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Peptoids on Steroids: Precise Multivalent Estradiol – Peptidomimetic Conjugates Generated *via* Azide – Alkyne [3 + 2] Cycloaddition Reactions

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Abstract
We have developed a family of functionalized peptidomimetic oligomers for the multivalent display of bioactive ligands in a site-directed manner. Sequence-specific *N*-substituted glycine peptoid oligomer scaffolds were synthesized on solid phase to include up to six azidoalkyl sidechains. These constructs were used as substrates for Cu(I)-catalyzed azide – alkyne [3 + 2] cycloaddition reactions. 17α-ethynylestradiol was conjugated at up to six positions along the peptoid backbone, generating estradiol – peptidomimetic conjugates in good yield. We evaluate how the binding avidities of these compounds to the estrogen receptor are enhanced when the valency of hormone ligand presentation is increased.

1 Introduction

Multivalent binding events may exhibit unique collective properties fundamentally different from the corresponding additive monovalent interactions [1]. Multivalent presentation of a receptor ligand on a polymer scaffold can improve the strength of ligand – receptor interactions by increasing the effective local concentration of the ligand, giving rise to enhanced binding avidity [2]. In addition, multivalent displays can be used to establish constructs with multiple low-affinity ligand – receptor interactions. Such constructs have been demonstrated to yield enhanced specificity for targeted receptors, thus mimicking the highly selective natural cell recognition events that are typical in living systems. Oligomeric or polymeric species that multivalently display bioactive ligands can be used to study the topology of clustered receptors and adhesion proteins present on the cellular membrane [3]. Consequently, multivalent oligomers that precisely display an array of recognition elements are of particular interest to bioorganic and medicinal chemists [3b]. Many current synthetic techniques used to develop multivalent constructs, such as block copolymerization and dendrimer synthesis can result in polydisperse products or random disposition of the displayed groups [2b]. Thus, there is a need to develop general synthetic strategies for improving the precision for display of binding elements on synthetic scaffolds [2b, 2c]. In this report, we outline a "click chemistry" protocol facilitating the generation of monodisperse, multivalent oligomers that display bioactive ligands in a precise, site-directed fashion.

The Cu-catalyzed azide – alkyne [3 + 2] cycloaddition reaction has emerged as a powerful tool in the fields of medicinal and bioorganic chemistry (reviewed in Ref. [4a]). This regiospecific and high-yielding reaction can be used in conjunction with a myriad of synthetic procedures under mild conditions. Owing to the broad orthogonality of azide – alkyne cycloadditions to most biotic chemical functionalities, click chemistry approaches have been developed to functionalize biomolecules, synthesize macromolecular architectures, and to perform conjugation reactions onto the surfaces of viral capsids and bacterial cells [4]. Due to the high yields for multisite modifications, Cu-catalyzed azide – alkyne cycloaddition reactions have been effective for the construction of multivalent displays [5]. We have previously demonstrated that Cu-catalyzed azide – alkyne [3 + 2] cycloaddition reactions are compatible with multisite conjugation procedures that allow for diverse chemical functionalities to be multivalently displayed along linear oligomer scaffolds [6].

N-substituted glycine "peptoids" are a class of sequence-specific oligomers that have proven valuable for biomedical research due to their peptide-like composition

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and proteolytic resistance [7]. Peptoid oligomers can be synthesized on solid-phase support using facile, automated synthesis procedures and can incorporate an extremely diverse array of sidechain chemical functionalities [7a]. Recently, we reported that peptoid sidechains including azide or alkyne functionalities can be modified in good yield using a Cu(I) catalyzed azide–alkyne [3+2] cycloaddition reaction [6]. This conjugation technique can be utilized to multivalently display bioactive ligands along the oligomer scaffold and allows for precise control over the spacing of the displayed functionalities. We now seek to develop ligand–peptoid conjugates to address targets of therapeutic importance.

