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(54) Title: ASSAYS FOR LIGANDS FOR NUCLEAR RECEPTORS

(57) Abstract

The present invention includes new nuclear receptor heterodimer and nuclear receptor–coactivator peptide assays for identifying ligands for nuclear receptors, utilizing scintillation proximity and fluorescence resonance energy transfer (FRET).

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ASSAYS FOR LIGANDS FOR NUCLEAR RECEPTORS

Cross reference to Related Applications

This application claims benefit under 35 U.S.C.119(e) of US provisional applications 60/150,390 filed 23 October 1998; 60/135,097 filed 23 December 1998; and 60/134,836 filed 19 May 1999.

Background of the Invention

Identification of ligands for orphan nuclear receptors has traditionally made use of either cell-based reporter gene assays or, in those cases where some ligand is known, competition binding assays. Both the transactivation and ligand-binding assays typically used to determine activity of putative nuclear receptor activators assess the effect of test ligands on an isolated receptor. The available evidence, however, indicates that a large proportion of the known orphan nuclear receptors, including the LXRs and FXR (1,2,8), function as heterodimeric receptor complexes with the common dimerization partner, RXR (reviewed in 6). Recently, it has been demonstrated that certain synthetic RXR ligands exhibit preferential activation of RXR heterodimers, and that this preference is determined by the receptor partner bound to RXR (7). These findings suggested the possibility that ligand binding to, and therefore subsequent activation of, nuclear receptors may be modulated by the receptor's dimerization state. Further, microscopic reversibility implies that ligand binding should modulate heterodimer affinity. Therefore, the ability of a ligand to induce changes in the degree of receptor dimerization could be used as the basis for a novel assay for the discovery of nuclear receptor ligands.

FXR is an orphan nuclear receptor initially identified from a rat liver cDNA library (8) that is most closely related to the insect ecdysone receptor. The ligand binding domain of the receptor was cloned and expressed in support of an effort to develop a robust assay to identify a novel ligand. The

availability of ligands for FXR will aid in the elucidation of the physiological role of this receptor. In addition, the information gained will further increase understanding of nuclear receptors as a target class.

For both FXR and LXR, a basal level of nuclear receptor-RXRα heterodimer formation is observed in the absence of added ligand. Ligands that promote heterodimer formation induce a concentration-dependent increase in time-resolved fluorescent signal. Compounds which bind equally well to both monomeric receptor and to the receptor: RXRα heterodimer would be expected to give no change in signal, whereas ligands which bind preferentially to the monomeric receptor would be expected to induce a concentration-dependent decrease in the observed signal.

Summary of the Invention

The present invention includes a generic approach to assay development for nuclear receptors, utilizing purified ligand binding domains. The concept of generic assay development has been extended to develop in vitro assays that detect ligand binding by monitoring ligand induced changes in receptor heterodimerization. This approach has been demonstrated using both scintillation proximity and homogenous time-resolved fluorimetry (HTRF) in the accompanying examples but it is not restricted to these methods. Other marking and measuring techniques may also be used. However, the use of scintillation proximity or HTRF provides a simpler and more practical methodology.

Another aspect of the invention is a new nuclear receptor-peptide assay for identifying ligands. This assay utilizes fluorescence resonance energy transfer (FRET) and was used to test whether putative ligands bound to FXR. The FRET assay is based upon the principle that ligands induce conformational changes in nuclear receptors that facilitate interactions with coactivator proteins required for transcriptional activation.

In FRET, a fluorescent donor molecule transfers energy via a non-radiative dipole-dipole interaction to an acceptor molecule (which is usually a fluorescent

molecule). FRET is a standard spectroscopic technique for measuring distances in the 10-70Å range. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's fluorescence is reduced, and the acceptor fluorescence is increased, or sensitized. FRET is frequently used in both polymer science and structural biology and has recently been used to study macromolecular complexes of DNA, RNA, and proteins. In addition, Mathis has used europium cryptates with the multichromophoric Allophycocanin to achieve an extremely large R_0 of 90Å Mathis, G. (1993) Clin. Chem. 39, 1953-1959.

Brief Description of the Drawings

Fig. 1. As shown in Fig 1, ligand binding to LXRβ measured by modulation of LXRβ:RXR heterodimer formation.

Fig.2. Fig. 2 shows ligand binding to FXR measured by modulation of FXR:RXR heterodimer formation.

Detailed Description of the Invention

In the assay of the present invention a method is provided for the rapid and simple determination of a ligand for a nuclear receptor which comprises contacting a component to be tested with an isolated nuclear receptor ligand binding domain which may be associated with a marking component, and a dimerization partner for the nuclear receptor ligand binding domain which is also associated with a marker; and measuring the interaction between the marking components to determine whether the component to be tested modifies heterodimerization. Various known markers may be used in the process of the present invention such as radioactive markers. The marker could also be a fluorescent dye. When the marker is radioactive, scintillation proximity may be used to measure the marker. When the marker used is a fluorescent dye, homogenous time-resolved fluorimetry may be used to detect the marker. Other known marking and measuring techniques may be used

depending on the marker. However, the markers need to be in close proximity to indicate heterodimerization. That is, to indicate that the component to be tested functions as a ligand for the dimerization pair.

RXRs, such as RXRα, RXRβ, and RXRγ, PPARs, such as PPARα, PPARγ, and PPARδ, LXR α, β, ERα, ERβ, CAR α, HNF4 α,β,γ, NGFIB α,β,γ, PXR, PHR, EAR-1, EAR-2, TR, RAR, and ERRs are examples of nuclear receptors that may be used as the dimerization partners and/or the nuclear receptors in the process of the present invention. RXRα is exemplified in some of the examples. Any nuclear receptor can be selected for use in an assay. More dimerization partners may be known or later discovered which can readily be utilized in the assay. It is preferable that the dimerization partners and the nuclear receptor ligand binding domains are recombinant proteins and preferably are bacterial expressed.

This method for the rapid determination of a ligand for a nuclear receptor comprises contacting a component to be tested with an isolated nuclear receptor ligand binding domain which is associated with a first marking component, and a heterodimeric partner for the nuclear receptor ligand binding domain associated with a second marking component, and measuring the interaction between the marking components to determine whether the component to be tested modifies hetero-dimerization.

