Biochemical Pharmacology 80 (2010) 1221–1229

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Identification and characterization of a novel estrogenic ligand actinopolymorphol A

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ARTICLE INFO

Article history:
Received 10 May 2010
Accepted 16 June 2010

Keywords:
Estrogen receptor α
Estrogen receptor β
BRET
Xenoestrogens
Soil bacteria

ABSTRACT

Xenoestrogenic compounds are abundant in the modern environment including phytoestrogens from plants, chemical by-products from industry, and secondary metabolites from microbes; all can profoundly affect human health. Consequently mechanism-based screens are urgently needed to improve the rate at which the xenoestrogens are discovered. Estrogen Receptor (ER) dimerization is required for target gene transcription. The three ER dimer pairs (ERα/α homodimers, ERβ/β homodimers, and ERα/β heterodimers) exhibit diverse physiological responses in response to ligand-dependent activation with ERα/α homodimers being pro-proliferative and ERβ/β homodimers being anti-proliferative. The biological role of the ERα/β heterodimer remains unclear. We previously developed a cell-based, bioluminescence resonance energy transfer (BRET) assay that can distinguish natural estrogenic compounds based on their abilities to activate the three diverse ER dimer pairs. Using BRET assays, we sought to identify novel xenoestrogens from soil bacteria that preferentially activate ERα/β heterodimer with hopes of shedding light on the biological function of this elusive dimer pair. Here we describe the application of BRET assays in high throughput screens of crude bacterial extracts not previously screened for ER modulatory function and originating from unique ecological niches. Here we report the discovery and biological evaluation of a new natural product, actinopolymorphol A (1), that preferentially induces ERα/β dimerization. Actinopolymorphol A represents the first representative of a new ER modulatory scaffold.

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1. Introduction

The Estrogen Receptors (ERs) are hormone dependent transcription factors existing in two forms: ERα and ERβ. The binding of both endogenous (i.e. 17β-estradiol, also known as E2) and exogenous estrogenic ligands to these receptors induces conformational changes leading to dissociation from the Hsp90 molecular chaperone complex, subsequent receptor dimerization, interaction with coactivator proteins, and recognition of Estrogen Response Elements (EREs) in the promoter regions of target genes to activate target gene transcription. Transcriptional activity of ERs is strongly influenced by ligands at each step including (i) the binding of a given ligand for ERα vs. ERβ, (ii) the conformational changes induced upon ligand binding which influence dimer partner preference (i.e. ERα/α and ERβ/β homodimers, or ERα/β heterodimers), (iii) cofactor recruitment, and (iv) interaction with chromatin. The differential regulation of ERα and ERβ by endogenous and exogenous estrogenic compounds has extensive physiological implications, as transcriptional activation of ERα by these ligands is known to stimulate cellular proliferation, while transcriptional activation of ERβ inhibits cell growth [1–6].

In addition to the genomic transcriptional activities of ERα and ERβ in estrogenic signaling, ERs can also be regulated by growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) [7–10]. The effects of many of these growth factor pathways are believed to reflect their abilities to change the phosphorylation state of ERs, as well as that of coregulators and other proteins with which ERs interact to modulate gene expression. Furthermore, in addition to the well-documented

Abbreviations: BRET, bioluminescence resonance energy transfer; ERα, estrogen receptor α; ERβ, estrogen receptor β; LBD, ligand binding domain; FP, Fluorescence Polarization; HTS, high throughput screening; NPE, natural product extract; EDC, endocrine disrupting compound; MS, mass spectrum; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

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0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2010.06.030

synergistic effects of estrogens and growth factors on gene transcription, estrogens also exert rapid membrane-initiated effects that are known to massively impact cell signaling and may also influence gene transcription in the nucleus. Membrane-bound ERs have been shown to mediate estrogenic effects in ER-negative cells via activation of the MAPK pathway [11]. These non-genomic mechanisms of estrogenic signaling should therefore be carefully considered as important mechanisms of global estrogen action.

The cellular functions of ERα and ERβ homodimers are well established. However, the biological role of the ERα/β heterodimer remains a topic for intense study and debate, due largely to the lack of tools to study ERα/β heterodimerization in a physiological context. The co-expression of ERα and ERβ results in a heterogeneous pool of homodimers and heterodimers, and thus the activity of heterodimers cannot be deciphered from that of either homodimer, although evidence strongly suggests that ERβ antagonizes the proliferative action of ERα via formation of growth-inhibitory heterodimers [1,3,4,12,13]. To elucidate the biological role of these heterodimers, we have developed novel bioluminescence resonance energy transfer (BRET) assays in order to study ERα/β heterodimerization in a cell-based, physiological environment in real time [14]. We have used these assays to study the basic intermolecular mechanism of ERα/β heterodimerization. Another important application of the BRET assay involves the identification of selective ERα/β heterodimer-inducing small molecules. Of particular significance here is the application of the BRET assay to identify new natural products able to activate ERα/β heterodimerization.

Exogenous estrogenic ligands are ubiquitous in the natural environment and include xenoestrogenic industrial by-products and phytoestrogens such as genistein, a principle constituent of soy. Metabolites of xenoestrogenic and phytoestrogenic compounds have been demonstrated to be produced by several bacterial strains including those present in the intestinal flora [15–20] and soil bacteria [21,22]. For example, bisphenol A (BPA) can be metabolized by many organisms ranging from microorganisms to animals, and these transformations represent an important pathway for its detoxification [23]. Other studies have shown that many natural products produced by bacteria serve as xenoestrogens [24,25]. Screening of such compounds for their ability to selectively activate ER homodimers and heterodimers is important in order to determine the physiological effects of these environmental ligands as they may act through pro-proliferative ERα/α homodimers or anti-proliferative ERβ/β heterodimers. Moreover, the identification of such compounds represents an important undertaking as compounds displaying selective ER activation may serve as scaffolds which may be used for the development of novel therapeutics or biochemical tools. Inspired by the realization that the chemical structures of natural products remain either the source of, or the basis for, the majority of drug discovery and synthesis [26], this study sought to identify new natural products able to induce selective ER heterodimer formation leading to subsequent transcriptional activity with the rationale that these structures may be useful as a basis for chemical synthesis of therapeutically-useful ER dimer-selective ligands. Natural products of interest were produced by actinomycetes of terrestrial origin.

The application of our novel ER dimer-specific BRET assay [14,27] for high throughput screening (HTS) of a microbial library of crude extracts resulted in the identification of actinopolymorphol A from the actinomycete *Actinopolymorpha rutilus* whose structure has not previously been reported or characterized as an ER ligand. This discovery was enabled by the novelty of the BRET assay with its rapid in-cell format which circumvents the need for tissue-culture grade crude extracts and serves as an excellent assay for activity-guided chemical fractionation of crude extracts containing an assortment of natural products.