The Estrogen Receptor (ER) is a ligand-mediated transcription factor that binds the natural female sex hormone 17β-estradiol (E2) [8]. Alterations in ER-mediated responses have been associated with a variety of diseases including cancer and osteoporosis [9]. The ER exists as two distinct isoforms, ERα and ERβ, which bind E2 with similar affinity and can function differentially in both homo- and heterodimeric form [10]. The classic mechanism of ER action involves E2 binding to the ER, after which the receptor undergoes a conformational rearrangement, allowing the receptor to dimerize and bind DNA at specific gene promoter sites in the nucleus [11]. In addition, it has recently been reported that functional ERs localize at the cell membrane and in the cytoplasm where they are involved in "non-genomic" actions, such as regulation of protein phosphorylation cascades (reviewed in Ref. [12]). A complex relationship between membrane, cytosolic, and nuclear actions of E2 has been described. However, the interaction between ER-mediated genomic and non-genomic pathways remains poorly understood [11, 13]. Further elucidation of the varying mechanisms of ER action is a priority for enhancing knowledge of ER-related cancer pathogenesis and molecular pharmacology. One promising approach is to develop estrogenic ligands that are capable of selectively activating one specific pathway [14]. For example, macromolecular forms of estradiol, such as E2 conjugated to bovine serum albumin or to dendrimers, have been used in studies aiming to activate the membrane-associated ER exclusively [15]. However, the stability and activity of E2–macromolecule conjugates is highly variable, establishing a demand for optimized estrogen conjugates.

We have adapted our synthetic techniques using Cu-catalyzed azide–alkyne [3+2] cycloaddition reactions [6] to site-specifically conjugate steroid hormones at multiple positions onto peptoid scaffolds through 1,4-disubstituted-1,2,3-triazole linkages. By multi-site cycloaddition of unprotected 17α-ethynylestradiol (EE2), we have generated a family of oligomers we term estradiol–peptidomimetic conjugates. In this report, we outline the synthesis, characterization, and *in vitro* binding activity of a small library of multivalent conjugates to the ER. Using similar reaction techniques, we have also synthesized a set of multivalent ethisterone-peptidomimetic conjugates, demonstrating the general applicability of this approach for generating displays of bioactive ligands suitable for tailored multivalent interactions with corresponding receptors.

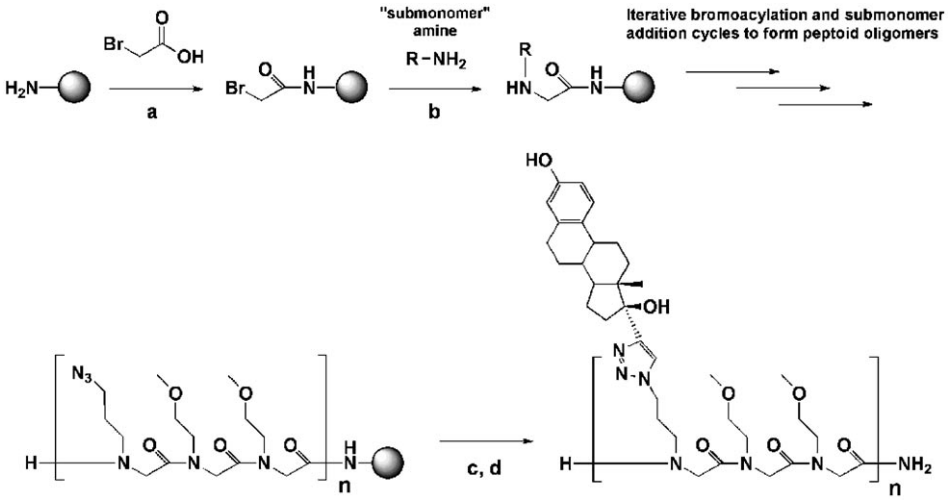
2 Results and Discussion

Using modifications of previously described solid-phase peptoid synthesis techniques [7], linear peptoid oligomers of up to 18 monomer units long were synthesized in good purity (>85%) on Rink Amide® resin (Scheme 1). These peptoids incorporate reactive azidoalkyl sidechains site-specifically positioned along the oligomer scaffold. These groups were installed utilizing 3-azido-1-aminopropane [16] as a "submonomer" reagent by coupling to the growing peptoid chain. In order to enhance overall hydrophilicity and water solubility, methoxyethyl sidechains were incorporated at all other positions using 2-methoxyethylamine as a submonomer [6b]. All peptoids described herein were identified by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) and Mass Spectrometry (MS) [6].

