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Synthesis and functional analysis of novel bivalent estrogens

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ABSTRACT

The steroid hormone estrogen plays a critical role in female development and homeostasis. Estrogen mediates its effects through binding and activation of specific estrogen receptors alpha (ERα) and beta (ERβ), members of the steroid/nuclear receptor family of ligand-induced transcription factors. Due to their intimate roles in genomic and nongenomic signaling pathways, these hormones and their receptors have been also implicated in the pathologies of a variety of cancers and metabolic disorders, and have been the target of large therapeutic development efforts. The binding of estrogen to its respective receptors initiates a cascade of events that include receptor dimerization, nuclear localization, DNA binding and recruitment of co-regulatory protein complexes. In this manuscript, we investigate the potential for manipulating steroid receptor gene expression activity through the development of bivalent steroid hormones that are predicted to facilitate hormone receptor dimerization events. Data are presented for the development and testing of novel estrogen dimers, linked through their C-17 moiety, that can activate estrogen receptor alpha (ERα)-mediated transcription events with efficacy and potency equal to or greater than that of ERα's cognate ligand, 17β-estradiol. These bivalent estrogen structures open the door to the development of a variety of steroid therapeutics that could dramatically impact future drug development in this area.

1. Introduction

Estrogens regulate gene expression events through their ability to bind either of two intracellular receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) [1,2]. This binding event facilitates dimerization of the receptor and modulation of both nongenomic and genomic signaling pathways [3–5]. Nongenomic signaling pathways are mediated through a subpopulation of estrogen-bound ER dimers that bind peripheral membranes and activate G protein and extracellular signal-regulated kinase (ERK)-mediated events. The classical genomic signaling pathway is mediated by estrogen-ER binding events that result in receptor nuclear localization, binding to specific DNA response elements motifs (EREs) in gene promoters, and regulation of transcription of these genes [6]. Furthermore, as might be predicted, recent studies suggest the convergence of these two signaling pathways in the mediation of their signaling events [6,7].

Estrogen binding activates its intracellular receptors by altering the conformation of its ligand-binding domain [8–10]. Unliganded estrogen receptor adopts a mobile and partially disordered globular state that rearranges upon estrogen binding and goes through multiple conformational changes that finally allow it to dimerize and mediate genomic and non-genomic signaling events [11,12]. These data suggest that the specific targeting and manipulation of the dimeric state of ERs might serve as a mechanism for regulating their activity if suitable agents were available to modulate this dimerization in either a positive or negative sense.

The above mechanisms of activity permit estrogens to coordinate the expression of a large number of genes in a variety of tissues. This is a positive facet of estrogen activity in that it orchestrates the large pleiotropic effects that are needed, but it can also have serious negative impacts on an organism in situations where the hormone or its receptors are improperly expressed, improperly regulated, or expressed at the wrong time. Accordingly, estrogen, its receptor and its coregulatory proteins, has been implicated in the pathologies of a variety of diseases and cancers [13–16]. Furthermore, these large pleiotropic effects are a serious complication in using hormone or hormone derivatives as potential therapeutics [17–19]. Technologies that increase specificity for estrogen activity or possibly target it to cells, tissues or independent genes, have tremendous applicability in therapeutics. In this manuscript, we investigate the potential for developing steroid-based molecules that would directly impact the critical steroid receptor dimerization event. Using estrogen as a model system, evidence is presented for the development of novel estrogen dimers (bivalent estrogens), linked through their C-17 moiety, that can function as potent agonists of transcriptional activation events mediated by human ERα. It is further shown that these bivalent estrogens retain both their speci-

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ficity and stability throughout the *in vitro* analyses employed. These data foster a host of subsequent studies in which the linker moiety might be manipulated to create targeted agonists and antagonists of ERα-mediated events.