2. Materials and methods

2.1. High throughput screening BRET of the UWCCC SMSF Discovery Library

HTS BRET was performed at the University of Wisconsin Small Molecule Screening Facility. ERα/α homodimerization was examined using ERα-RLuc and ERα-YFP, ERβ/β homodimerization was examined using RLuc-ERβ and YFP-ERβ, and ERα/β heterodimerization was examined using ERα-RLuc and YFP-ERβ using the optimized conditions described previously [14]. Cells were transfected with these fusion proteins (0.73 μg RLuc fusion + 2.8 μg YFP fusion) in batches on 10 cm plates to reduce well-to-well variation in phenol red free DMEM + 5% SFS. Empty vector (pCMX-pL2) and RLuc fusions were also transfected alone in order to calculate the Correction Factor (CF) portion of the BRET ratio [14]. Twenty-four hours after transfection, the cells were trypsinized from their 10 cm plates and resuspended to 10,000 cells per well of 384-well white bottom plates in PBS. On each plate, dimer pairs were plated by quadrant (i.e. ERα/α homodimers were plated in quadrant 1, ERβ/β homodimers were plated in quadrant 2, and ERα/β heterodimers were plated in quadrants 3 and 4). Thus, all three dimer pairs were present within the same plate in order to avoid confounding plate-to-plate variation. Cells were treated with a final concentration of 5 μM library compounds for 1 h, and each condition was performed in triplicate for each compound. The RLuc substrate coelenterazine h was then added to a final concentration of 5 μM. The RLuc and YFP emission signals were detected at 470 nm and 530 nm, respectively, on a Victor Wallac V plate reader (PerkinElmer).

2.2. Cell-based assays

2.2.1. Cells and culture

MDA-MB-231 breast cancer cells were purchased from ATCC (cat. no. HTB-26) and were maintained in DMEM + 10% FBS. PC3 human prostate cancer cells were kindly provided by the laboratory of Dr. Douglas McNeel (Department of Medicine, UW-Madison) and were maintained in DMEM + 10% FBS, and HC11 normal mouse mammary cells were kindly provided by the laboratory of Dr. Caroline Alexander (Department of Oncology, UW-Madison) and were maintained in RPMI1640 + 10 ng/mL EGF, 5 μg/mL insulin, and 10% FBS.

2.2.2. HEK293 ERE-luciferase reporter assays

HEK293 cells were transfected in batches in 48-well plates using 2.5 ng of each indicated ER and 50 ng tk-ERE-luc vector per well as described above. After allowing 48 h for protein expression and incubating with the indicated ligands for 24 h, cells were lysed, and firefly luciferase emission was detected upon addition of the firefly luciferase substrate (Promega) on a PerkinElmer Victor 3-V plate reader using a luminescence detection setting. β-gal was analyzed using the Tropix β-galactosidase detection kit (Tropix), and emission was detected on a PerkinElmer Victor 3-V plate reader using a luminescence detection setting. Luciferase counts were normalized to β-gal counts in each well.

2.2.3. Cell growth and viability assays

1×10^5 PC3 or HC11 cells were seeded onto 6 cm plates in phenol red free DMEM + 5% SFS and allowed to attach overnight. The next day, media was replaced with media containing the indicated concentration of ligands or 0.1% DMSO, and the total amount of DMSO per plate was kept constant at 0.1%. Time points

were harvested at 24 h, 48 h, 72 h, and 96 h by trypsinizing cells, inactivating the trypsin with DMEM + 10% FBS and transferring to 2 mL eppendorf tubes, and centrifuging at 3000 rpm for 5 min. Media + trypsin was then removed from the cell pellets, and the pellet was resuspended in 200 μL PBS + 200 μL of a 1:1 dilution of Trypan blue and PBS. Cells were then loaded onto cell counting chambers (18 μL per chamber side) in duplicate and each side was read in triplicate using the Cellometer. The Cellometer protocol was "initial cell type" with a dilution of 2, and cell viability was measured on each read. The cell number per mL was then extrapolated to total cell number based on the total 400 μL volume.

2.2.4. MTT assays for cellular metabolic activity

This assay measures mitochondrial activity when yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to its purple formazan metabolic product [28]. Thus, the ability of a cell to metabolize MTT to formazan is correlated to its metabolic activity. These assays were performed in a variety of cell types following the same protocol. Cells were seeded to a confluency of ~10% on Day 1 in phenol red free DMEM supplemented with 5% FBS stripped 6 times with charcoal and dextran (SFS) in 48-well plates. Cells were allowed to attach overnight, and on Day 2 the appropriate dilution of DMSO vehicle, 10 nM E2, or actinopolymorphol A was added with a final DMSO concentration of 0.1%. Four consecutive time points were then harvested on Days 3, 4, 5, and 6 (24 h, 48 h, 72 h, and 96 h post-treatment), and ligands were refreshed every 48 h by replacing the media. Each time point was harvested by adding MTT to a final concentration of 500 μg/mL and incubating for 30 min at 37 °C, 5% CO_2 in humidified air. The media + MTT solution was removed from each well with suction and 50 μL of DMSO was added to each well, incubated at room temperature with shaking to solubilize the purple formazan crystals, and transferred to a clear flat-bottom 96-well plate. The plate was then read at 595 nm for formazan absorbance and 650 nm for background absorbance, and these values were normalized by subtraction. Each condition was performed in triplicate.

2.3. Statistical analysis

T-Tests were employed to statistically analyze data. Comparisons were made between each ligand treatment condition concentration and treatment with the vehicle DMSO.

2.4. Quantitation of proteins and mRNA

2.4.1. Western blots

Cells were lysed with Triton X-100 lysis buffer with added protease inhibitors and 50 μg total protein was loaded onto an 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes for 1 h (for ERα) or 2 h (for ERβ) at 0.35 A. Membranes were blocked overnight in 5% nonfat milk (for ERα) or 10% nonfat milk (for ERβ) in PBS Tween. ERα was detected with a 1:10,000 dilution of Santa Cruz HC-20, and ERβ was detected with a 1:5000 dilution of Santa Cruz H-150. β-Actin was detected with a 1:5000 dilution of Sigma A5441.