Resin-bound linear peptoid oligomers were used as substrates for Cu(I) catalyzed azide–alkyne [3+2] cycloaddition reactions. Using a modification of our published synthetic techniques [6], we were able to site-specifically conjugate EE2 at up to six positions along the oligomer scaffolds (Scheme 1, Table 1). EE2 conjugations were performed at every third position along the oligomer scaffold to alleviate steric congestion during the cycloaddition reaction and facilitate the formation of multivalent products. The resultant peptoid conjugates (Figure 1, compounds **1**–**4**) were synthesized in good crude purity (Table 1). The peptoid conjugates were cleaved from the resin as described previously [6, 7] and purified to >95% by RP-HPLC. Purified products were lyophilized and dissolved in 100% ethanol to a final concentration of 10 mM. In addition, using similar reaction conditions shown in Scheme 1 and Table 1, we were successful in conjugating ethisterone at up to three positions along a linear peptoid scaffold (Figure 2, compounds **5**–**7**).

In order to investigate the effects of multivalency on binding avidity of our conjugates to the ER, we employed an *in vitro* radiometric competitive binding assay using ER positive MCF-7 whole cell lysates (see Section 4) [17]. Competitive binding assays were performed by incubating cell lysate with tritium-labeled E2 (^{3}H-E2, Sigma) in the presence or absence of varying concentrations of compounds **1**–**4.** Concentrations tested were based on the molar concentration of the conjugate molecule and not E2 equivalencies. Following incubation, unbound ligand was removed with activated charcoal and tritium decay was counted using a scintillation counter. Binding curves were generated by computing counts *per* minute (cpm) in the absence of unlabeled peptoid conjugate minus cpm in its presence (Figure 2).

Competitive binding experiments were conducted to determine the 50% effective concentration (EC50) for E2



Scheme 1. General scheme for the solid-phase synthesis of estradiol–peptidomimetic conjugates. Reagents and conditions: a) bromoacetic acid (1.2 m), diisopropylcarbodiimide (1.2 m) in DMF, 20 min, rt; b) submonomer amine (1.0 m) in DMF, 20 min, rt; c) EE2, CuI, ascorbic acid, DIPEA (see Table 1 and Section 4 for reactant concentrations), 2-butanol-DMF-pyridine (5:3:2 by vol.), 45 °C, 18 h, d) 95% TFA in H_2O, rt, 10 min.

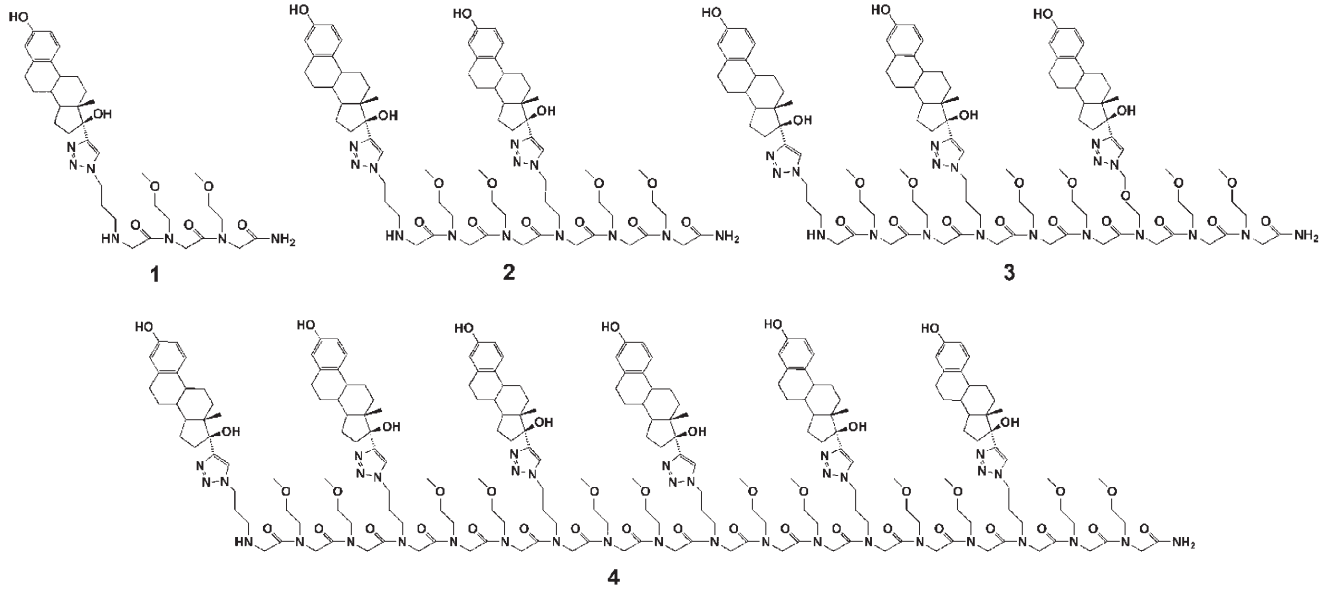


Figure 1. Estradiol–peptidomimetic conjugates showing precise multivalent display of estrogen receptor ligands.