2. Experimental

2.1. Chemicals and reagents

Estrone (**1**), estrone 3-methyl ether (**2**), and equilenin (**3**) were purchased from Sigma–Aldrich. 3-Hydroxyestra-1,3-5(10)-triene-17-oxime (**4a**) [20] and 3-methoxyestra-1,3-5(10)-triene-17-oxime (**4b**) [21] were synthesized according to literature procedures. Solvents were used from commercial vendors without further purification unless otherwise noted. Infrared spectra were determined on an Avatar 360 FT. Nuclear magnetic resonance spectra were determined on a Varian 200 or 400 MHz instrument. LRMS electron-impact (EI) ionization mass spectra were recorded at 70 eV on a ThermoFinnigan PolarisQ (ion trap mass spectrometer). Samples were introduced *via* a heatable direct probe inlet. MALDI mass spectra were obtained on a Bruker Autoflex time-of-flight mass spectrometer (Billerica, MA), using various matrices (*i.e.*, CHCA: alpha-cyano-4-hydroxycinnamic acid; SA: sinapinic acid; DHB: 2,5-dihydroxybenzoic acid), as noted in the following experimentals. HRMS electron impact (EI) ionization mass measurements were recorded at 25 eV on a JEOL JMS-700T MStation (magnetic sector instrument) at a resolution of greater than 10,000. Samples were introduced *via* heatable direct probe inlet. Perfluorokerosene (PFK) was used to produce reference masses. Elemental analyses were determined by Atlantic Microlabs, Inc., Norcross, GA. Compounds were chromatographed on preparative layer Merck silica gel F254 unless otherwise indicated.

2.2. Synthesis of 3-hydroxyestra-1,3-5(10)-triene-17-oximinyl 17-laurate (*4c*)

To 50 mg (0.175 mmol) of oxime **4a** in 0.9 mL anhydrous pyridine was added 67 mg (0.175 mmol) of lauric anhydride. The mixture was stirred for 72 h at 25 °C and quenched with ice to give a white precipitate. The mixture was diluted with ethyl acetate, washed successively with saturated copper sulfate solution, and brine, and dried over anhydrous $MgSO_4$. The product was chromatographed in 1:2 ethyl acetate:hexane and recrystallized from ethyl acetate to give 18 mg (23%) of **4c**: mp, 72–74 °C; ^{1}H NMR (400 MHz, DMSO, ppm) δ 7.28–7.31 (1H, d, J = 8.8), 7.82–7.84 (1H, dd, $J_{1,4}$ = 2.4, $J_{1,2}$ = 8), 6.70–6.80 (1H, s), 2.8–2.85 (2H, m), 2.25–2.55 (4H, m, overlaps with DMSO peak), 1.8–2.0 (3H, m), 1.2–1.65 (26H, m), 0.8–0.87 (6H, m); ^{13}C NMR (200 MHz, $CDCl_3$, ppm) δ 172.88, 171.32, 148.80, 138.21, 137.71, 126.53, 121.76, 118.91, 53.14, 44.39, 38.01, 34.65, 34.21, 32.13, 29.82, 29.68, 29.56, 29.48, 29.34, 27.25, 26.22, 25.37, 25.22, 23.15, 22.91, 17.40, 14.34; LRMS m/z (intensity): 467 (M^+, 40), 468 (MH^+, 17), 268 (M^+-199, 83); HRMS Calcd. for $C_{30}H_{45}O_3N$ 467.3399, found 467.3392 (mean of five determinations, SD 2.1 ppm; error −0.7 ppm).

2.3. Synthesis of bis-(3-hydroxyestra-1,3,5(10)-triene-17-oximinyl) 1,12-dodecanedioate (*5a*)

The procedure described for the preparation of **5c** was repeated using 137 mg (0.48 mmol, 1.2 equiv.) of oxime 4a, 46 mg (0.2 mmol) of 1,12-dodecanedioic diacid, 65 mg (0.48 mmol, 1.2 equiv.) of 1-hydroxybenzotriazole hydrate, and 92 mg (0.48 mmol, 1.2 equiv.) of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride in 1.2 mL anhydrous THF to afford, after stirring at 25 °C for 24 h, a product that was chromatographed in 2:1 ethyl acetate:hexane and recrystallized from 1:10 dichloromethane:methanol to afford 45 mg (29%) of **5a**: mp 182–183 °C; ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 7.1–7.2 (2H, d, J = 8.8 Hz), 6.55–6.68 (4H, m) 4.6–4.7 (2H, s, OH), 2.8–2.9 (4H, m), 2.5–2.72 (4H, m), 2.35–2.42 (6H, m), 2.2–2.3 (4H, m), 1.9–1.98 (4H, m), 1.6–1.8 (6H, m), 1.4–1.6 (10H, m, overlaps with H_2O peak), 1.26–1.4 (12H, m) 1.1–1.4 (6H, s); ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ 178.73, 171.81, 153.68, 138.17, 132.35, 126.77, 115.45, 113.04, 52.96, 45.60, 44.02, 38.36, 33.91, 33.31, 29.66, 29.59, 29.43, 29.38, 27.36, 27.37, 26.31, 25.19, 23.03, 17.26; m/z (MALDI-TOF, DHB matrix) m/z (intensity): 787.4 (M+Na+, 600), 803.4 (M+K+, 240). Anal. Calcd. for $C_{48}H_{64}N_2O_6\cdot 3H_2O$: C, 70.39; H, 8.61. Found: C, 70.78; H, 8.14.