The first marking component may be a radioactive marker and the second marking component (or second marker) may be a SPA bead. The interaction of the markers in this case is determined by scintillation proximity. Alternatively, the first marking component may be a first fluorescent dye emitting at an emitting wavelength which excites the second marking component which may be a second fluorescent dye. The interaction of the markers in this case is determined by homogenous time-resolved fluorimetry.

The interaction of the marking components in either case is measured by comparing a signal produced by a combination of the heterodimeric partner, the isolated nuclear receptor binding domain and the component to be tested with a signal produced by a combination of the heterodimeric

partner and the isolated nuclear receptor ligand binding domain in the absence of the component to be tested.

Example 1

This example describes the use of ligand mediated heterodimerization to quantify ligand binding to the nuclear receptor, Liver X Receptor (LXR).

Liver X receptor alpha (LXRα) is an orphan nuclear receptor initially identified from a rat liver cDNA library (1). Human LXRα (2) and LXR β (3) have also been identified. The ligand binding domains of these receptors were cloned and expressed in support of an effort to develop a robust assay to identify a novel ligand. Oxysterols, including 24(S),25- epoxycholesterol have been identified as weak activators for these receptors (4,5). The availability of more potent and selective ligands for the LXRs may aid in the elucidation of the physiological role(s) of these receptors. In addition, the information gained will further increase understanding of nuclear receptors as a target class.

This example describes the use of ligand mediated heterodimerization to quantify ligand binding to the nuclear receptor Liver X Receptor beta (LXRβ). The method measures the ability of putative ligands to mediate the heterodimerization between the purified bacterial expressed LXRβ, and RXRα, ligand binding domains (LBD). Detection of the associated LBD's are measured by time resolved fluorimetry (TRF). The purified LBD of LXRβ is labeled with biotin then mixed with stoichiometric amounts of europium labeled streptavidin (Wallac Inc). The purified LBD of RXRα is labeled with CY5^{Tm}. Equimolar amounts of each modified LBD are mixed together and allowed to equilibrate for at least one hour prior to the addition to either variable or constant concentrations of the sample for which the affinity is to be determined. After equilibration, the time-resolved fluorescent signal is quantitated using a fluorescent plate reader. The affinity of the test compound is estimated from a plot of fluorescence versus concentration of test compound added.

A basal level of LXRβ:RXRα heterodimer formation is observed in the absence of added ligand. Ligands that promote heterodimer formation induce a concentration-dependent increase in time-resolved fluorescent signal. Compounds which bind equally well to both monomeric LXRβ and to the LXRβ:RXRα, heterodimer would be expected to give no change in signal, whereas ligands which bind preferentially to the monomeric receptor would be expected to induce a concentration-dependent decrease in the observed signal.

METHODS & MATERIALS

Advance Preparation:

Human LXRβ Ligand Binding Domain (LXR β LBD; Genbank accession number U 07132, amino acids 185-461) was expressed in E.coli strain BL21 (DE3) as an amino-terminal polyhistidine tagged fusion protein. Expression was under the control of an IPTG inducible T7 promoter. DNA encoding this recombinant protein and a modified polyhistidine tag was subcloned into the expression vector pRSETa (Invitrogen).

Ten-liter fermentation batches were grown in Rich PO_4 media with 0.1 mg/mL Ampicillin at 25°C for 12 hours, cooled to 9°C and held at that temperature for 36 hours to a density of OD_{600}=14. At this cell density, 0.25 mL IPTG was added and induction proceeded for 24 hours at 9°C, to a final OD_{600} =16. Cells were harvested by centrifugation (20 minutes, 3500g, 4°C), and concentrated cell slurries were stored in PBS at -8°C.

Purification of Receptor Ligand Binding Domain

Routinely, 30-40 g cell paste (equivalent to 2-3 liters of the fermentation batch) was resuspended in 300-400 mL TBS, pH 8.5 (25mM Tris, 150 mM NaCl). Cells were lysed by passing three times through a homogenizer (Rannie) and cell debris was removed by centrifugation (30 minutes, 20,000g, 4°C). The cleared supernatant was filtered through coarse pre-filters, and TBS, pH 8.5, containing 500 mM imidazole was added to obtain a final

imidazole concentration of 50mM. This lysate was loaded onto a column (6 x 8 cm) packed with Sepharose (Ni $^{++}$charged) Chelation resin (Pharmacia) and pre-equilibrated with TBS pH 8.5/ 50mM imidazole. After washing to baseline absorbance with equilibration buffer, the column was developed with a linear gradient of 50 to 275 mM imidazole in TBS, pH 8.5. Column fractions were pooled and dialyzed against TBS, pH 8.5, containing 5% 1,2-propanediol, 5mM DTT and 0.5mM EDTA. The protein sample was concentrated using Centri-prep 10K (Amicon) and subjected to size exclusion, using a column (3 x 90 cm) packed with Sepharose S-75 resin (Pharmacia) pre-equilibrated with TBS, pH 8.5, containing 5% 1,2-propanediol, 5mM DTT and 0.5mM EDTA.

Biotinylation of LXRβ

Purified LXRβ, LBD was desalted/buffer exchanged using PD-10 gel filtration columns into PBS [100mM Na Phosphate, pH 7.2, 150 mM NaCl]. LXRβ LBD was diluted to approximately 10μM in PBS and five-fold molar excess of NHS-LC-Biotin (Pierce) was added in a minimal volume of PBS. This solution was incubated with gentle mixing for 30 minutes at room temperature. The biotinylation modification reaction was stopped by the addition of 2000x molar excess of Tris-HCl, pH 8. The modified LXR β LBD was dialyzed against 4 buffer changes, each of at least 50 volumes, PBS containing 5mM DTT 2mM EDTA and 2% sucrose. The biotinylated LXRβ LBD was subjected to mass spectrometric analysis to reveal the extent of modification by the biotinylation reagent. In general, approximately 95% of the protein had at least a single site of biotinylation; and the overall extent of biotinylation followed a normal distribution of multiple sites, ranging from one to nine.