displacement by compounds **1**–**4** (Table 1). EC50 values reported are representative of three separate experiments from one batch of cell lysate (see Supporting Information for representative binding curves). As a positive control, we measured that the unlabeled natural ligand E2 binds the ER with an EC50 of 0.42 nM, in agreement with previous observations [15b]. Monovalent estradiol–peptoid conjugate **1** exhibits an EC50 of 2.74 μM, which indicates a *ca.* 6500-fold decrease in affinity when compared to unconjugated E2 (Table 2, entries 1 and 2). Divalent compound **2**, with an EC50 of 44.1 nM (Table 2, entry 3), shows a *ca.* 60-fold greater binding affinity compared to **1** when binding the ER. These results demonstrate that binding to ER can be substantially enhanced through divalent presentation of ER ligand. Estrogen receptors have been documented to exist in active form as dimers [11]. Interestingly, augmenting the valency to tri- and hexavalent displays (Figure 1, compounds **3, 4**, respectively), results in only minor enhancements of binding affinity to the ER (Table 2, entries 4 and 5). Out of the four compounds tested, hexavalent compound **4** demonstrated the greatest binding affinity to the ER with an EC50 of 10.9 nM in this system. Competitive binding assays in ER positive MCF-7 whole cell lysates demonstrate that this class of molecules can compete with natural ligand for binding to the ER and that binding affinity is enhanced when the valency of receptor ligand is presented in a multivalent fashion along the peptoid backbone.

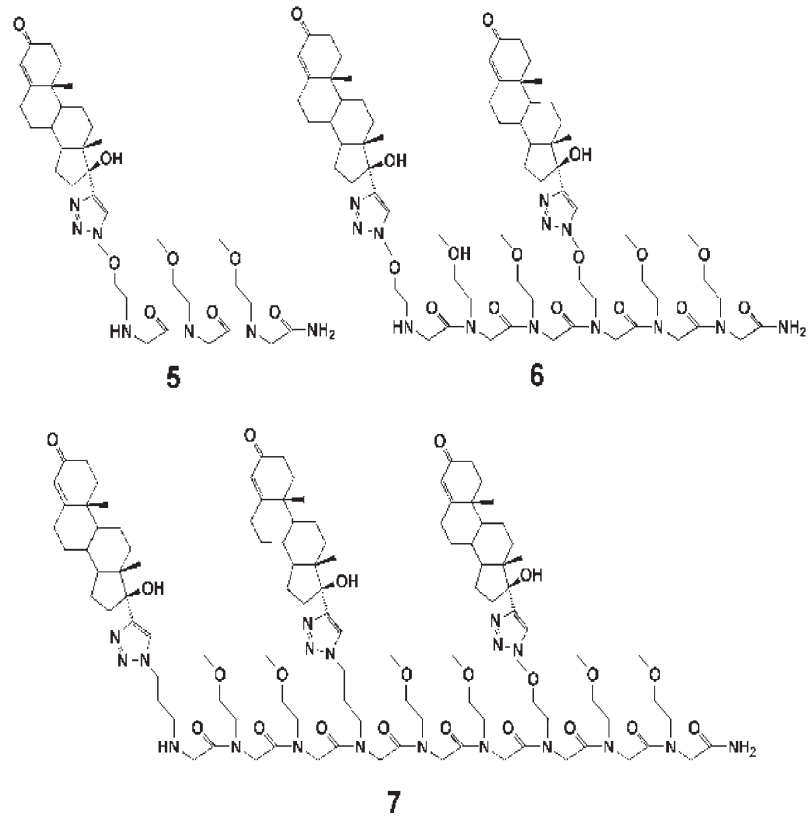


Figure 2. Ethisterone – peptidomimetic conjugates showing precise multivalent display of progesterone receptor ligands.

Table 1. Reactant concentrations for estradiol – peptidomimetic conjugate synthesis.

Entry	Compound	Valency (*n*)	[EE2][a] (m)	[CuI] (m)	[AA][b] (m)	[DIPEA] (m)	Purity[c] (%)
1	**1**	1	0.05	0.10	0.05	0.12	87
2	**2**	2	0.125	0.24	0.125	0.30	69
3	**3**	3	0.15	0.29	0.15	0.36	58
4	**4**	6	0.15	0.29	0.15	0.36	54

[a] 17α-Ethynylestradiol.