2.4. Synthesis of bis(3-hydroxyestra-1,3,5(10)-triene-17-oximinyl) 1,22-docosanedioate (*5b*)

The procedure described for **5c** was repeated using 138 mg (0.486 mmol, 1.2 equiv.) of oxime **4b,** 75 mg (0.202 mmol) of 1,22-docosanedioic acid, 66 mg (0.486 mmol, 1.2 equiv.) of 1-hydroxybenzotriazole, and 93 mg (0.486 mmol, 1.2 equiv.) of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride in 1 mL of 1:1 acetonitrile:tetrahydrofuran to afford, after stirring at reflux for 24 h, a product that was chromatographed using 2:1 ethyl acetate/hexane, to afford 131 mg (72%) of **5b**: ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 7.13–7.15 (2H, d, J = 8.4 Hz), 6.57–6.66 (4H, m), 5.22 (2H, s, OH), 2.8–2.87 (4H, m), 2.5–2.72 (4H, m), 2.35–2.42 (6H, m), 2.18–2.3 (4H, m), 1.88–1.96 (4H, m) 1.64–1.78 (9H, m), 1.4–1.58, (9H, m), 1.2–1.4 (34H, m), 1.01 (6H, s); ^{13}C NMR (400 MHz, $CDCl_3$, ppm) δ 178.92, 172.03, 153.93, 138.00, 132.03, 126.65, 115.50, 113.12, 52.92, 45.62, 43.98, 38.34, 33.83, 33.30, 29.92, 29.90, 29.86, 29.79, 29.64, 29.42, 29.34, 27.37, 26.28, 25.17, 23.00, 17.22; LRMS m/z: 816 (M+Na, 3000), 534 (M+Na−258, 1200).

2.5. Synthesis of bis-(3-methoxyestra-1,3-5(10)-triene-17-oximinyl) 1,12-dodecanedioate (*5c*)

To 123 mg (0.411 mmol, 0.8 equiv.) of oxime **4b** and 57 mg (0.247 mmol, 1 equiv.) of 1,12-dodecanedioic acid in 1 mL of 1:2 methanol:dichloromethane was added 67 mg (0.494 mmol, 1 equiv.) of 1-hydroxybenzotriazole hydrate followed immediately by 158 mg (0.823 mmol, 1.7 equiv.) of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride. The mixture was stirred at 25 °C for 48 h, evaporated to dryness, and diluted with dichloromethane. The solution was washed with water; the resulting emulsion was broken with brine; and the product was dried over anhydrous $MgSO_4$. The crude product was chromatographed using 1:1 ethyl acetate/hexane and recrystallized twice from ethyl acetate/hexane to give 15.4 mg (8%) of **5c**: mp 119–121 °C; IR (KBr) 1766 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 7.18–7.24 (2H, d, J = 8.4 Hz), 6.7–6.73 (2H, dd, $J_{1,4}$ = 2.4 Hz $J_{1,2}$ = 8.4 Hz), 6.64 (2H, s), 3.8 (6H, s), 2.84–2.9 (4H, m), 2.5–2.8 (4H, m), 2.2–2.5 (12–14H, m), 1.8–2.0 (4H, m), 1.4–1.8 (25H, m), 1.2–1.4 (12H, m), 1.03 (6H, s); ^{13}C NMR (400 MHz, $CDCl_3$, ppm) δ 178.707, 171.769, 157.778, 137.867, 132.273, 126.557, 114.10, 114.06, 111.754, 55.421, 52.977, 45.591, 44.027, 38.372, 33.923, 33.308, 29.854, 29.589, 29.437, 29.39, 27.349, 26.309, 25.193, 23.30, 17.268; LRMS m/z (intensity): 793 ($M+H^+$, 87), 815 ($M+Na-H^+$, 100), 816 ($M+Na^+$, 57), 831 ($M+K-H^+$, 12), 832 ($M+K^+$, <1). Anal. Calcd. for $C_{50}H_{68}N_2O_6$ hydrate: C, 74.04; H, 8.70; N, 3.45. Found: C, 74.24; H, 8.68; N, 3.44.

