

In the present reporter assay, EYFP and ECFP were selected as a reporter protein and an internal control for normalization of transfection, respectively. These two fluorescent proteins were chosen, because the peaks of their excitation and emission wavelengths are different sufficiently (a difference of 80 nm, and 50nm, respectively) so that they can be detected simultaneously without cross-detection. The considerable cross-detection between EYFP and ECFP could be prevented using a set of the optical filters (see Materials and Methods). The EYFP/ECFP ratio was calculated after the autofluorescence of COS-7 cells was subtracted from the fluorescence intensities of EYFP and ECFP, because the autofluorescence was not negligible.

The reporter plasmids were constructed as shown in Fig.1A. As hormone response elements (HREs) for FXR (FXR-RXR heterodimer), RAR (RAR-RXR heterodimer) and RXR (RXR homodimer), the fragments shown in Fig.1B were used. In order to amplify signals, we employed 3 copies of DR5 (direct repeat with 5 bp of spacing) and 4 copies of

DR1 as RAR and RXR response elements (RARE and RXRE). For the FXR response element (FXRE), 4 copies of the response element (inverted repeat) existing in the upstream region of the phospholipid transfer protein (PLTP) gene were employed. The tandem repeats in HREs elevated the response to a sufficient degree to detect the chemicals that activated the receptor. Then, an appropriate promoter for enhancing the fluorescent signal while retaining the response to the chemicals was selected from among seven promoters (Fig. 1C). Since the SV40 or CMV promoter caused a high fluorescence intensity with or without ligands, the responses to the ligands were not strong. The response of the RAR reporter plasmid with SV40 promoter was about ten-fold. However, the apparent rate of the response was enhanced by interference of the expression of ECFP by the expression of EYFP, because the same promoter was employed for the reporter plasmid and the internal control plasmid. Therefore, the rate did not reflect a real response, and had a large SD. The TK promoter, the 3' region of the TK promoter and the minimal CMV promoter caused strong responses, but the expression in the control plasmid was too low for quantitative measurement. The expression of reporter proteins with the 3' region of the CMV promoter was higher than that with TK or the minimal CMV promoter, maintaining the induction rate by the ligands. Based on a comparison between the 3' regions of the CMV promoters, we selected the CMV201 (201 bp of the CMV promoter) promoter for use in the experiments below, since the response of CMV201 was stronger than that of CMV265.

In addition to the promoter for reporter plasmids, the promoter for the internal control plasmid and the expression plasmids of nuclear receptors were examined in order to establish an appropriate assay system of the nuclear receptor ligands. When the SV40 promoter was employed for the expression of ECFP in the internal control plasmid, the SV40 promoter for nuclear receptor expression interfered with the expression of ECFP (data not shown). Therefore, the CMV promoter was employed for nuclear receptor expression

plasmids. Finally, we established the following plasmid set as the reporter assay system: a reporter plasmid containing the EYFP gene, whose expression was regulated by the hormone response element and CMV201 promoter; an internal control plasmid containing the ECFP gene expressed by the SV40 promoter; and the expression plasmid of the nuclear receptor containing each nuclear receptor gene expressed by the CMV promoter.

Figure 2A shows the response to typical agonists for FXR, RARs and RXRα in the screening system. For screening of RAR ligands, three subtypes of RARs (RARα1, RARβ2, RARγ1) were expressed in the cells independently. Although endogenous RARs co-exists in the cell, the preference for the subtype of compounds could be detected. Figures 2B and 2C show the dose-dependence of the assay system of FXR and RAR ligands, respectively. RARs were activated by 100 pM of ATRA. ED_{50} values were estimated to be about 1-10 nM for RARα and 0.1-1 nM for RARβ and RARγ (only the result of RARα is shown in Fig. 2B). On the other hand, activation of FXR was seen in 3-10 μM CDCA and greater activation was observed at 100 μM CDCA (Fig. 2C). These dose-dependent response patterns were comparable to those reported previously (Brand et al. 1988; Parks et al. 1999), indicating that these assays could be used for quantitative measurement of the activation by ligands. The established method of the reporter assay was described in the Materials and Methods.