2.4.2. RT-PCR

Cellular RNA was isolated using the Trizol extraction method. DNA was digested with DNase I, and RNA was quantitated using the Nanodrop (Thermo Scientific, Wilmington, DE). cDNA was reverse transcribed from RNA using the Superscript II Reverse Transcription kit according to the manufacturer's instructions, and PCR amplification was performed using the following primers: ERα Forward 5′-TTATGGAGTCTGGTCCTGTG-3′, Reverse 5′-CATCATTCCCACTTCGTAGC-3′; ERβ Forward 5′-TTTGGGTGATTGCCAAGAGC-3′, Reverse 5′-AGCACGTGGGCATTCAGC-3′; β-actin Forward 5′-AGGCACCAGGGCGTGATGGT-3′, Reverse 5′-GGTCTCAAACATGATCTGGG-3′.

2.5. Fluorescence Polarization Assays for measuring ligand binding affinity to ERα and ERβ

The binding affinity of ligands for ERα and ERβ was measured using Estrogen Receptor Competitor Assays from Invitrogen (PanVera) (Part # P2614, P2698 for ERα; Part # P2615, P2700 for ERβ) according to the manufacturer's instructions. Using purified ERα or ERβ provided in the kit, serial dilutions of test compounds were prepared ranging in concentration from 2 mM to 20 pM by preparing 1:10 dilutions in provided screening buffer. The concentration of DMSO was kept below 1%. Compounds were diluted 2-fold in the final reaction with a mixture of 30 nM purified ERα, 2 nM Fluormone, and screening buffer such that the final concentration of test ligand was serially diluted 1 mM to 10 pM, the final concentration of ERα was 15 nM, and the final concentration of Fluormone was 1 nM. Test ligands were prepared in a similar fashion for ERβ using a final concentration of 10 nM ERβ. This mixture was incubated in the dark at room temperature for 2 h, and polarization values were read in individual glass tubes using a Beacon 2000 instrument.

2.6. Molecular modeling

Crystal structure of ERα-estradiol (2OCF.pdb) and ERβ-estradiol (2J7X.pdb) complex was retrieved from PDB databank (www.rcsb.org). The structure of actinopolymorphol A was built and minimized with OPLS 2005 force field in Maestro (Maestro, version 8.5, Schrodinger, LLC, New York, NY, 2008) interface. All the molecular files were prepared by Maestro in Schrodinger program. The grid-enclosing box was centered on the E2 present in the LBD with approximate dimension of 25 Å × 25 Å × 25 Å. A scaling factor of 1.0 was set to van der Waals (VDW) radii of those receptor atoms with the partial atomic charge less than 0.25. The minimized actinopolymorphol A was docked into the ligand binding pocket of each ER using Glide (Glide, version 5.0, Schrödinger, LLC, New York, NY, 2008) software package using with standard parameters (SP). All structure figures were prepared using PyMOL (DeLano Scientific).

3. Results

3.1. Identification of ERα/β heterodimer-inducing compound actinopolymorphol A by high throughput screening

3.1.1. High throughput BRET screening of the University of Wisconsin Discovery Library (WDL)

In order to identify ligands capable of differentially inducing ERα/α and ERβ/β homodimerization and ERα/β heterodimerization, a BRET assay was developed and optimized [14]. Distinct from the existing ER reporter assay, the BRET assay is exquisitely sensitive and allows the formation of different dimer types to be detected independently, including ERα/α, ERβ/β, and ERα/β dimers. This is especially important in the case of the ERα/β heterodimer, as the co-expression of ERα and ERβ allows the formation of all three dimer pairs, which prevents a clearly delineated understanding of heterodimer function *in vivo*. The BRET assay allows the visualization of ERα/β heterodimers and downstream function without interference from either homodimer. Moreover, because the BRET assay takes place in a physiological environment, it allows selection for small molecules that penetrate into the appropriate intracellular compartments. This assay involved the transfection of DNA encoding an ERα-Renilla Luciferase (RLuc) fusion protein and an ERβ-YFP fusion protein into ER-negative HEK293 cells. The high

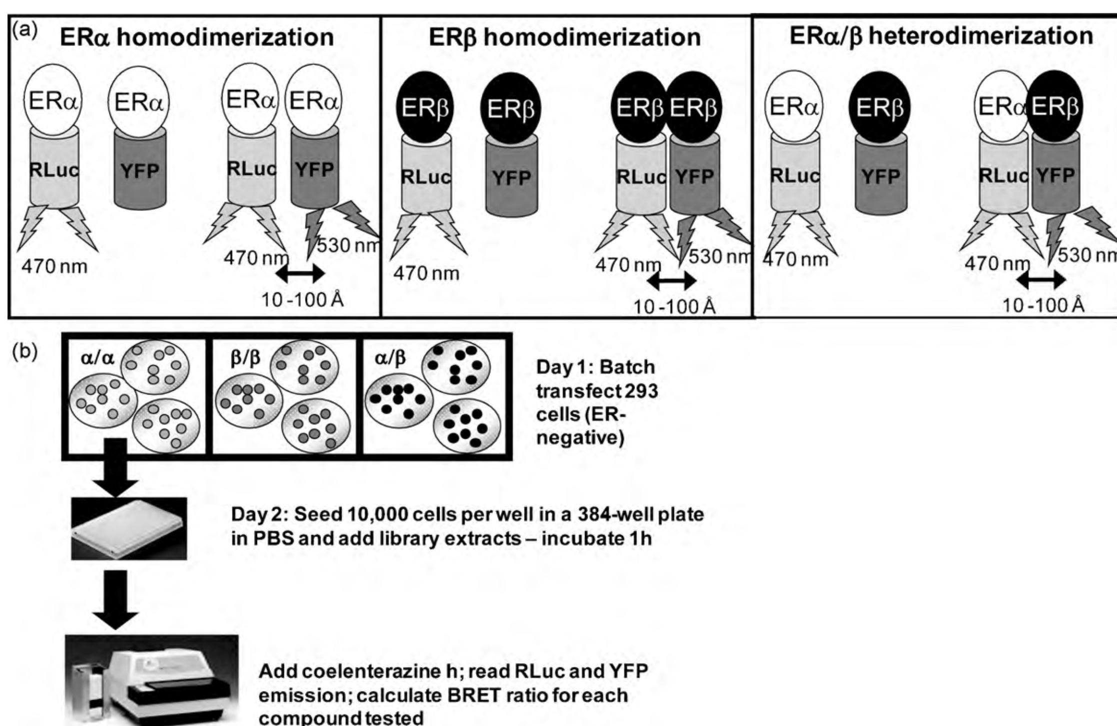

Fig. 1. BRET assay methodology. (a) Schematic representing ligand-dependent dimerization and resonance energy transfer between RLuc and YFP fusions via BRET. (b) Schematic representing the BRET assay 96-well format protocol.