2.6. Synthesis of bis(3-methoxyestra-1,3-5(10)-triene-17-oxime) 1,22-docosanedioate (**5d**)

The procedure described for **5c** was repeated using 185 mg (0.617 mmol, 0.9 equiv.) of oxime **4b**, 137 mg (0.371 mmol, 1 equiv.) of 1,22-docosanedioic acid, 101 mg (0.741 mmol, 1 equiv.) of 1-hydroxybenzotriazole hydrate, and 237 mg (1.23 mmol, 1.6 equiv.) of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride to afford, after stirring at 25 °C for 72 h, a product that was chromatographed using 1:1 ethyl acetate:hexane and recrystallized from ethyl acetate:hexane to afford 71 mg (21%) of **5d**: mp 63–66 °C; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 7.20–7.26 (2H, d, J = 8.4 Hz), 6.7–6.73 (2H, dd, $J_{1,4}$ = 2 Hz, $J_{1,2}$ = 8.4 Hz), 6.63–6.64 (2H, d, J = 2.4 Hz), 3.77 (6H, s), 2.87–2.91 (4H, m), 2.5–2.7 (4H, m), 2.36–2.42 (5H, m), 2.20–2.32 (4H, m), 1.9–1.98 (4H, m), 1.2–1.8 (60H, m), 1.02 (6H, s); ^{13}C NMR ($CDCl_3$) δ 178.669, 171.761, 157.763, 137.844, 132.257, 126.557, 114.069, 111.724, 55.406, 52.946, 45.568, 44.019, 38.364, 33.908, 33.316, 29.908, 29.839, 29.665, 29.460, 29.391, 27.425, 27.327, 26.287, 25.201, 23.007, 17.246; m/z (MALDI-TOF, DHB matrix) 955.8 (M+Na, 2400), 971.8 (M+K, 1400). Anal. Calcd. for $C_{60}H_{88}N_2O_6$: C, 77.21%; H, 9.50%; N, 3.00%. Found: C, 77.10%; H, 9.54%; N, 2.97%.

2.7. Synthesis of Girard P adduct (**6a**)

The procedure described for the preparation of **6b** was repeated using 25 mg (0.092 mmol) of estrone (**1**), 17 mg (0.092 mmol, 1 equiv.) of Girard's reagent P, and 10 mg of Dowex 50 W × 2 acid resin to afford, after refluxing for 16 h and filtering, a crude solid. The product was recrystallized from abs EtOH to give 33 mg (81%) of **6a**: mp 199–201 °C (with decomposition); ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 10.92 (0.5H, s), 9.05–9.06 (2H, d, J = 5.6 Hz), 8.65–8.69 (1H, t, J = 7.6 Hz), 8.19–8.23 (2H, t, J = 7.6 Hz), 7.04–7.09 (1H, t, J = 8.4 Hz), 6.47–6.54 (2H, m), 5.79–5.91 (2H, q, J = 16.8 Hz), 1.8–2.8 (10H, m, overlaps DMSO peak), 1.3–1.6 (6H, m), 0.82–0.92 (3H, m); ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ 169.18, 166.75, 137.10, 129.97, 127.37, 126.06, 114.97, 112.79, 61.74, 55.99, 51.98, 44.75, 43.65, 37.93, 34.05, 29.08, 26.98, 26.80, 25.86, 22.76, 18.57, 16.92; IR (KBr) 3199 (phenol); m/z 404 ($M-Cl^-$, 100); Anal. Calcd for $C_{25}H_{30}N_3O_2Cl{\cdot}H_2O$: C, 65.56%; H, 7.04%; N, 9.17%. Found: C, 65.21%; H, 7.27%; N, 8.77%.

2.8. Synthesis of Girard P adduct (**6b**)

To 50 mg (0.176 mmol) of estrone 3-methyl ether (**2**) in 1.8 mL abs EtOH was added 33 mg (0.176 mmol, 1 equiv.) of Girard's reagent P followed immediately by 20 mg of Dowex 50 W × 2 acid resin. The mixture was refluxed for 16 h, cooled, and filtered through Celite. The crude product was recrystallized from hexane/ethanol to give 38 mg (48%) of **6b**: mp 218–220 °C (with decomposition); ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 10.92 (0.5H, s), 9.04–9.05 (2H, d, J = 6 Hz), 8.65–8.69 (1H, t, J = 7.6 Hz), 8.19–8.22 (2H, t, J = 7.6 Hz), 7.18–7.20 (1H, d, J = 7.6 Hz), 6.63–6.70 (2H, m), 5.78–5.90 (2H, q, J = 17.2 Hz), 3.69 (3H, s), 2.79–2.85 (2H, m), 2.16–2.6 (8H, m, overlaps with DMSO peak), 1.86–2.04 (4H, m), 1.2–1.6 (8H, m), 0.9–0.91 (3H, m); ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ 169.11; 166.72; 157.12; 146.45, 146.06, 137.37, 131.72, 127.43, 126.18, 113.46, 111.54, 61.73, 54.89, 51.95, 44.72, 43.61, 37.80, 34.01, 29.16, 26.96, 26.69, 25.79, 22.74, 16.89; m/z (MALDI-TOF, DHB Matrix) 418 ($M-Cl^-$, 2700), 137 (MNa^+-114, 275); Anal. Calcd. for $C_{26}H_{32}N_3O_2Cl{\cdot}H_2O$: C, 65.27%; H, 7.45%. Found: C, 65.66%; H, 7.39%.