Screening of a novel ligand for nuclear receptors

Using the established screening system, we found some natural compounds and their derivatives which acted as agonists for RARs and FXR. In the screening, there was a possibility that unexpected factors changed the signal responses (in the present assay system, the transcriptional efficiency may be changed irrespective of the nuclear receptor, the tested chemicals may have their own fluorescence, and so forth). Therefore, another reporter plasmid without HRE was also constructed to eliminate these unexpected factors. As this

plasmid was used in place of the reporter plasmid, the compounds that regulated the expression of EYFP without HRE were eliminated. Some results of the response for each nuclear receptor are shown in Fig. 3 (RAR, upper panel; FXR, middle panel; Control, lower panel). The results for RARβ are presented as representative of those for RARs. Ten mM of each compound in DMSO was added to the culture medium of the transfected COS-7 cells at a final concentration of 30 μM (Fig.3, No.1 to 26). Compounds No. 27, 28, and 29 were 3 μM ATRA, 30 μM CDCA, and vehicle, respectively. ATRA also slightly activated the FXR-RXR heterodimer, due to the activation of RXR. Although, for example, Nos.16, 18, 19, and 25 enhanced the relative EYFP/ECFP ratio, these compounds also enhanced the control that was used with the reporter plasmid without HRE. Thus it was concluded that these compounds were not ligands for the nuclear receptors.

As the result of screening more than 140 compounds (a part of theresults was shown in Fig.3), five compounds were found as ligands for the nuclear receptors. Caffeic acid phenethyl ester (CAPE, compound No.20 in Fig. 3), geranyl caffeate (No.21), and farnesyl caffeate (not shown in Fig. 3), were found as RAR agonists. ginkgolic acid 15:1 (No.12), geranyl caffeate (No. 21), and grifolin (No.26) were found as FXR agonists.

The structures of the caffeic acid derivatives tested in the screening are shown in Fig.4A. CAPE, known as active compound of propolis from honeybee hives, was synthesized from caffeic acid and β-phenylethyl bromide and other caffeic acids are purified and synthesized as described in Materials and Methods. Three of these compounds (i.e., all of those tested except for bazzanenyl caffeate) activated RARs (Fig. 4B). The cells treated with over 30 μM of these compounds were removed from wells by washing of the reporter assay, because these compounds had toxicity for cell. Therefore, the results shown are for a reporter assay conducted using lower concentrations. Although the activation of RARs could be hardly detected by low concentration of caffeic acid-derivatives, the activation by the

compounds 10-30 μM was comparable to maximum activation by ATRA. As shown in Fig. 4B, CAPE activated RARβ to a greater degree than RARα or RARγ.

As FXR agonists, geranyl caffeate, ginkgolic acid 15:1 and grifolin were found. Geranyl caffeate, the RAR agonist, highly activated FXR (Fig.3, No. 21), but the activation of RXR homodimer was not detected (data not shown). It could not be determined whether or not farnesyl caffeate, a compound similar to geranyl caffeate, acitivated FXR, because 30 μM of these compounds showed toxicity for cells. The structures of ginkgolic acids and grifolin are shown in Fig. 5A. It has been reported that ginkgolic acid 15:1 was contained in ginkgolic leaves (Ahlemeyer et al. 2001), and grifolin in mushrooms (Hirata and Nakanishi, 1949; Sugiyama et al. 1992). The activations of FXR by ginkgolic acid 15:1 and geranyl caffeate were comparable to that by CDCA, the most potent endogenous bile acid. Ginkgolic acids 17:1, 15:0 and 13:0 (described in Fig. 5A) were also investigated as the other ginkgolic acids of ginkgo leaves (Fig. 5B). Ginkgolic acid 17:1 activated FXR more strongly than did 15:1, and ginkgolic acids with an alkyl chain (13:0, 15:0) activated FXR at concentrations of more than 20 μM. It seemed that the double bond and length of the carbon chain had an influence on FXR activation. Moreover, the structures except for the carbon chain were also important for FXR activation, because the methylated compound of ginkgolic acid 15:1 (2-methyl ginkgolic acid methyl ester, Fig. 5A) had no potency for FXR activation (Fig. 3, No. 13).