RXRα LBD

Human Retinoid X Receptor alpha Ligand Binding Domains:
RXR-alpha LBD (amino acids 225-462) was expressed in E. coli strain BL21 (DE3) as an amino-terminal polyHistidine tagged fusion protein. Expression was under the control of an IPTG inducible T7 promoter. DNA encoding this

recombinant protein and a modified histidine tag was subcloned into the expression vector pRSETa (Invitrogen). The sequence used in the construction of RXR-alpha LBD was derived from Genbank accession number X52773.

Ten-liter fermentation batches were grown in Rich PO_4 media with 0.1 mg/mL Ampicillin at 25°C for 12 hours, cooled to 9°C and held at that temperature for 36 hours to a density of OD_{600} =14. At this cell density, 0.25 mM IPTG was added and induction proceeded for 24 hours at 9°C, to a final OD_{600} =16. Cells were harvested by centrifugation (20 minutes, 3500g, 4°C), and concentrated cell slurries were stored in PBS at -8°C.

Protein purification

Routinely, 40-50 g frozen cell paste (equivalent to 2-3 liters of the fermentation batch) was thawed and resuspended in 300mL TBS, pH 7.2, (25mM Tris, 150mM NaCl). Cells were lysed by three passages through a homogenizer (Rannie) and cell debris was removed by centrifugation (30 minutes, 20,000g, 4°C). The cleared supernatant was filtered through coarse pre-filters and TBS, pH 7.2, containing 500 mM Imidazole was added to obtain a final imidazole concentration of 50 mM. This lysate was loaded onto a column (3 x 8 cm) packed with Sepharose [Ni^{++} charged] Chelation resin (Pharmacia) and pre-equilibrated with TBS, pH 7.2, containing 50mM imidazole. After washing to baseline absorbance, the column was developed with a linear gradient of 50 to 500 mM imidazole in TBS, pH 7.2. Column fractions were pooled and dialyzed against TBS, pH 7.2, containing 5 mM DTT and .5mM EDTA. After dialysis the sample was concentrated using Centri-prep 10K (Amicon) and subjected to size exclusion with a column (3 x 90 cm) packed with Sepharose S-75 resin (Pharmacia) pre-equilibrated with the same buffer.

Biotinylation of Human Retinoid-X Receptor Ligand Binding Domain

Purified RXRα LBD biotinylation was carried out in a manner similar to that described for LXRβ LBD.

Labeling of RXRα with CY5™

Purified RXRα LBD was diluted to approximately 10μM in PBS and approximately five-fold molar excess of Cy5™ monofunctional reactive dye [NHS ester] (Amersham Life Sciences) was added in a minimal volume of PBS. This solution was incubated in the dark with mixing for 30 minutes at ambient room temperature (approximately 23°C). The modification reaction was stopped by the addition of an excess of Tris-HCl, pH 8. Fluorescent dye modified RXRα LBD was dialyzed at 4°C, with minimal exposure to light, against 4 buffer changes, each of at least 50 volumes, PBS containing 5mM DTT, 2mM EDTA, and 2% (w/v) sucrose. Aliquots were frozen on dry ice and stored at -80°C.

Preparation of CY5™ -RXRα:Streptavdin-(Europium Chelate)-LXRβ Complex

Equimolar concentrations of biotinylated LXRβ, and streptavidin-conjugated europium chelate were incubated in assay buffer containing 10 mM DTT for at least 10 minutes. To this solution equimolar concentrations of Cy5™ labeled RXRα was added and allowed to equilibrate for at least 30 minutes. The premixed receptor was then added in a one-step addition to the compound plate, utilizing e.g., a Titertek Multidrop 384.

Materials:

Cy5™ RXRα and Europium labeled streptavidin LXRβ Complex

Assay Buffer: 50 mM KCl, 0.1 mg/mL BSA, 10 mM DTT and 50 mM Tris (pH 8) The stock buffer is made by dissolving 2.853g Tris base, 4.167 g Tris hydrochloride, 3.73 g KCl, and 0.1 g fatty acid free bovine serum albumin, in 1 L of deionized water. The pH is checked and adjusted to 8.0, if necessary, before adjusting to final volume. 0.154 g of solid DTT is added per 100 mL of

buffer just before the start of an experiment.

BSA, fatty acid free

DTT

KCl

Europium labeled Streptavidin: (Wallac CR28-100)

Tris Hydrochloride

96 well plates: polypropylene for intermediate dilutions (Costar #3794) and either a clear-bottomed white SPA plates (Costar #3632) or a black Polyfiltronics plate (UP350 PSB) for assays.

Methods:

Experimental Details:

Each well to be assayed contained a previously prepared solution of CY5 ™ RXRα and Europium labeled LXR and the desired concentration of test samples or controls (100µL total volume). In general, the total volume was held constant by varying the concentration and volume of premixed receptors to compensate for any changes in the volume of a particular set of samples. The plates were incubated for at least 2 hours at room temperature and the fluorescent signal determined in a Wallac Victor Multilabel Fluorescence Reader.

Data Reduction:

For single concentration assays, the results of each test well were expressed as % of control, C, calculated according to eq. 1.

$$C = 100 * \frac{F_{sample} - F_{basal}}{F_{std} - F_{basal}} \qquad (1)$$

where F_{sample} is the signal observed in a particular sample well, F_{total} is the signal observed in the presence of control inhibitor and F_{basal} is the count rate observed in the presence of no ligand. The values used for F_{std} and F_{basal} were averages of the corresponding control wells included on every plate.

For Dose response assays, the data were first normalized to % of control using eq. (1). A plot of C_L, the % of control observed at ligand concentration L, versus ligand concentration, L was constructed. The data were fit to equation (2) to obtain best-fit parameters for the EC_{50}, F_{max} and F_{basal}.

$$C_L = F_{basal} + \frac{F_{max} * L}{EC_{50} + L} \quad (2)$$

Note that F_{max}, the maximal amplitude observed at saturating ligand concentrations, can be either a positive or negative value. The sign of this parameter indicates whether a particular test compound favors binding to the LXR:RXR complex (positive F_{max}) or to either of the component receptors in a non-heterodimeric state (negative F_{max}). Furthermore, both F_{max} and F_{basal} are expressed in units of % of a standard compound.