2.9. Synthesis of Girard P adduct (**7**)

The procedure described for the preparation of **6b** was repeated using 25 mg (9.4 μmol) of equilenin (**3**), 18 mg (9.4 μmol, 1 equiv.) of Girard's reagent P, and 10 mg Dowex 50 W × 2 acid resin in 940 μL of methanol to afford, after refluxing for 16 h and filtering, a crude solid. The product was recrystallized from methanol to yield 27 mg (66%) of **7**: mp 210–212 °C (with decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.99 (1H, s), 9.67 (1H, s), 9.05–9.08 (2H, t, J = 5.6 Hz), 8.67–8.71 (1H, t, J = 8 Hz), 8.20–8.24 (2H, t, J = 7.2 Hz), 7.82–7.87 (1H, t, J = 9.2 Hz), 7.54–7.56 (1H, d, J = 4.8 Hz), 7.19–7.21 (1H, d, J = 8.8), 7.10–7.12 (1H, m), 5.85–5.97 (m, 2H), 3.12–3.47 (m, overlaps with H_2O), 2.95–2.99 (1H, m), 2.4–2.8 (4H, m), 2.25–2.29 (1H, m), 1.81–2.00 (2H, m), 0.74–0.78 (3H, m); ^{13}C NMR (200 MHz, DMSO-d_6) δ 173.30, 172.24, 159.981, 151.92, 151.72, 151.53, 138.97, 136.81, 132.91, 131.55, 130.12, 129.98, 124.01, 114.92, 67.197, 53.68, 49.79, 36.78, 33.194, 29.112, 28.55, 21.45; m/z (MALDI-TOF, DHB matrix) 400 ($M-Cl^-$, 5000); Anal. Calcd. for $C_{25}H_{26}N_3O_2Cl{\cdot}2H_2O$: C, 63.62%; H, 6.41%. Found: C, 63.52%; H, 6.05%.

2.10. Synthesis of bis-Girard adduct (**8a**)

Suberoyl chloride (0.586 g, 2.776 mmol) was added dropwise to a solution of 4-aminopyridine (0.627 g, 6.66 mmol), triethylamine (0.773 mL, 5.55 mmol), and 10 mL MeCN. After 60 min of stirring at room temperature, TLC of the reaction indicated completion; the reaction was refluxed for an additional 30 min to ensure full conversion of the starting materials. The reaction mixture was then dumped into an excess (75 mL) of saturated $NaHCO_3$, and allowed to stir for 30 min. The aqueous solution was then filtered, and the solids rinsed with additional saturated $NaHCO_3$. Solids were dried overnight under vacuum, and then recrystallized from boiling methanol to give 0.835 g (92%) of bis-N-(4-pyridyl) 1,8-octanediamide as a white solid: mp 195–196; ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 10.20 (2H, s, NH), 8.33–8.35 (4H, dd, J_a = 1.6 Hz, J_b = 4.8 Hz), 7.49–7.51 (4H, dd, J_a = 1.6 Hz, J_b = 4.8 Hz), 2.27–2.31 (4H, t, J = 7.2 Hz), 1.52–1.56 (4H, m), 1.26–1.28 (4H, m); ^{13}C NMR (400 MHz, DMSO-d_6, ppm): δ 172.47, 150.29, 145.75, 113.02, 36.44, 28.37, 24.65; HRMS Calcd. for $C_{18}H_{22}N_4O_4$ 326.1742, found 326.1744 (mean of 5 determinations, SD 1.0 ppm; error 0.6 ppm).