Discussion

To discover ligands for the nuclear receptors, we developed a battery of reporter assay system incorporating the advantages of fluorescent proteins. The disadvantage of GFP (low sensitivity) could be overcome by modifications. The present screening system using fluorescent proteins has the clear merits of high efficiency, convenience and low cost,

because the two fluorescent signals can be measured simultaneously without addition of any co-factors. Moreover, the fluorescent signal was stable for more than 2 h after the wash. Considering these merits, this reporter assay system with fluorescent proteins might be advantageous for automatic high-throughput screening. If the expression of the fluorescent protein can be increased, the measurement of fluorescence can be carried out in culture medium, and the signal can be measured by time-course without any treatment. Moreover, the use of three fluorescent proteins (for example, DsRed with EYFP and ECFP) would enable us to carry out more efficient measurement.

Using this assay system, several compounds that induce expression of the reporter gene for RARs and/or FXR were identified. These compounds were described as ligands in this report, though there is a possibility that these compounds are metabolized and their metabolites bind to the receptors as ligands.

Three new ligands for RARs were identified: CAPE, geranyl caffeate, and farnesyl caffeate. The whole structure of these compounds may be needed for RAR-activation, because caffeic acid, a constituent compound of the compounds, did not activate RARs (data not shown). CAPE has been reported to have antioxidant, antiviral, antiinflammatory and immunomodulatory activities (Grunberger et al. 1988), and also has been shown to inhibit the growth of different types of oncogene-transformed cells and to induce apoptosis (Grunberger et al. 1988; Burke et al. 1995; Su et al. 1994, Watabe et al. 2004). Since RARs have been reported to mediate many biological processes, it is possible that some of the diverse activities are due to their binding to RARs. Since geranyl and farnesyl caffeate have also been reported to exert antioxidant effects and to inhibit the growth of cancer cells (Inoue et al. 2004), the three compounds may suppress the growth of cancer by at least two pathways: induction of RAR and antioxidant effects. Considering its preferential activation of RARβ (Fig. 4B), CAPE may inhibit cancer (e.g., lung cancer) growth more selectively

without substantial toxicity, such as the triglyceride elevation associated with RARα, and the skin, bone and teratogenic toxicity associated with RARγ. Thus, especially CAPE could be assumed to be a seed for development of anti-cancer drug.

We also found that two natural compounds, ginkgolic acids and grifolin, activated FXR. Grifolin was first isolated as an antibiotic constituent of a mushroom, *Grifola confluens* (Hirata and Nakanishi, 1949). In 1992, it was reported that grifolin decreased liver cholesterol content, plasma total cholesterol levels, and plasma (very low-density lipoprotein (VLDL) + low-density lipoprotein (LDL))-cholesterol levels, and increased plasma HDL-cholesterol and plasma triglyceride levels (Sugiyama et al. 1992). It has been suggested that the effect of grifolin might be elicited, at least in part, by the augmented excretion of cholesterol into the faces (Sugiyama et al. 1994). On the other hand, FXR controls the expression of critical genes in bile acid and cholesterol homeostasis. In fact, FXR-null mice show elevated serum cholesterol and triglyceride levels (Sinal et al. 2000), and an FXR agonist has been shown to reduce serum triglyceride levels (Maloney et al. 2000). Moreover, FXR induces the expression of the gene of phospholipid transfer protein (PLTP), which plays a role in high-density lipoprotein (HDL) metabolism (Urizar et al. 2000). It seems that the cholesterol-lowering and HDL-cholesterol-increasing effects of grifolin are relate to FXR activation, though grifolin's enhancement of triglyceride production was not consistent with its down-regulation of FXR agonists.