RESULTS

Both the magnitude and sign of Fmax (the maximal ligand-induced amplitude) must be considered. Note that the definition of the maximal response observed for 24(S), 25-epoxycholesterol = 100% is an arbitrary assignment. The purpose of the normalization is only to allow comparison of values obtained for different compounds on different days and/or using different fluorescence plate readers. The results are shown in Figure 1.

Example 2

Determination of Ligand Binding to Farnasoid X Receptor: Retinoid X Receptor Heterodimer utilizing Time Resolved Fluorimetry.

This example describes the use of ligand mediated heterodimerization to quantify ligand binding to the nuclear receptor Farnasoid X Receptor (FXR).

The method measures the ability of putative ligands to mediate the heterodimerization between the purified bacterial expressed FXR and RXRα

ligand binding domains (LBD). Detection of the associated LBD's are measured by time resolved fluorimetry (TRF). The purified LBD of FXR is labeled with biotin then mixed with stoichiometric amounts of europium labeled streptavidin (Wallac Inc). The purified LBD of RXRα is labeled with CY5™. Equimolar amounts of each modified LBD are mixed together and allowed to equilibrate for at least 1 hour prior to the addition to either variable or constant concentrations of the sample for which the affinity is to be determined. After equilibration, the time-resolved fluorescent signal is quantitated using a fluorescent plate reader. The affinity of the test compound is estimated from a plot of fluorescence versus concentration of test compound added.

A basal level of FXR:RXRα heterodimer formation is observed in the absence of added ligand. Ligands that promote heterodimer formation induce a concentration-dependent increase in time-resolved fluorescent signal. Compounds which bind equally well to both monomeric FXR and to the FXR:RXRα heterodimer would be expected to give no change in signal whereas ligands which bind preferentially to the monomeric receptor would be expected to induce a concentration-dependent decrease in the observed signal.

METHODS & MATERIALS

Advance Preparation:

Human Farnasoid X Receptor alpha Ligand Binding Domain

Human FXRα Ligand Binding Domain (FXRα LBD) was expressed in E.coli strain BL21 (DE3) as an amino-terminal polyhistidine tagged fusion protein. Expression was under the control of an IPTG inducible T7 promoter. DNA encoding this recombinant protein was subcloned into the pRSET-A expression vector (Invitrogen). The coding sequence of Human FXRα LBD was derived from Genbank accession number U 68233 (amino acids 222 to 472).

Ten-liter fermentation batches were grown in Rich PO_4 media with 0.1 mg/mL Ampicillin at 25°C for 12 hours, cooled to 9°C and held at that

temperature for 36 hours to a density of OD_{600} =14. At this cell density, 0.25 mM IPTG was added and induction proceeded for 24 hours at 9°C, to a finalOD_{600} = 16. Cells were harvested by centrifugation (20 minutes, 3500g, 4°C), and concentrated cell slurries were stored in PBS at -8°C.

Purification of Receptor Ligand Binding Domain

Routinely, 30-40 g cell paste (equivalent to 2-3 liters of the fermentation batch) was resuspended in 200-250 mL TBS, pH 7.2 (25mM Tris, 150 mM NaCl). Cells were lysed by passing 3 times through a French Press and cell debris was removed by centrifugation (30 minutes, 20,000g, 4°C). The cleared supernatant was filtered through course pre-filters, and TBS, pH 7.2, containing 500 mM imidazole was added to obtain a final imidazole concentration of 50mM. This lysate was loaded onto a column (6 x 8 cm) packed with Sepharose [Ni^{++}charged] Chelation resin (Pharmacia) and pre-equilibrated with TBS pH 7.2/ 50mM imidazole. After washing to baseline absorbance with equilibration buffer, the column was washed with one column volume of TBS pH 7.2 containing 90mM imidazole. FXRαLBD was eluted directly with 365 mM imidazole. Column fractions were pooled and dialyzed against TBS, pH 7.2, containing 0.5mM EDTA and 5mM DTT. The dialyzed protein sample was concentrated using Centri-prep 10 K (Amicon) and subjected to size exclusion, using a column (3 x 90 cm) packed with Sepharose S-75 resin (Pharmacia) pre-equilibrated with TBS, pH 7.2, containing 0.5mM EDTA and 5mM DTT.

Biotinylation of FXR

Purified FXRα LBD was desalted/buffer exchanged using PD-10 gel filtration columns into PBS [100mM NaPhosphate, pH 7.2, 150mM NaCl]. FXRα LBD was diluted to approximately 10 μM in PBS and five-fold molar excess of NHS-LC-Biotin (Pierce) was added in a minimal volume of PBS. This solution was incubated with gentle mixing for 30 minutes at room temperature. The biotinylation modification reaction was stopped by the addition of 2000x molar excess of Tris-HCl, pH 8. The modified FXRα LBD

was dialyzed against 4 buffer changes, each of at least 50 volumes, PBS containing 5mM DTT, 2mM EDTA and 2% sucrose. The biotinylated FXRα LBD was subjected to mass spectrometric analysis to reveal the extent of modification by the biotinylation reagent. In general, approximately 95% of the protein had at least a single site of biotinylation; and the overall extent of biotinylation followed a normal distribution of multiple sites, ranging from one to nine.

RXRα LBD

RXRα LBD was prepared and labeled with CY5 ^{Tm} in accordance with the procedures set forth in example 1.

Preparation of CY5 ^{TM} -RXR:Streptavdin-(Europium Chelate)-FXR Complex

Equimolar concentrations of biotinylated FXR and streptavidin-conjugated europium chelate were incubated in assay buffer containing 10 mM DTT for at least 10 minutes. To this solution an equimolar concentrations of Cy5^{TM} labeled RXRα was added and allowed to equilibrate for at least 30 min. The premixed receptor was then added in a one-step addition to the compound plate, utilizing e.g., a Titertek Multidrop 384.