The recrystallized bis-N-(4-pyridyl) 1,8-octanediamide (0.350, 1.07 mmol) was added slowly to a solution of ethyl chloroacetate (0.228, 2.14 mmol) in 1 mL absolute ethanol at room temperature. The mixture was heated to reflux, at which point the solids fully dissolved. After refluxing for 4 h, the reaction was removed from heat and allowed to cool to room temperature, and then cooled to 0 °C with an ice/water bath. Hydrazine (0.067 mL, 2.14 mmol) was added dropwise, resulting in a very thick suspension. The solids were obtained by filtration, and then recrystallized in 75 mL of boiling methanol.

This recrystallized bis-Girard reagent (0.200 g, 0.368 mmol) was then suspended in 5 mL absolute ethanol. Estrone (0.199 g, 0.736 mmol), and catalytic Dowex 50 W × 2 resin (50 mg) were added and the reaction was brought to reflux. After 24 h the reaction was filtered, solids were washed with hot methanol, and the liquids were concentrated to give a crude solid. The resulting solid was brought up in a minimum of methanol, and dropped slowly into 100 mL of cold diethyl ether. The fine white precipitate was collected from the ether by gravity filtration through a sintered glass filter, giving bis-Girard adduct **8a**: mp >230 °C; ^{1}H NMR (400 MHz, MeOD-d_4, ppm): δ 7.19–7.21 (4H, d, J = 8 Hz), 6.28–6.31 (2H, d, J = 8.8 Hz), 6.04–6.06 (4H, d, J = 7.2 Hz), 5.73–5.76 (2H, d, J = 8.8 Hz), 5.69–5.70 (2H, d, J = 2.8), 4.5–4.6 (4H, q, AB), 4.25 (2H, s), 1.95–2.10 (4H, m), 1.1–1.9 (20H, m), 0.6–0.9 (18H, m), 0.17 (6H,

s); ^{13}C NMR (400 MHz, MeOD-d_4, ppm): δ 168.28, 166.68, 158.32, 153.41, 142.83, 142.59, 136.03, 129.49, 124.48, 113.41, 111.15, 107.56, 56.67, 51.11, 43.77, 42.82, 37.21, 32.83, 27.92, 25.73, 24.79, 21.51, 14.76; m/z (MALDI, SA matrix): 975 (M, 220), 841 (M−134, 400), 643 (M−332, 50).

2.11. Synthesis of bis-Girard adduct (**8b**)

To 2.26 g (24 mmol, 2.4 equiv.) of 4-aminopyridine and 2.02 g (20 mmol, 2 equiv.) of triethylamine in 40 mL of acetonitrile at 0 °C was added 2.67 g (10 mmol) of 1,12-dodecanedioyl dichloride dropwise. The reaction was stirred at 25 °C for 30 min, refluxed for 16 h, and quenched with saturated $NaHCO_3$. The resulting white solid was collected and recyrstallized from acetone to yield 2.8 g (72%) of bis-N-(4-pyridyl) 1,12-dodecanediamide: mp 138–139 °C; IR (KBr) 3312 (NH), 1697 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.3 (2H, s), 8.35–8.45 (4H, d, J = 6.3 Hz), 7.5–7.6 (4H, d, J = 6.3 Hz), 2.3–2.38 (4H, t, J = 7.48), 1.5–1.6 (4H, m), 1.2–1.35 (12H, m); ^{13}C NMR (DMSO-d_6) δ 172.55, 150.30, 145.77, 113.00, 36.47, 28.85, 28.74, 28.58, 24.77; m/z (EI) 383 (MH^+, 100), (ESI-MS–MS) 95 (MH^+−289, 100), 289 (MH^+−94, 100); HRMS calcd for $C_{22}H_{40}N_4O_2$: 382.2363, found 382.2377 (mean of five determinations, error 0.0014 ppm). Anal. Calcd. for $C_{22}H_{30}N_4O_2$–CH_3OH: C, 66.64%; H, 8.27%; N, 13.52%. Found: C, 66.35%; H, 8.10%; N, 13.77%.

To 1 g (8.16 mmol, 1 equiv.) of ethyl chloroacetate in 3.5 mL of absolute EtOH was added 1.56 g (4.08 mmol) of bis-N-(4-pyridyl) 1,12-dodecanediamide. The reaction was refluxed for 2.5 h and cooled to 25 °C. To this solution at 0 °C was added 262 mg (8.16 mmol, 1 equiv.) of hydrazine. The solid product was collected, dried under high vacuum, recrystallized from methanol to afford 699 mg (29%) of the bis-Girard Reagent: mp 233–250 °C with decomposition; ^{1}H NMR (200 MHz, D_2O) δ 8.5–8.6 (d, 4H, J = 7.4 Hz), 8.05–8.15 (d, 4H, J = 7.4 Hz), 5.2–5.3 (s, 4H), 2.45–2.6 (t, 4H, J = 7.2 Hz), 1.5–1.8 (m, 4H), 1.1–1.4 (br m, 12H); ^{13}C NMR (D_2O) δ 179.99, 155.42, 148.60, 118.20, 99.80, 39.87, 31.12, 31.02, 30.85, 27.13; m/z (ESI) 264.1 (MH^+−Cl_2/2, 100). Anal. Calcd. for $C_{26}H_{40}N_8O_4Cl_2$–CH_3OH: C, 51.35%; H, 7.02%; N, 17.74%. Found: C, 51.69%; H, 6.79%; N, 18.04%.