The FXR agonist found in this study are all non-steroidal compounds, whereas the well-known ligand of FXR, bile acid, is a steroidal one. The common characteristic of the structure of the ligands is their long carbon chains (i.e., geranyl, farnesyl and pentadecenyl), and farnesol has been shown to be a FXR ligand (Forman et al. 1995). However, aspects of the structures other than the carbon chains also appear to be important for FXR activation, because geraniol, a constituent compound of geranyl caffeate, has been reported not to

activate FXR (Forman et al. 1995), and the methylated compound of ginkgolic acid 15:1 had no potency for FXR activation in the present study.

Several compounds, such as TTNPB, GW4064, Farnesoid, Forskolin, Fexaramine, AGN29 and AGN31, have been reported as non-steroidal agonists (Maloney et al. 2000; Howard et al. 2000; Downes et al. 2003; Dussault et al. 2003). The non-steroidal ligands may be important tools for studying the pharmacology of the receptor, because they may not have the property of bile acids and are not metabolized to form harmful lithocholic acid (Fischer et al. 1996; Javitt, 1966). In the present study, ginkgolic acids and geranyl caffeate strongly activated FXR, and both have structures quite different from bile acids, so that they will be good tools in this sense. Moreover, the importance of identifying gene-selective modulators that regulate a subset of FXR-specific genes as therapeutic agents has been recognized (Cui et al. 2003; Dussault et al. 2003). The gene-selective modulators of estrogen receptor, selective estrogen receptor modulators (SERMs), have been well studied (reviewed in McDonnell et al. 2002), and some compounds with a structure divergent from that of estrogen have been identified and applied to therapies of breast cancer and osteoporosis. The non-steroidal compounds could also be good tools for studying the selective response of FXR target genes.

In this report, we developed a new method for screening novel nuclear receptor agonists, and used it to identify new candidate ligands for FXR and RARs. We expect that these new ligands will be good pharmacological tools. Since the compound whose structure is much different from bile acids is expected to possess specific effect as a ligand, we continue to screen various ligands from natural compounds with a wide variety of structures.

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Footnotes

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Figure Legends

Fig.1 **Reporter plasmids for the assay of nuclear receptors.** (A) Model of the constructed reporter plasmids. (B) The sequences for HREs of RAR, RXR and FXR (RARE, RXRE, and FXRE). (C) Effect of different promoters on the reporter assay. Seven species of promoter were employed in the reporter plasmid containing the HRE and EYFP genes. The activation of RARα (a) and FXR (b) are shown. The transfected cells were treated with ligands (black bar), 1 μM of ATRA for the RAR reporter assay or 100 μM of CDCA for FXR, or DMSO as a vehicle (white bar). The vertical axis indicates the ratio of fluorescence of EYFP (signal) to ECFP (internal control). The fold response relative to vehicle-treated cells is shown above the bars. Data are shown as the means+SD derived from six experiments.

Fig. 2 **Response in the reporter expression.** (A) The responses in the reporter assay system by typical agonists for RAR, RXR, and FXR. COS-7 cells were transfected with appropriate set of the plasmids (e.g. for assay of RARα ligand, the reporter plasmid containing RARE, the expression plasmids of RARα and RXRα, and the internal control plasmid; for assay of RXRα ligand, the reporter plasmid containing RXRE, the RXRα expression plasmid, and the internal control plasmid). The transfected cells were treated with 1 μM of ATRA, 1 μM of 9CRA, or 100 μM of CDCA as ligands (black bar), or DMSO as a vehicle (white bar). The response rate is shown above the bars. Data are shown as the means+SD derived from three experiments. (B), (C) Dose-response analyses of ATRA and CDCA on the reporter assay of RAR and FXR. Data are shown as the means+SD derived from four experiments

Fig. 3 **Screening of ligands for RAR and FXR.** COS-7 cells were transfected with the reporter plasmid, the receptor expression plasmid, and the internal control plasmid as shown in Fig.2. The cells were treated with 30 μM of each compound. The results of the screening

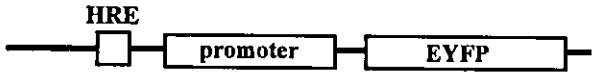
for RAR are shown in the upper panel, those for FXR in the middle panel, and those for the control (no HRE) in the lower panel. The results for RARβ are presented as representative of those for RARs.