Materials:

Assay Buffer: 50 mM KCl, 0. 1 mg/mL BSA, 10 mM DTT, and 50 mM Tris (pH 8) The stock buffer is made by dissolving 2.853g Tris base, 4.167 g Tris hydrochloride, 3.73 g KCl, and 0. 1 g fatty acid free bovine serum albumin, in 1 L of deionized water. The pH is checked and adjusted to 8.0, if necessary, before adjusting to final volume. 0.154 g of solid DTT is added per 100 mL of buffer just before the start of an experiment.

BSA, fatty acid free

DTT

KCl

Europium labeled Streptavidin: (Wallac CR28-1 00)

Tris Hydrochloride

96 well plates: polypropylene for intermediate dilutions (Costar #3794)

and either a clear-bottomed white SPA plates (Costar #3632) or a black Polyfiltronics plate (UP350 PSB) for assays.

Methods: The methods, data reduction and interpretation of results are as described in example 1 and the results are shown in Figure 2.

Example 3

A novel fluorescence resonance energy transfer (FRET) assay as described further infra, was used to test whether putative ligands bound to a nuclear receptor, using FXR as an example. The FRET assay is based upon the principle that ligands induce conformational changes in nuclear receptors that facilitate interactions with nuclear receptor coactivator proteins required for transcriptional activation. In an example, the ligand binding domain of FXR labeled with fluorophore allophycocyanin (APC) was incubated with a peptide containing the nuclear receptor interaction domain from the SRC-1 coactivator labeled with europium cryptate.

Both the transactivation and ligand-binding assays typically used to determine activity of putative nuclear receptor activators assess the effect of test ligands on isolated receptor. However, a large proportion of the known orphan nuclear receptors interact with cofactor or coactivator proteins as a complex. Therefore, the present invention suggests the possibility that ligand binding to nuclear receptors may be modulated by the receptor's complexation with a cofactor peptide. The ability of ligand to induce changes in the degree of this complex was then used as a basis for an inventive assay for the discovery of nuclear receptor ligands. Certain sequences of the cofactor may only be required to interact with the nuclear receptor. Various sequences of the two cofactor proteins SRC-1 and CBP were synthesized and tested in HTRF and Biacore to determine the best sequences to use. The peptide, CPSSHSSLTERHKILHRLLQEGSPS-$CONH_2$ (SEQ ID NO.:1), i.e., SRC-1(LCD2,676-700) was used in screening efforts with FXR and this forms a further aspect of this invention.

Coactivator proteins interact with nuclear receptors in a ligand-

dependent manner and augment transcription (9). A short amphipathic α-helical domain that includes the amino acid motif LXXLL (L is Leu and X is any other amino acid) serves as the interaction interface between these coactivator molecules and the ligand-dependent activation function (AF-2) located in the COOH-terminus of the nuclear receptor LBD (10). To test whether ligands would induce a conformation of FXR that favors coactivator binding, a cell-free ligand-sensing assay utilizing fluorescence resonance energy transfer (FRET) to monitor allosteric interaction of a peptide based on the sequence of the steroid receptor coactivator 1 (SRC1) with the receptor was established. The use of FRET to monitor macromolecular complex formation is well established, particularly for immunoassays (11), and this detection methodology has recently been extended to characterize ligand binding to nuclear receptors (12).

Human FXR LBD was prepared and fluorescently labeled as described in Example 2. The LBD of human FXR was labeled with the fluorophore allophycocyanin and incubated with a peptide derived from the second LXXLL (SEQ ID NO.:1) motif of SRC1 (amino acids 676 to 700) that was labeled with europium chelate. The FRET ligand-sensing assay was performed by incubating 10 nM of the biotinylated FXR LBD that was labeled with streptavidin-conjugated allophycocyanin (Molecular Probes) and 10 nM of the SRC1 peptide [amino acids 676 to 700, 5'-biotin-CPSSHSSLTERHKI**L**HR**LL**QEGSPS-$CONH_2$] (SynPEP) that was labeled with streptavidin-conjugated europium chelate (Wallac), in 50 mM Tris pH 8, 50 mM KCl, 0.1 mg/ml BSA, 1 mM EDTA, and 10 mM DTT, in the presence of test compound for 2 hours at 22°C. Data were collected using a Wallac Victor™ fluorescence reader in a time-resolved mode. The relative fluorescence was measured at 665nM and the data reduction was as described in Example 1.

Preparation of Streptavidin-(Europium Chelate)-SRC1:Steptavidin-(APC)-FXR Complex

Biotinylated SRC-1(LDC2,676-700) peptide and a ½ stoichiometric amount of streptavidin-conjugated europium chelate were incubated in assay buffer containing 10mM DTT for at least 30 minutes. A second solution of stoichiometric amounts of biotinylated FXR and streptavidin-conjugated APC were incubated in assay buffer containing 10mM DTT for at least 30 minutes. Each solution was then blocked with a 5 fold molar excess of biotin and allowed to equilibrate for at least 15 minutes. The labeled receptor and labeled peptide were mixed and again allowed to equilibrate for at least 30 minutes, then added in a one-step addition to the compound plate, utilizing, e.g., a Titertek Multidrop 384.

Materials:

APC-labeled streptavidin FXR and Europium labeled streptavidin SRC-1(LDC2,676-700)

SRC-1(LDC2,676-700:(SynPEP)

APC-Labeled streptavidin FXR and Europium labeled streptavidin SRC1(LCD2,676-700)

Biotinylated Human Farnasoid-X receptor LBD:

Biotinylated SRC-1(LDC2,676-700):Biotin-CPSSHSSLTERHKILHRLL-QEGSPS-$CONH_2$ (SynPEP)

Assay Buffer: 50 mM KCl, 2mM EDTA, 0.1 mg/mL BSA, 10 mM DTT, and 50 mM Tris (pH 8). The stock buffer is made by dissolving 2.853g Tris base, 4.167 g Tris hydrochloride, 3.73 g KCl, 0.74 g EDTA (disodium salt, dihydrate) and 0.1 g fatty acid free bovine serum albumin, in 1 L of deionized water. The pH is checked and adjusted to 8.0, if necessary, before adjusting to final volume. 0.154 g of solid DTT is added per 100 mL of buffer just before the start of an experiment.