[b] Ascorbic acid.

[c] Integration monitored at $\lambda = 214$ nm.

Table 2. Estradiol – peptidomimetic conjugate binding data. EC50 values were calculated from competitive binding curves generated by GraphPad Prism Software. E2, 17β-estradiol.

Entry	Compound	Valency (*n*)	EC50
1	**E2**	1	0.42 nM
2	**1**	1	2.74 μM
3	**2**	2	44.1 nM
4	**3**	3	43.2 nM
5	**4**	6	10.9 nM

3 Summary

We have utilized a modification of the Cu-catalyzed azide – alkyne [3 + 2] cycloaddition reaction to generate robust multivalent steroid – peptidomimetic conjugates. We demonstrate that these compounds can be conveniently synthesized in good purity on solid phase. Notably, the broad orthogonality of this conjugation reaction mitigates the requirement for protecting groups when conjugating steroids to the oligomer scaffold. Despite the substantial hydrophobic bulk of multiple steroid units, the conjugated species display good solubility in ethanol and aqueous systems. Estradiol – peptidomimetic conjugates were shown to compete with natural ligand for binding the estrogen receptor in a radiometric competitive binding assay. The binding avidity of these molecules to the ER was shown to be substantially enhanced when the valency of the estradiol pendant groups was increased from a monovalent to a divalent display. The synthetic technique outlined herein allows for precise control over ligand valency and spacing along the oligomer scaffold, facilitating the generation of

estradiol–peptoid conjugate libraries that can be evaluated for optimized binding to the estrogen receptor. It has been observed previously that the size and charge of synthetic estradiol conjugates perturbs their ability to localize in the nucleus [15b]. Due to our ability to tune the physicochemical properties of the peptoid scaffold by modification of other sidechain types, compounds **1**–**4** have the potential to be targeted for extracellular or various intracellular localization. In this regard, we anticipate that these compounds may be exploited to activate specific subsets of ER-mediated pathways based on subcellular localization. Additionally, these molecules may prove effective for blocking the association of protein coactivators with ER or as a neuroprotective agent against membrane-localized oxidative insults [18].

4 Experimental Section

4.1 General

Peptoid oligomers were characterized by analytical RP-HPLC using a C18 analytical column (Peeke Scientific, Ultro 120 5 μm C18, 50×2 mm^2) on a Beckman Coulter System Gold HPLC system. HPLC chromatograms were monitored at 214 nm using a System Gold 166 detector. Linear gradients were conducted from 5 to 95% solvent B [0.1% trifluoroacetic acid (TFA) in HPLC grade acetonitrile] over solvent A (0.1% TFA in HPLC grade water) in 10 min with a flow rate of 0.7 mL/min.

Additional characterization of peptoid oligomers was conducted using LC/MS. All peptoids described herein (mass range: 684.84–4018.28 Da) were analyzed using an Agilent 1100 Series LC/MSD Trap XCT equipped with an electrospray ion source in positive ion mode.

4.2 Synthesis of Linear Peptoid Scaffolds on Solid-Phase Support

Using modifications of the standard submonomer synthesis procedures [7a], peptoid oligomers were synthesized on Rink Amide resin (0.69 mmol/g) (Novabiochem, San Diego, CA). Typically, 100 mg of resin was swollen in 3 mL of dichloromethane (DCM) for 45 min before Fmoc deprotection. Multiple washing steps using *N*,*N*′-dimethylformamide (DMF) (4×2 mL) and DCM (3×2 mL) were performed between each synthetic addition step. Resin was Fmoc deprotected by treatment with 20% piperidine in DMF (15 mL/g resin) for 20 min at rt. Deprotection reagents were washed with the resin and approximately 1.2 m bromoacetic acid (8.5 mL/g resin) and 1.2 m diisopropylcarbodiimide (2 mL/g resin) in DMF were added to the resin and the suspension was agitated at rt for 20 min. Following washing, 1.0 m azidoaminopropane or 2-methoxyethylamine in DMF (10 mL/g resin) was added and the reaction was agitated for 20 min. Bromoacetylations and monomer amine displacements were repeated until peptoid oligomers of desired length were obtained.