transfectability (>90%), low doubling time, and ER-negative status of this cell line made it an attractive candidate for the high throughput ER BRET screening, as interference from endogenous ERs was not a concern. Cells were transfected with the fusion proteins described in the Methods section in batches in phenol red free DMEM + 5% SFS on 10 cm plates to reduce well-to-well variation. Twenty-four hours after transfection, the cells were trypsinized and resuspended to 10,000 cells per well in white 384-well plates. 1 μL Library extract was then added and incubated with cells for 1 h, at which point the RLuc substrate coelenterazine h was added to a final concentration of 5 μM. Coelenterazine h induces RLuc emission at ~470 nm; if ER dimerization has occurred, YFP is in close proximity to RLuc, which results in resonance energy transfer to YFP and its emission at 530 nm. Thus, YFP emission is indicative of dimerization (Fig. 1). After the addition of coelenterazine h, RLuc and YFP signals were detected at 470 nm and 530 nm, respectively, on a Victor Wallac V plate reader (PerkinElmer). These values were used to calculate the BRET ratio described previously [14,29]. A schematic of the BRET assay format is shown in Fig. 1b. E2 was used as a positive control. Because the ER antagonist ICI 182,780 also induces dimerization [14], vehicle (DMSO) served as the sole negative control. Internal positive and negative controls were included on each plate. HEK293 cells transfected with the ER-RLuc fusion alone (in the absence of YFP) were included on each plate and treated with the vehicle DMSO in order to calculate the Correction Factor portion of the BRET ratio [14]. Each compound was tested in an ERα/α homodimer BRET assay, an ERβ/β homodimer BRET assay, and an ERα/β heterodimer BRET assay. On each plate, dimer pairs were plated by quadrant (i.e. ERα/α homodimers were plated in quadrant 1, ERβ/β homodimers were plated in quadrant 2, and ERα/β heterodimers were plated in quadrants 3 and 4). Thus, all three dimer pairs were present within the same plate in order to prevent confounding plate-to-plate variation. Each condition was performed in triplicate for each compound. This assay setup allowed verification of the specificity of each primary hit compound based on its ability to activate ERα/β heterodimers in comparison with its ability to activate each respective homodimer.

3.1.2. Rationale for choice of University of WDL (natural products and microbial extracts library)

The University of Wisconsin is intensely interested in the discovery of new small molecules possessing novel and useful bioactivities. Reflective of this interest, researchers at UW have established a library of small molecules and microbial extracts referred to as the Wisconsin Discovery Library (WDL). The WDL is composed of a wide assortment of privileged natural products and natural product-inspired synthetic compounds in addition to >1000 natural product extracts from un- and underexplored microorganisms. The molecular diversity displayed by WDL members is extraordinary particularly among the natural product extracts which likely possess heretofore unknown structural scaffolds with, as yet, unidentified bioactivities. For this reason, we applied the BRET assay to a selection of natural product extracts available to us through the WDL. Beyond the molecular diversity presented by natural product extracts, a strong motivator for interest in the screening of natural product extracts is their ready availability via scaled up fermentation of the producing organism and our ability to produce analogs via the application of combinatorial biosynthesis methods. Finally, it is well established that terrestrial microbes are a rich source for xenoestrogens [21,22,30–33]. This realization supported our hypothesis that crude extracts from the WDL might be an excellent source of ER modulators.

3.1.3. Characterization of hit crude extracts from the WDL

HTS ERα/β heterodimer BRET assays were performed on selected members of the WDL, and crude extracts capable of inducing ERα/β heterodimerization were re-tested alongside each homodimer pair. A total of 25% of the extracts induced ERα/β heterodimerization, and the strongest 1% (10 extracts) were re-tested alongside each homodimer pair. This high number of heterodimer-inducing compounds is likely due to the large size and flexibility of the ERα and ERβ LBD, which is accommodating for a variety of chemical structures. Because heterodimerization only was initially tested, this number is not reflective of dimer selectivity. Because of the unrefined and unknown composition of

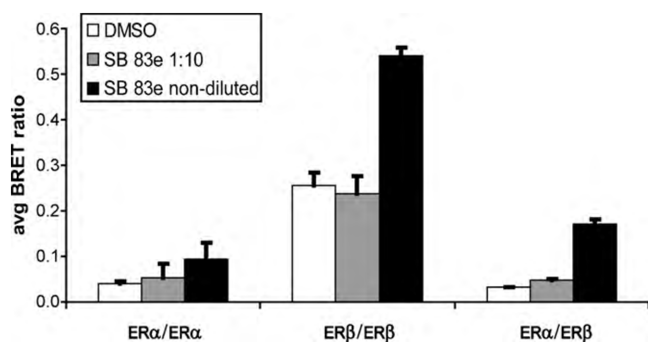

Fig. 2. BRET HTS identifies one natural product extract SB83e capable of preferentially inducing anti-proliferative ERβ/β and ERα/β dimers while minimally inducing pro-proliferative ERα/α homodimers. Error bars represent standard deviation from the mean. SB83e 1:10 diluted or non-diluted was incubated with HEK293 cells transfected with different pairs of ER fusion plasmids for 1 h before reading of Rluc and YFP signals.

crude extracts, the concentration of specific components could not be determined. Therefore extracts were tested at 1 μL per well to avoid any toxicity from DMSO. Natural product extract SB83e appeared to exhibit a preference for inducing ERα/β heterodimerization relative to either homodimer pair (Fig. 2), and was therefore chosen for subsequent investigation. While a low level of ERα/α homodimerization was also induced by this extract, the crude mixture of multiple compounds did not preclude the possibility of a heterodimer-specific compound coexistent with a dimer non-selective compound.