BSA, fatty acid free

DTT

EDTA, disodium salt dihydrate

KCl

Allophycocyanin labeled streptavidin: (Molecular Probes S-868)

Europium labeled Streptavidin: (Wallac CR28-100)

Tris Hydrochloride

96 well plates: polypropylene for intermediate dilutions (Costar #3794) and either a clear-bottomed white SPA plates (Costar #3632) or a black Polyfiltronics plate (UP350 PSB) for assays.

RESULTS

Ligands increased the interaction between FXR and the SRC1 peptide as determined with time-resolved FRET. Dose response analysis showed that the ligands increased the amount of SRC1 peptide bound to the FXR LBD. A typical saturable concentration response curve characteristic of specific interaction was observed.

As used in the claims for this assay, "nuclear receptor coactivator peptide" means a peptide whose affinity for the receptor is changed in the presence of ligand and which has a LXXLL motif. Examples of coactivator peptides useful for ligand identification by this method that have been demonstrated to interact with FXR include SRC-1 and those listed below:

1. B-QEQLSPKKKENNALLRYLLDRDDPS-$CONH_2$ (SEQ ID NO.: 2), ACTR (734-758), RAC3 (724-748), SRC-3 (724-748), AIB1 (724-748), pCIP (716-740)

2. B-QEPVSPKKKENALLRYLLDKDDTKD-$CONH_2$ (SEQ ID NO.:3), TIF2 (732-756)

3. B-GSTHGTSLKEKHKILHRLLQDSSSPVD-$CONH_2$ (SEQ ID NO.:4), TIF2 (676-702)

4. B-SNMHGSLLQEKHRILHKLLQNGNSPAE-$CONH_2$ (SEQ ID NO.:5), pCIP (664-690), RAC3 (671-697), ACTR (681-707), AIB1 (671-697)

The sequences of peptides 1 and 4 appear in a number of coactivators, hence the multiple names. The "B" in the sequences stands for biotinylated, which is a modification that allows attachment of the peptide during the analysis.

The abbreviations used in the examples are included below.

ACTR	Activator for Thyroid Hormone and Retinoid Receptors
AIB1	Amplified in Breast Cancer
APC	Allophycocyanin
APMSF	p-Amidinophenylmethylsulfonylfluoride, HCl
Bestatin	[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanoyl]-Leucine
BSA	bovine serum albumin
CHAPS	(3-[3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CPM	counts per minute
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FXR	Farnasoid X Receptor
IBTG	isopropyl-β-D-thiogalactopyranoside
LBD	ligand binding domain
LXR	Liver X Receptor
OD_{600}	optical density at 600 nm
PBS	phosphate buffered saline [100mM NaPhosphate, pH 7.2, 50mM NaCl]
pCIP	Co-Integrator Protein
RAC	Receptor Activated Cofactor
RPM	revolutions per minute
RXR	Retinoid X Receptor
SA-APC	Streptavidin Crosslinked Allophycocyanin
SPA	Scintillation Proximity Assay
SRC	Steroid Receptor Cofactor
TIF	Transcriptional Intermediary Factor
Tris	tris-(Hydroxymethyl)-aminomethane

The following references are noted and the entire disclosure of each is herein incorporated by reference

1. Apfel., R.H., Benbrook; D., Lernhardt, E., Ortiz, M.A., Salbert, G. And Pfahl, M. "A novel orphan receptor specific for a subset of TREs and its interaction with the retinoid/thyroid hormone receptor superfamily" Mol. Cell. Biol. 14, 7025-7035 (1994)

2. Willy PJ. Umesono K. Ong ES. Evans RM. Heyman RA. Mangelsdorf DJ. "LXR, a nuclear receptor that defines a distinct retinoid response pathway" Genes & Development. 9:1033-45 (1995)

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Heyman R.A. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 386: 407-410 (1997)

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9. C. K. Glass, D. W. Rose, M. G. Rosenfeld, *Curr. Opin. Cell Biol.* **9**, 222 (1997); D. Moras and H. Gronemeyer, *Curr. Opin. Cell Biol.* **10**, 384 (1998).

10. B. Le Douarin et al., *EMBO J.* **15**, 6701 (1996); D. M. Heery, E. Kalkhoven, S. Hoare, M. G. Parker, *Nature* **387**, 733 (1997); G. Krey et al., *Mol. Endocrinol.* **11**, 779 (1997).

11. E. Soini, I. Hemmila, P. Dahlen, *Ann. Biol. Clin.* **48**, 567 (1990); E. F. Gudgin Dickson, A. Pollak, E. P. Diamandis, *J. Photochem. Photobiol.* **27**, 3 (1995).

12. G. Zhou et al., *Mol. Endocrinol.* **12**, 1594 (1998); L. Paige et al., *Proc. Natl. Acad. Sci USA.* **96**, 3999 (1999).

We claim:

1. A method for the rapid determination of a ligand for a nuclear receptor which comprises contacting a component to be tested with an isolated nuclear receptor ligand binding domain which is associated with a first marking component, and a nuclear receptor coactivator peptide associated with a second marking component, and measuring the interaction between the marking components to determine whether the component to be tested modifies binding between the nuclear receptor ligand binding domain and the nuclear receptor coactivator peptide.

2. The method of claim 1, wherein the first marking component is a radioactive marker and the second marking component is a SPA bead.

3. The method of claim 1, wherein the first marking component is a first fluorescent dye emitting at an emitting wavelength which excites the second marking component which is a second fluorescent dye.

4. The method of claim 1, wherein the nuclear receptor is Farnesoid X Receptor ligand binding domain.

5. The method of claim 1 wherein the nuclear receptor coactivator peptide is SEQ ID NO.:1, SEQ ID NO.:2, SEQ ID NO.:3, SEQ ID NO.:4, SEQ ID NO.:5.