(No.1, hydrangeic acid; No.2, ethyl 4'-ethylhydrangenate; No.3, hydrangenol; No.4, 8,3'-Dimethoxyphylodulcin; No.5, macrophyllaside A; No.6, yashabashiletodiol A; No.7, lycogarbin C; No.8, lycogarbin A; No.9, polygodial; No.10, sacculatal; No. 11, ptychantin A; No.12, ginkgolic acid 15:1; No.13, 2-methyl ginkgolic acid methyl ester; No.14, bilobal dimethyl ether; No.15, 3-tridecanyl-*m*-cresol; No.16, [11]-cytochalasa-6(12),13-diene-1,21-dione-7,18-dihydroxy-16,18-dimethyl-19-methoxy-10 -phenyl-(7*S**,13*E*,16*S**,18*S**,19*R**); No.17, hispidin; No.18, costunolide, No. 19, beta-cyclocostanolide; No.20, caffeic acid phenethyl ester; No. 21, geranyl caffeate; No.22, atroctylon)

Fig. 4 **Ligands for RARs.** (A) The structures of caffeic acid derivatives tested in the screening. (B) Response in the RAR reporter assay. The responses in the COS-7 cells expressing RARα, RARβ or RARγ are indicated by black, gray, and white bars, respectively. Data are expressed as the fold response relative to vehicle (0.1% DMSO)-treated cells and are shown as the means+SD derived from four experiments.

Fig.5 **Ligands for FXR.** (A) The structure of candidates for FXR agonists and their related compounds (2-methyl ginkgolic acid methyl ester). (B) The activation of FXR by ginkgolic acids. COS-7 cells were transfected with the reporter plasmid containing FXRE, the expression plasmids of FXR and RXRα, and the internal control plasmid. The transfected cells were treated with each compound. Data are shown as the means+SD derived from four experiments.

(A)



(B)

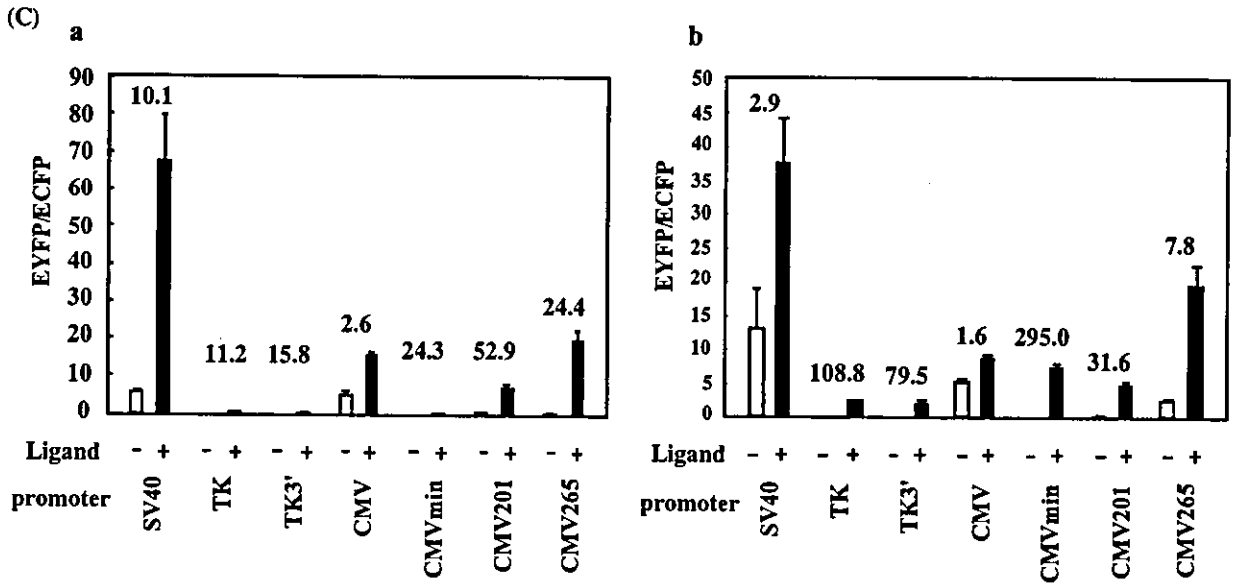
Hormone response element (HRE)