Bioactivity-guided fractionation of the crude extract SB83e led to the identification of one discrete compound, actinopolymorphol A, responsible for ERα/β heterodimerization observed in BRET assays [41]. Actinopolymorphol A (Fig. 3a, inset) was found to activate the transcriptional activity of ERβ alone and ERα + ERβ, but was not able to activate the transcriptional activity of ERα alone. The dimer selectivity was subsequently confirmed using the BRET assay in a dose response from 1 μM to 100 μM (Fig. 3a). These BRET assays showed an optimal 5-fold increase in ERα/β heterodimerization at 100 μM purified actinopolymorphol A, and this concentration minimally induced either homodimer (less than 1.5-fold). 10 μM purified actinopolymorphol A likewise induced 3-fold induction for ERα/β heterodimers; at this concentration neither ERα/α or ERβ/β homodimerization was induced (Fig. 3a). Similarly, both 10 μM and 100 μM exhibited a preference for the transcriptional induction of both ERβ alone and ERα + ERβ, but not ERα alone (Fig. 3b). The fold induction of both the BRET ratio and transcriptional activity of ERα + ERβ was greater than that obtained with ERβ alone, indicating that this compound retains some level of heterodimer selectivity. Furthermore, this transcriptional activity on an ERE-luciferase reporter was shown to be ER-specific in the presence of the ER pure antagonist ICI 182,780 (Fig. 3b). ICI 182,780 completely abrogated actinopolymorphol A-dependent reporter activity in the presence of ERα + ERβ (Fig. 3b). This is in contrast to the failure of ICI 182,780 to reduce the basal transcriptional activity of ERβ homodimer alone (Fig. 3b). ERβ was previously found to display a high level of ligand-independent dimerization [14] and transcriptional activity [34]. The apparent discrepancy between BRET and reporter assays for ERβ/β homodimers at 10 μM purified actinopolymorphol A is likely due to the lower sensitivity of the ERβ/β homodimer BRET assay relative to the reporter assay because the BRET assay only captures receptor dimerization transiently whereas the reporter assay detects accumulated product formation over time. Synthetic actinopolymorphol A was confirmed to retain its ability to selectively induce ERα/β dimerization (Fig. 4a) and to enhance transcriptional activity (Fig. 4b) of ERs in a fashion comparable to the natural product from *Actinopolymorpha rutilus*. Thus, because this compound selectively induced the activity of anti-proliferative

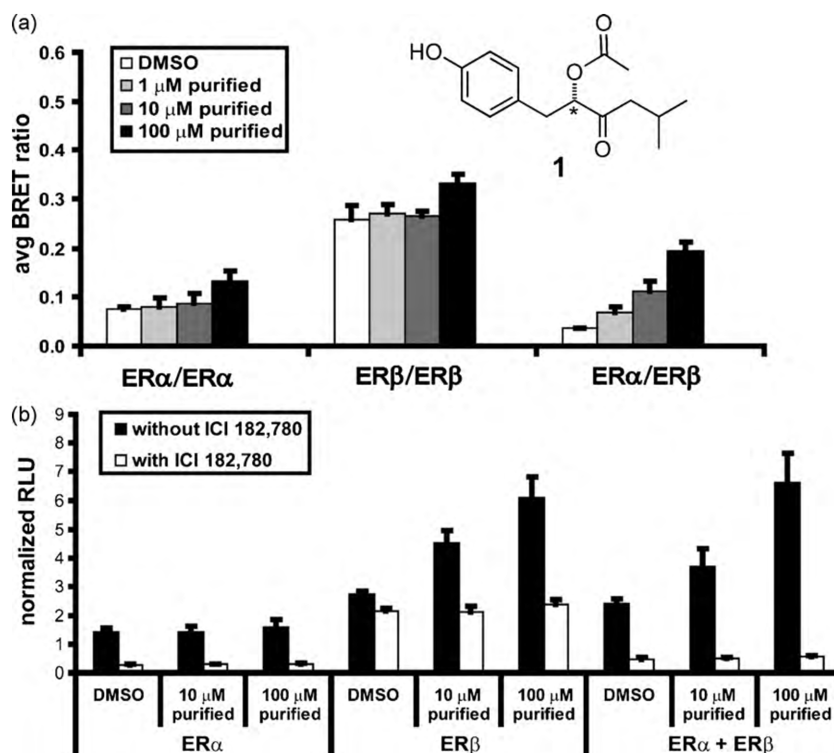

Fig. 3. Dimer-selective natural product extract SB83e was fractionated, and the constituent compound responsible for dimer selectivity was identified and purified. (a) BRET assays showing the dimer selectivity of actinopolymorphol A for ERβ/β and ERα/β. (b) ERE-luciferase assays in HEK293 cells showing the ability of actinopolymorphol A to induce the transcription of ERα alone (ERα homodimers), ERβ alone (ERβ homodimers), or ERα + ERβ (all three dimer pairs). The transcriptional response is ablated by the ER antagonist ICI 182,780. Error bars represent standard deviations from the mean.

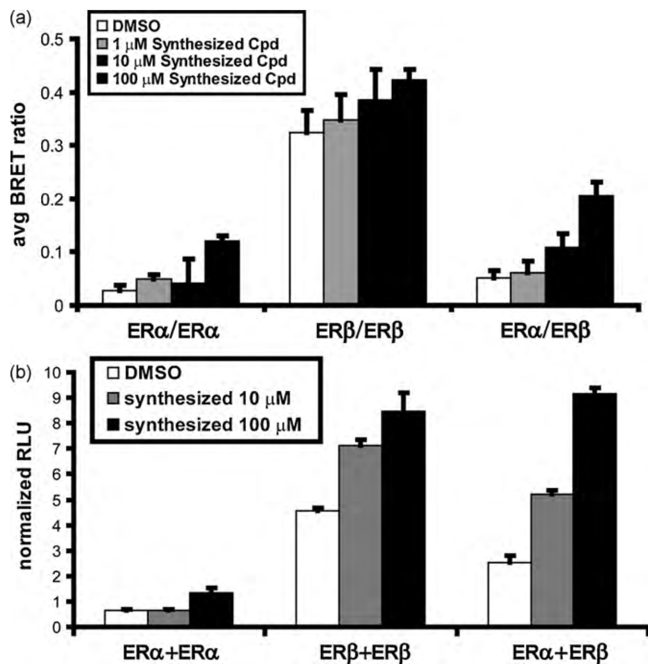

Fig. 4. The dimer selectivity for synthetic actinopolymorphol A was confirmed via BRET assays (a) and ERE-luciferase assays in HEK293 cells (b). Error bars represent standard deviations from the mean.

ERβ/β homodimers and ERα/β heterodimers while not having a pronounced effect on the activity of proliferative ERα/α homodimers, we hypothesized that this compound may be able to inhibit cell growth by enhancing ERβ dimerization leading to a dampening of the proliferative effects of ERα.