6. The method of claim 2, wherein the interaction of the markers is determined by scintillation proximity.

7. The method of claim 3, wherein the interaction of the markers is determined by homogenous time-resolved fluorimetry.

8. The method of claim 1, wherein the interaction of the marking

components is measured by comparing a signal produced by a combination of the nuclear receptor coactivator peptide, the isolated nuclear receptor binding domain and the component to be tested with a signal produced by a combination of the nuclear receptor coactivator peptide and the isolated nuclear receptor ligand binding domain in the absence of the component to be tested.

9. A method of identifying compounds for the treatment of diseases or disorders modulated by FXR, comprising the step of determining whether the compound interacts directly with FXR, wherein a compound that interacts directly with FXR is a compound for the treatment.

10. A method for the rapid determination of a ligand for a nuclear receptor which comprises contacting a component to be tested with an isolated nuclear receptor ligand binding domain which is associated with a first marking component, and a heterodimeric partner for the nuclear receptor ligand binding domain associated with a second marking component, and measuring the interaction between the marking components to determine whether the component to be tested modifies heterodimerization.

11. The method of claim 10, wherein the first marking component is a radioactive marker and the second marking component is a SPA bead.

12. The method of claim 10, wherein the first marking component is a first fluorescent dye emitting at an emitting wavelength which excites the second marking component which is a second fluorescent dye.

13. The method of claim 10 wherein the heterodimeric partner is an RXR, a PPAR, LXRα, LXRβ, ER α, ERβ, CAR α, an HNF4 , an NGFIB , PXR, PHR, EAR-1, EAR-2, TR, RAR, ERRs or RAR.

14. The method of claim 11, wherein the interaction of the markers is determined by scintillation proximity.

15. The method of claim 12, wherein the interaction of the markers is determined by homogenous time-resolved fluorimetry.

16. The method of claim 10, wherein the interaction of the marking components is measured by comparing a signal produced by a combination of the heterodimeric partner, the isolated nuclear receptor binding domain and the component to be tested with a signal produced by a combination of the heterodimeric partner and the isolated nuclear receptor ligand binding domain in the absence of the component to be tested.

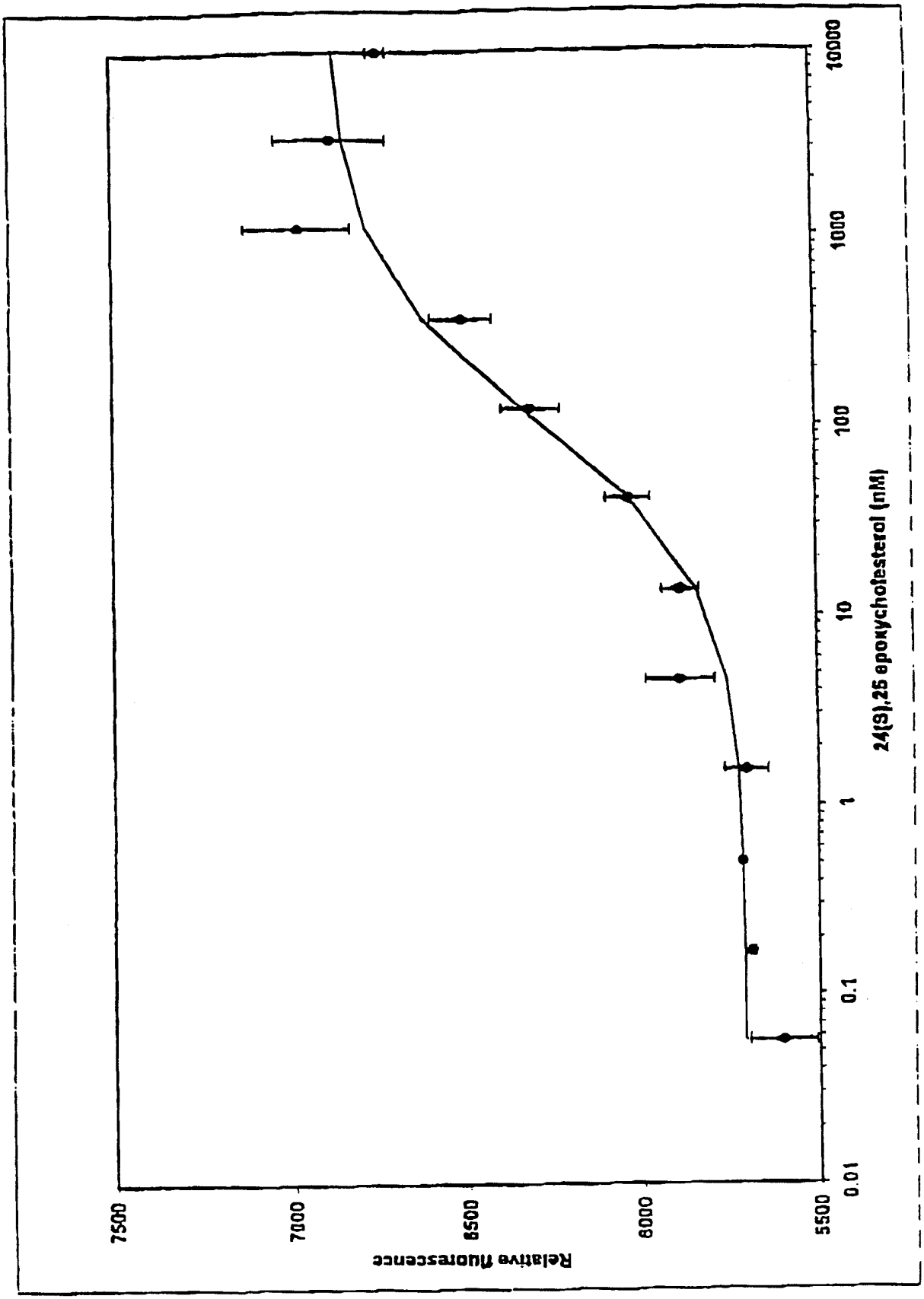
Relative fluorescence
7500
7000
6500
6000
5500
24(S),25 epoxycholesterol (nM)
0.01
0.1
1
10
100
1000
10000