To 75 mg (0.13 mmol) of bis-Girard reagent in 0.65 mL methanol was added 81 mg (0.3 mmol, 1.2 equiv.) of estrone (**1**), followed by 10 mg of Dowex 50 W × 2 acid resin. The reaction was refluxed for 24 h and filtered through a warm sintered-glass funnel. The funnel was rinsed several times with warm methanol. The methanolic solutions were concentrated to give a solid product. The product was recrystallized from 1:1 acetonitrile:methanol to give 14 mg (28%) of bis-Girard adduct **8b**: mp >240 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.81 (s, 2H), 10.83 (s, 2H), 9.04 (s, 2H), 8.70–8.74 (d, 4H, J = 7); 8.12–8.16 (d, 4H, J = 7.4), 7.05–7.09 (m, 2H), 6.45–6.54 (m, 4H), 5.62–5.65 (m, 4H), 3.8–4.2 (br s, 4H); 2.70–2.80 (m, 4H), 1.65–2.60 (m, 21H, overlaps with DMSO peak); 1.28–1.62 (m, 29H); 0.82–0.90 (m, 6H); ^{13}C NMR (200 MHz, DMSO-d_6) δ 179.54, 174.41, 172.56, 160.49, 157.69, 152.17, 142.52, 135.40, 131.47, 120.41, 119.44, 118.24, 65.40, 57.42, 50.15, 49.09, 48.98, 42.15, 39.45, 34.48, 34.27, 34.14, 33.90, 32.30, 32.22, 31.34, 29.81, 28.20, 22.31; m/z (MALDI-TOF, SA matrix) 1032 (M−2Cl$^-$, 2500); 707 (M−325, 1500).

2.12. Synthesis of bis-Girard reagent (**8c**)

A flask containing 1,18-octadecanedioic acid (1.50 g, 4.77 mmol), thionyl chloride (10 mL), and DMF (1 drop) was stirred at room temperature for 30 min. The suspension was then brought to reflux, at which point the solids dissolved. Reaction was refluxed for 2 h. The solvents were removed under reduced pressure, and the remaining white solid was re-suspended in MeCN and evaporated several times. When all the thionyl chloride was removed, the solid was added slowly to a flask containing 1.12 g (11.92 mmol) 4-aminopyridine, 1.66 mL (11.92 mmol) triethylamine, and 25 mL MeCN. The mixture was stirred at room temperature for 30 min, then refluxed for 2 h, then cooled to room temperature. The reaction mixture was dumped into an excess (100 mL) of saturated $NaHCO_3$, and allowed to stir for 30 min. The aqueous solution was then filtered, and the solids rinsed with additional saturated $NaHCO_3$. Solids were dried overnight under vacuum, and then recrystallized from boiling methanol to give an off-white solid. One recrystallization gave 1.89 g (4.05 mmol, 85%) of bis-N-(4-pyridyl) 1,18-octadecanediamide: mp 141–143 °C; ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ 10.22 (2H, s, NH), 8.38–8.40 (4H, d, J = 6.4 Hz), 7.53–7.55 (4H, d, J = 6.8 Hz), 2.31–2.35 (4H, t, J = 7.2 Hz), 1.55–1.59 (4H, m), 1.15–1.30 (24H, m); ^{13}C NMR (400 MHz, DMSO-d^6, ppm): δ 172.53, 150.30, 145.75, 113.00, 36.48, 28.98 (several), 28.88, 28.74, 28.57, 24.76; HRMS: Calcd. for $C_{28}H_{42}N_4O_2$ 466.3307, found 466.3307 (mean of 5 determinations, SD 1.8 ppm, error 0.0 ppm).