3.2. Molecular characterization of actinopolymorphol A

3.2.1. The novel ER heterodimer-inducing actinopolymorphol A inhibits cellular proliferation

Synthetic actinopolymorphol A was used in cell proliferation and viability assays in ERα and ERβ positive cell lines to determine its effect on cell growth. Fig. 5a shows confirmation of ERα and ERβ expression in these cell lines by RT-PCR (left panel) and Western blotting (right panel). MDA-MB-231 was used as a negative control. Consistent with previous reports, HC11 mouse mammary epithelial cells and PC3 human prostate cancer cells have both ERα and ERβ co-expressed [2,35]. Fig. 5b shows the effect of the novel compound in HC11 cells (top panels) and PC3 cells (bottom panels) on cell number (left panels) and viability (right panels). In HC11 cells, a statistically non-significant decrease in cell number was observed in the presence of 10 μM compound, but these decreases are statistically significant at 100 μM ($p = 0.06$ and 0.02, respectively). Neither concentration was generally cytotoxic compared to the vehicle DMSO in this cell line, as observed by Trypan blue staining. In PC3 cells, statistically significant decreases in cell number are observed in the presence of both 10 μM and 100 μM actinopolymorphol A ($p = 0.03$ and $p = 0.002$, respectively). Similarly to HC11 cells, neither concentration was generally cytotoxic to PC3

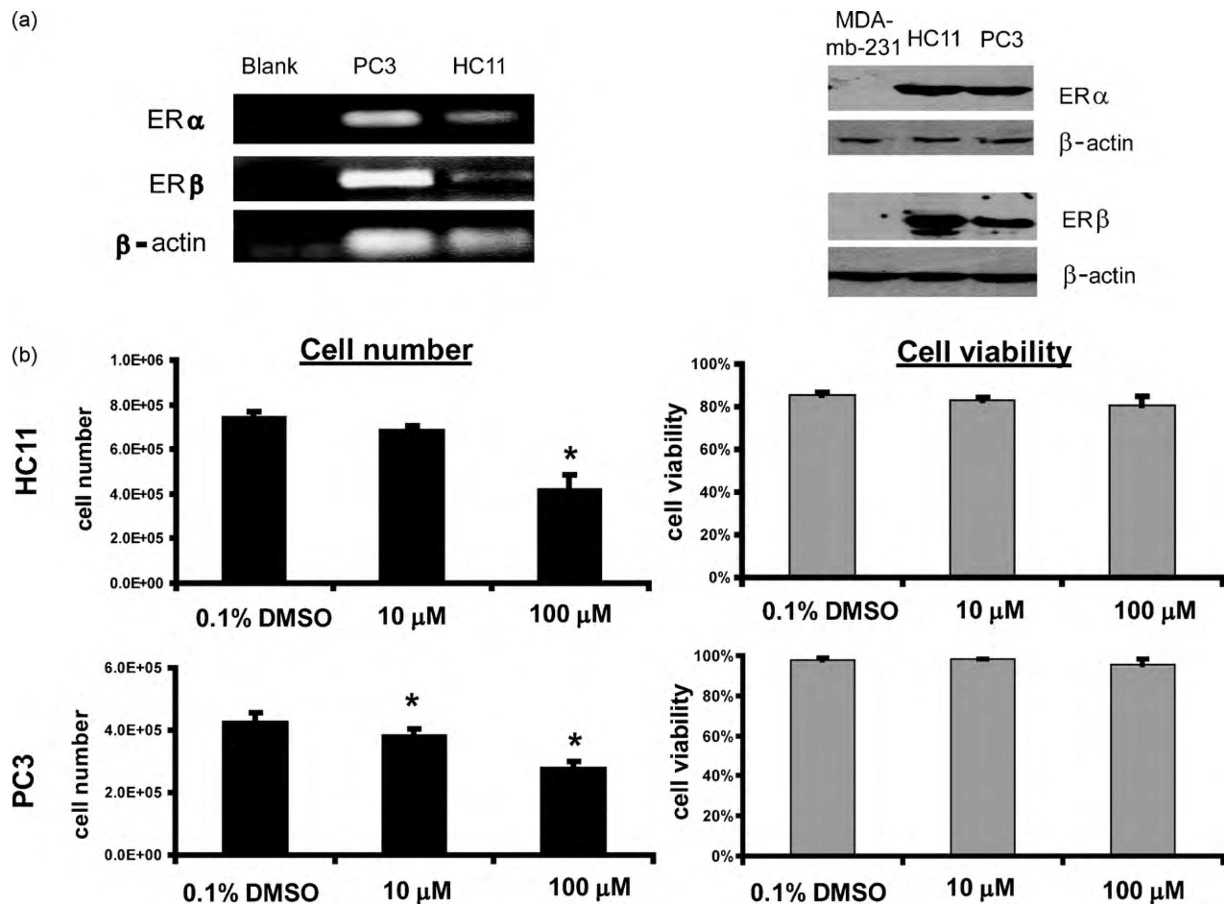

Fig. 5. The synthetic actinopolymorphol A inhibits cell growth. (a) ERα and ERβ expression in PC3 and HC11 cells was confirmed via semi-quantitative RT-PCR (left panel) and Western blotting (right panel). β-Actin served as a loading control. (b) Cell growth and viability were decreased by treatment with the synthetic actinopolymorphol A in both cell lines as determined by cell counting and Trypan blue staining. Error bars represent standard deviations from the mean.

cells. We hypothesize that the difference in the compound's ability to influence cell growth in these two cell lines depends on the relative expression level of ERα and ERβ as well as the expression levels of coactivator and corepressor proteins. The effect of actinopolymorphol A on growth and viability cannot be completely reversed by the antagonist ICI 182,780 in ER-positive HC11 and PC3 cells (Fig. S1), suggesting that other pathways, including non-genomic estrogenic signaling pathways, could be partially responsible for this compound's effects on cell growth. Thus, actinopolymorphol A is a weak estrogenic ligand which may exert cellular effects through both genomic and non-genomic pathways.

3.2.2. Actinopolymorphol A binds differentially to both ERα and ERβ

In order to determine the binding affinity of actinopolymorphol A to ERα and ERβ, we employed *in vitro* Fluorescence Polarization (FP) Competition Binding Assays. The basis of this assay lies in the capturing of polarized light in horizontal and vertical planes if a fluorescent ligand remains stationary by binding to a protein during plane-polarized light excitation. A non-fluorescently labeled compound is then titrated to a saturating concentration of fluorescently labeled ligand, and the degree of competition is measured as the fluorescently labeled ligand is competed off and freely tumbled during the period of excitation, the emitted light will be random or depolarized. Measurements of the binding affinity of actinopolymorphol A for recombinant ER proteins using this assay were validated commercially [36,37] and in our experiment using the characterized compound genistein (Fig. 6a). Genistein has been reported to have a ~10-fold greater binding affinity for ERβ over ERα [38], and our results showed a ~15-fold greater binding affinity for ERβ (Fig. 6a). As shown in Fig. 6b, actinopolymorphol A was able to effectively compete with fluorescently labeled E2 for binding to both ERα and ERβ with a ~2-fold higher affinity for binding to ERβ. This data strongly suggests the ability of this compound to bind within the ligand binding domain (LBD) of ERα and ERβ, and furthermore, the differential binding affinity likely contributes to the compound's ability to selectively activate ERβ/β homodimers relative to ERα/α homodimers. The IC_{50} values for actinopolymorphol A binding to ERα and ERβ were 29 μM and 15 μM, respectively, correlating to K_i values of 6.2 μM and 4.3 μM, respectively.