Fig. 1

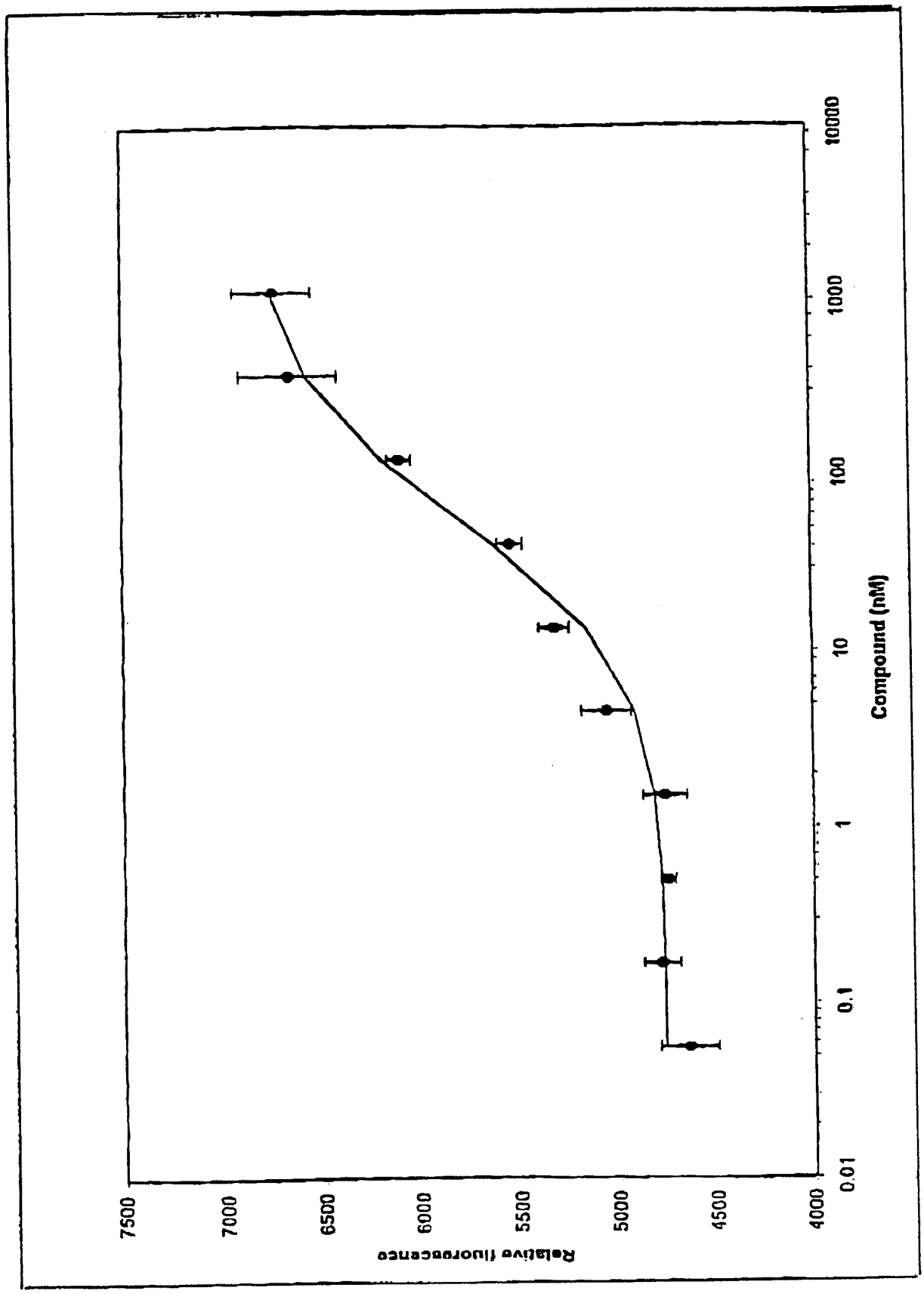
Relative fluorescence
7500
7000
6500
6000
5500
5000
4500
4000
Compound (nM)
0.01
0.1
1
10
100
1000
10000

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/24956

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 33/53; C12Q 1/70, 3/00
US CL :435/3, 5, 7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/3, 5, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US. 5,776,699 A (KLEIN et al) 07 July 1998, col. 6-col. 7.	1, 2, 4-6 and 8
A	MANGELSDORF. D.J. The RXR Heterodimers and Orphan Receptors. Cell. 15 December 1995, Vol. 83. pages 841-850, see entire document.	1, 2, 4-6 and 8
Y	MUKHERJEE. R. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agaonists. Nature.27 March 1997, Vol. 386. pages 407-410, especially page 410.	1, 2, 4-6 and 8
Y	US 4,568,649 A (BERTOGLIO-MATTE) 04 February 1986, col. 3-col. 4.	1, 2, 4-6 and 8
Y	US 5,670,360 A (THORENS) 23 September 1997, see col. 11, col. 12.	1, 2, 4-6 and 8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
08 FEBRUARY 2000	29 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRETT L NELSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/24956

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2, 4-6 and 8

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/24956

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, DIALOG, EMBASE, SCISEARCH, MEDLINE, BIOSIS
search terms: nuclear receptor, ligand binding domain, label, radioactive, coactivator protein, SPA bead, FXR, Farnesoid X Receptor, Scintillation

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-8, drawn to a method for the rapid determination of a ligand for a nuclear receptor employing an isolated receptor ligand binding domain and a nuclear receptor coactivator peptide.

Group II, claim 9, drawn to a method for identifying compounds for the treatment of diseases or disorders.

Group III, claims 10-16, drawn to a method for the rapid determination of a ligand for a nuclear receptor employing an isolated receptor ligand binding domain and a heterodimeric partner for the nuclear receptor ligand binding domain.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species correspond to Groups I and III and are as follows:

a)radioactive markers (claims 2, 6, 11, and 14); and

b)fluorescent markers (claims 3, 7, 12, and 15).

The following claims are generic: claims 1, 4, 5, and 8 are generic to Group I and claims 10, 13 and 16 are generic to Group III.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the groups recite different methods which employ different reagents, have different steps, and yield different results and PCT rules do not provide for multiple methods.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the species recite different products which have different structures and are detected differently.