RARE cgcgtagcttcAGGTCAccaggAGGTCAgagagctagcttcAGGTCAccaggAGGTCAgagagctagcttc AGGTCAccaggAGGTCAgagagcta

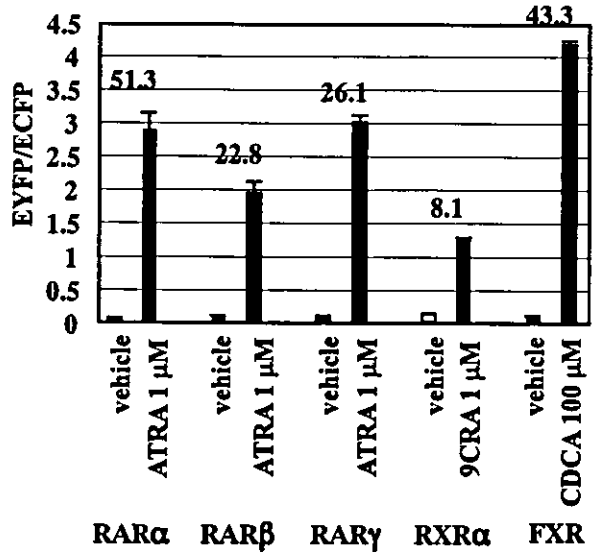
RXRE cgcgttgtcagAGGTCAgAGGTCAgAGGTCAgAGGTCAgAGGTCAgAGGTCAgAGGTCAg AGGTCAga

FXRE ggatccaaactgaGGGTCAgTGACCCaagtgaaaaactgaGGGTCAgTGACCCaagtgagaattcaaac tgagGGTCAgTGACCCaagtgaaaaactgaGGGTCAgTGACCCaagtgaagatct

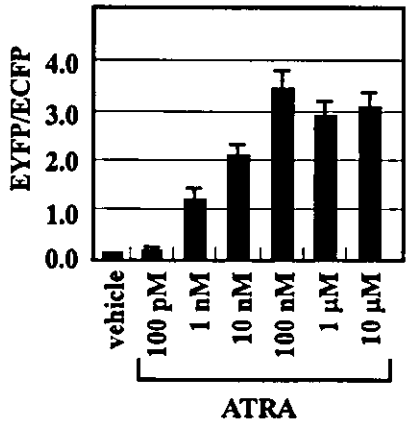
(C)



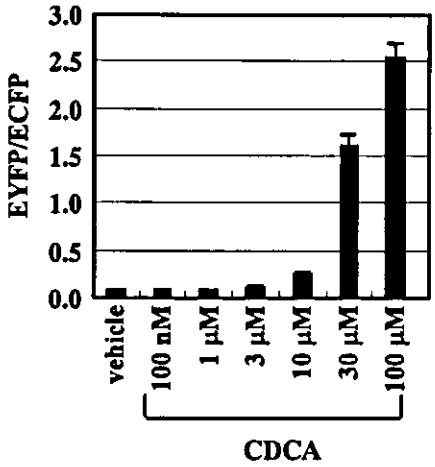
(A)


EYFP/ECFP
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
51.3
22.8
26.1
8.1
43.3
vehicle
ATRA 1 μM
vehicle
ATRA 1 μM
vehicle
ATRA 1 μM
vehicle
9CRA 1 μM
vehicle
CDCA 100 μM
RARα
RARβ
RARγ
RXRα
FXR

(B)


EYFP/ECFP
4.0
3.0
2.0
1.0
0.0
vehicle
100 pM
1 nM
10 nM
100 nM
1 μM
10 μM
ATRA

(C)


EYFP/ECFP
3.0
2.5
2.0
1.5
1.0
0.5
0.0
vehicle
100 nM
1 μM
3 μM
10 μM
30 μM
100 μM
CDCA