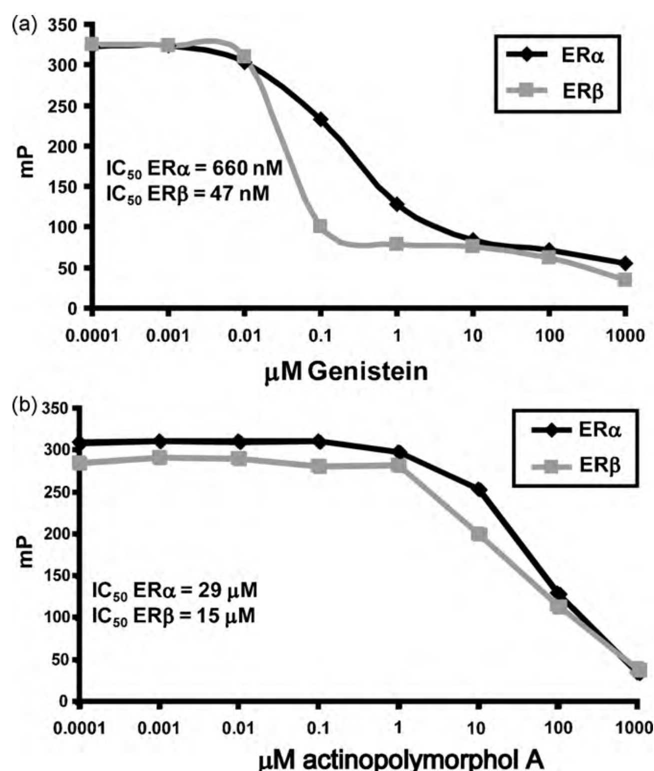

Fig. 6. Fluorescence Polarization Competition Binding Assays for ERα and ERβ. (a) Genistein binds to ERα and ERβ with different affinities consistent with previous reports and served as a positive control for the Fluorescence Polarization Competition Binding Assay. (b) The synthetic actinopolymorphol A binds to ERβ with 2-fold higher affinity than to ERα.

3.3. Actinopolymorphol A induces the agonist conformation of both ERα and ERβ

Molecular modeling indicated that actinopolymorphol A is well accommodated in the agonist conformation in the LBD of both ERα and ERβ (Fig. 7). The C-3 hydroxyl group of A-ring of 17β-estradiol forms strong hydrogen bonds with conserved residues Glu353 and Arg394 from one side of the ligand binding pocket of ERα (Glu260 and Arg301 in ERβ) (Fig. 7b and e). The 17β-hydroxyl of estradiol makes direct hydrogen bond with the residue His524 on the other side of the binding pocket of ERα (His430 in ERβ). The phenolic hydroxyl group of actinopolymorphol A mimics the C-3 hydroxyl group of 17β-estradiol and participates in the same kind of hydrogen bond interactions observed with residues Glu and Arg in both ERs' binding pockets. However, by virtue of its limited size and simple architecture, actinopolymorphol A cannot extend to the

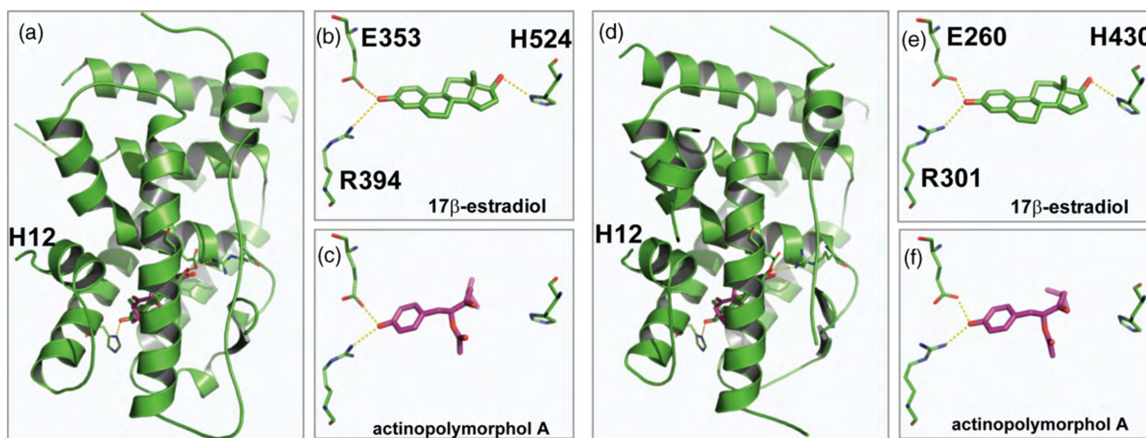

Fig. 7. Comparison of predicted binding mode of actinopolymorphol A and binding mode of 17β-estradiol from crystal structure. (a and d) Superimposition of actinopolymorphol A and 17β-estradiol in the ligand binding domain of ERα (a) and ERβ (d). (b and e) Important interaction between estradiol and residues in the ligand binding pocket of ERα (b) and ERβ (e). (c and f) Important interactions between actinopolymorphol A and residues in the ligand binding pocket of ERα (c) and ERβ (f). The hydrogen bonds were shown as yellow dash line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

right side of the LBD pocket to form tight interactions with residue His514 in ERα or His430 in ERβ, which is likely responsible for the lower docking scores compared to E2. The relatively similar docking scores of actinopolymorphol A to ERα (−8.13) and ERβ (−8.02) agree well with our findings of similar K_i values from Fluorescence Polarization Competition Binding Assays. These high docking scores indicate that actinopolymorphol A takes an acceptable low-energy conformation and is accommodated by the physic-chemical environment of the pocket. Thus, based on the binding mode predicted by this molecular modeling, more potent ER dimer-selective estrogenic compounds could be designed based on the structure of this scaffold.