4.3 Synthesis and Purification of Steroid Peptidomimetic Conjugates

Linear peptoid scaffolds containing one, two, three, or six site-specifically positioned azidopropyl sidechains were synthesized with high efficiency using standard submonomer synthesis techniques as described above. Resin-bound peptoid oligomers were taken up in 20 mL 2-butanol/DMF/pyridine (5:3:2 by vol.) and reacted with EE2, CuI, ascorbic acid, and *N*,*N*-Diisopropylethylamine (DIPEA) in a 20 mL scintillation vial (see Table 1 for reactant concentrations). A similar strategy was utilized to synthesize ethisterone–peptidomimetic conjugates compound **5**–**7** (see Supporting Information for synthesis details). The vessel was purged with gaseous nitrogen, tightly capped, sealed with paraffin film and vigorously shaken at 45 °C for 18 h. Following completion of the reaction, the resin was washed with DMF (7×3 mL), Cu scavenger cocktail (DMF/pyridine 6:5 v/v, ascorbic acid 0.02 g/mL) (7×3 mL) and DCM (7×3 mL). The resin was then dried under nitrogen gas flow and approximately 3 mg resin was removed for characterization. Mass data: compound **1**, calc. 684.84, obs. 684.52; compound **2**, calc. 1350.84, obs. 1350.91; compound **3**, calc. 2018.45, obs. 2018.32; compound **4**, calc. 2009.14 [$M+2H^+/2$], obs. 2009.46 [$M+2H^+/2$]; compound **5**, calc. 700.88, obs. 700.30; compound **6**, calc. 1382.73, obs. 1382.61; compound **7**, calc. 2065.58, obs. 2065.88.

For purification, resin-bound products were cleaved from solid support using 95% TFA in H_2O (10 mL/g resin) at rt for 10 min. The cleavage cocktail was evaporated under reduced pressure and crude EPC was resuspended in an appropriate volume of 50% H_2O in acetonitrile. This solution was frozen and lyophilized overnight. Crude EPC powder was purified by preparatory RP-HPLC using a C18 preparatory column (Waters) on a Beckman Coulter System Gold HPLC system. HPLC traces were monitored at 230 nm using a System Gold 166 detector. Linear gradients were conducted from 5 to 95% solvent B (0.1% TFA in HPLC grade acetonitrile) over solvent A (0.1% TFA in HPLC grade water) in 50 min with a flow rate of 5.0 mL/min. Collected fractions were combined, shell frozen and lyophilized overnight. Purified (>95%) EPCs were dissolved to a final concentration of l0 mM in 100% EtOH.

4.4 Preparation of Mammalian Cell Extracts

MCF-7 cells were grown to ca. 80% confluence in phenol-red containing Dulbeccco's Modification of Eagle's Medium (10% fetal bovine serum) supplemented with l-Glu and antibiotics. The media was aspirated and cells were washed $2 \times$ with $1 \times$ PBS. The cells were grown for an additional 24 h in phenol-red-free Dulbeccco's Modification of

Eagle's Medium (10% charcoal-stripped fetal bovine serum) supplemented with l-Glu. The cells were then harvested in ice cold $1 \times$ PBS. Cell suspensions were centrifuged and the pellets were snap frozen on dry ice to lyse. Cell pellets were resuspended in freshly prepared receptor buffer (50 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 15 mM $MgCl_2$, 20 mM sodium molybdate, 20% glycerol, 1 μg/μL protease inhibitor), and incubated on ice for 10 min. The lysates were centrifuged for 20 min at $15000 \times g$ for 20 min at 4 °C. Whole cell lysate samples were diluted with a suitable amount of receptor buffer to a final protein concentration of 1 μg/μL. Protein concentration was monitored using colorimetric dye binding assays (BioRad).

4.5 Competitive Binding Assays in Whole Cell Lysates

Cell extracts were incubated with 1.0×10^{-8} m radiolabeled ligand (^{3}H-Estradiol, Sigma) in the presence or absence of varying concentrations of unlabeled ligand in a final volume of 100 μL of receptor buffer. Following 18 h incubation at 4 °C, cell extracts were mixed with an equal volume of activated charcoal suspension (10 mg/mL in receptor buffer) and incubated on ice for 10 min. The activated charcoal/cell extract slurry was centrifuged at $12000 \times g$ for 3 min. Cell extract supernatant (180 μL) was added to 2 mL scintillation fluid and ^{3}H decay was counted for 3 min on a scintillation counter (Beckman Coulter). Binding was computed as scintillation cpm in the absence of unlabeled conjugate minus cpm in its presence. Non-linear regression analyses were performed using GraphPad Prism® software.

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