4. Discussion

It is well established that phytochemicals serve as recruitment signals resulting in the symbiotic activation of plant growth signals by soil bacteria, and that the presence of endocrine disrupting compounds (EDCs) contained in pesticides and other industrial by-products can disrupt this process [38–40]. The direct production of xenoestrogenic compounds by soil bacteria is not as well established and therefore represents an opportunity for discovery of new chemical scaffolds with possible utility as, or the potential for optimization of, ER modulators. Secondary metabolites have been identified through drug discovery methods and include valuable compounds such as antitumor and antibacterial agents. The University of Wisconsin's WDL contains crude bacterial extracts consisting of various natural products and was therefore screened using three highly optimized BRET assays to identify novel inducers of ERα/α and ERβ/β homodimerization as well as ERα/β heterodimerization. The application of these BRET assays to screen identify new ER modulators from crude extracts obtained from actinomycetes originating from unique ecological niches resulted in the identification of a novel, previously uncharacterized, dimer-selective ER agonist named actinopolymorphol A.

High throughput, mechanism-based assays are on call to advance the discovery of xenoestrogens and drug leads at a rapid pace. Two types of high throughput assays are popularly used for large-scale screening of ER structural scaffolds and agonists or antagonist ligands. A Fluorescence Polarization (FP) method that measures the capacity of a competitor chemical to displace a high affinity fluorescent E2 from purified, recombinant ERα or ERβ have been adapted for testing environmental chemicals for ER binding interactions [36]. However, this method requires pure preparation of receptor, and the fluorescence from the test compounds could interfere with fluorescence readout [36]. Thus far this method has been restricted to use with pure compounds and has not been applicable to whole cell extracts or bioassay-guided fractionation efforts.

Transcriptional reporter assays can be applied to library extracts or compounds but require 18–24 h incubations. Thus, these extracts or compounds must be sterile and of high-quality tissue-culture grade to avoid contamination and concomitant ablation of the transcriptional output signal. The BRET assays described herein circumvent this issue because the library extract or compound in question needs only to be incubated with cells expressing ER fusion proteins for 1 h to induce dimerization. Furthermore, the three possible ER dimer pairs (ERα/α homodimers, ERβ/β homodimers, and ERα/β heterodimers) may be directly examined in parallel yet in isolation from each other, thus providing an added layer of sensitivity and complexity to the library extract or compound's ability to act as an agonist or antagonist ligand. Thus, the utility of this cell-based assay for high throughput crude extract screening lies in its rapid assay time frame. In addition, this method does not require sterile tissue-culture grade extracts. Using this BRET screening method, the crude extract from *A. rutilus* was found to selectivity induce formation and transcriptional activity of ERβ/β homodimers and ERα/β heterodimers. This screening method allowed assay-guided fractionation of the extract, and the pure compound responsible for induction of dimerization and subsequent transcriptional activity was identified as actinopolymophol A, a previously unknown natural product. However, estrogenic compounds identified by BRET assays may activate both genomic and non-genomic signaling pathways. Different from the classical genomic signaling through EREs, these non-genomic signaling pathways initiated at the cell membrane may also require receptor dimerization and couples with a variety of other signaling partners that can eventually culminate in the phosphorylation of transcription factors and their partners, ultimately influencing transcriptional outcome, and thus physiological effects such as cell division and apoptosis. Thus, the physiological effects of BRET identified compound await further characterization.

It is worth noting that BRET assays measure the ligand's ability to induce receptor dimerization and the FP method measures ligand replacement of E2 in ER pocket; neither of these assays can distinguish agonists from antagonists. Transcriptional reporter assays measure the ability of the lead compound to induce or inhibit (in the presence of E2) transcription of ER subtypes, allowing determination of agonist or antagonist activity of a ligand. For example, while a low level of ERα/α homodimerization was induced by this compound and substantiated via the BRET assay, these proliferative dimers were not transcriptionally active in ERE-luciferase reporter assays. Because of the limitation of each assay, all three were employed in the present study leading to the discovery of the estrogenic natural product.

The structure of this novel ER dimer-selective natural product from *A. rutilus* was determined by NMR and mass spectroscopic analysis and its absolute stereochemistry was established by total synthesis using an optically pure starting material ((*S*)-2-hydroxy-3-(4-hydroxyphenyl)-propionic acid). [41]. Combined, the results of structural characterization and coordinated BRET assays reveal that actinopolymorphol A represents a novel scaffold for estrogenic small molecule design. Molecular modeling suggests that, although the phenolic hydroxyl group of actinopolymorphol A mimics the C-3 hydroxyl group of 17β-estradiol and makes the same hydrogen bond interactions with residues Glu and Arg in both ERs' binding pockets (Fig. 7), other structural elements of the natural product do not strictly adhere to predictions likely to be made on the basis of other ER ligands such as tamoxifen or raloxifene. The modeled structure explains how actinopolymorphol A may compete with E2 in binding to the same LBD in FP assay while also displaying a lower binding affinity due to the absence of functionalities needed to H-bond with histidine distal to the Glu and Arg end of the ER LBD. This modeling indicates that the agonist or partial agonist conformation is adopted by ERα and ERβ and that their ligand binding cavities are shaped into a low-energy conformation by actinopolymorphol A. Perhaps most significantly, actinopolymorphol A is, to the best of our knowledge, the first ER dimerization modulator identified from actinomycetes. As such, discovery of this natural product and subsequent association with ER modulatory function unveils a new molecular scaffold with a novel and potentially useful bioactivity. This structure may serve as a molecular scaffold upon which chemical modifications may be made in order to increase the selectivity and efficacy of this novel compound.

The application of ER BRET assays for high throughput screening of crude natural product extracts and subsequent bioassay-guided fractionation leading to the identification of actinopolymorphol A showcases a new molecular scaffold but also highlights the utility of BRET assays in discovering new, otherwise difficult to detect, natural products with ER modulatory activity. These ER modulatory compounds may function through genomic

or non-genomic signaling pathways. Follow-up assays showed that actinopolymorphol A is able to act as an agonist on ERs and can decrease the growth of ERα and ERβ positive cell lines while not adversely affecting their viability. Despite its low activity on ERs, this novel structure is able to compete with endogenous E2 for LBD binding in both ERα and ERβ. Competitive LBD binding by actinopolymorphol A is rationalized on the basis of molecular modeling which suggests the natural product can induce an agonist conformation upon binding to both ERs. Combined, these data reveal the unique application of BRET assays to find new ER modulators and reveal actinomycetes as a potentially rich source of such bioactive natural products, the apparent first example of which, highlights a unique molecular scaffold which may serve as a lead for drug discovery and therapeutic intervention in ER dependent diseases such as breast and prostate cancers.

Acknowledgements

We thank Erin Shanle for proofreading the manuscript and the Analytical Instrumentation Center of the School of Pharmacy, UW-Madison for support in obtaining MS and NMR data. This work is supported by NIH Grants R01CA125387 and R03MH089442 to W.X., T32 CA009135 to E.P. and CA113297 to B.S.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.06.030.

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