Bis-Girard reagent, **8c**, was prepared as described above for bis-Girard adduct, **8a**, using bis-N-(4-pyridyl) 1,18-octadecanediamide: ^{1}H NMR (400 MHz, CD_3OD, ppm): 7.00–8.01 (4H, d, J = 7.2 Hz), 7.08–7.10 (2H, d, J = 8.4 Hz), 6.84–6.86 (4H, d, J = 6.8 Hz), 6.54–6.56 (2H, d, J = 8.4 Hz), 6.49–6.50 (2H, d, J = 2.4 Hz), 5.3–5.41 (4H, q, AB), 5.06 (2H, s, OH), 2.7–2.9 (4H, m), 1.90–2.7 (20H, m), 1.3–1.75 (38H, m), 0.97 (6H, s); ^{13}C NMR (400 MHz, CD_3OD, ppm): d 168.25, 166.68, 158.31, 153.42, 142.69, 136.03, 129.48, 124.49, 113.29, 111.05, 107.49, 56.68, 51.09, 43.76, 42.71, 37.19, 35.52, 32.55, 27.52–28.06 (many), 25.81, 25.65, 24.82, 21.62, 14.70; m/z (MALDI-TOF, CHCA matrix): 1117 (M, 100), 982 (M−135, 400).

2.13. Plasmids

The development of the human estrogen (pRSV-ERα), progesterone (pRSV-PR), androgen (pRSV-AR), glucocorticoid (pRSV-GR) and beta galactosidase (pRSV-βGal) mammalian expression plasmids used in these studies were as described elsewhere [22–24]. The development and use of the estrogen (pBL-ERE-tk-Luc), androgen (pBL-ARE-tk-Luc), progesterone pBL-PRE-tk-Luc, and glucocorticoid (pBL-MMTV-tk-Luc) reporter plasmids have likewise been described elsewhere.

2.14. Transcription assays

The cell line used throughout these studies was the human embryonic kidney 293 (HEK) cell line obtained from ATCC (CRL-1573). These cells were grown and maintained at 37 °C/5% CO_2 in DMEM media plus 5% FBS. Mammalian transient co-transfections were performed in the HEK293 cell line as previously described [25]. To determine the efficiency of transfection and to standardize the expression of activity, a pRSV-βGal expression plasmid was included in all co-transfections and analyzed as previously described [25]. Each transfection was run in sextuplet and the average luciferase response for the 6 independent transfections was normalized to the average βGal rate [average luciferase response/(average βGal response/minute)]. Statistical evaluations of these data were expressed as standard error of their means (SEM) for the sextuplet wells of each treatment dose. Data were analyzed using the graphical and statistical program Prism (GraphPad, San Diego, CA).

2.15. Stability assays

To evaluate the chemical stability of the bis(3-hydroxyestra-1,3,5(10)-triene-17-oximinyl) 1,22-docosanedioate, the bivalent estrogen **5b**, HEK cells, aliquoted at 5 × 10^5 cells per well of a 96-

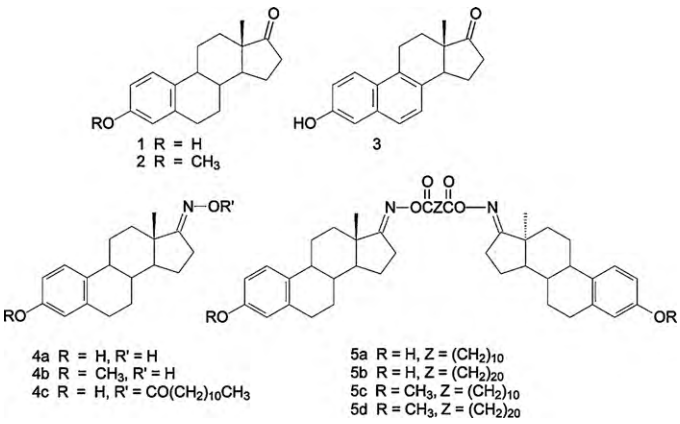

Fig. 1. Monovalent and bivalent 17-oxime esters. Presented are the 6 monovalent (**1**, **2**, **3**, **4a**, **4b** and **4c**) and 4 bivalent (**5a**–**d**) 17-oxime esters evaluated for their stability, specificity and ability to modulate ERα-mediated gene expression parameters.

well plate, were treated for 36 h in the presence and absence of 10 μM bivalent estrogen. The liquid in the wells was aspirated; the wells were treated with methanol to lyse the cells; and the contents of 1 well, 5 wells, 10 wells and 25 wells were collected and concentrated to dryness. Each of these samples was diluted with 500 μL of methanol. The samples were applied to a Merck silica gel F254 analytical plate, developed in 1:2 ethyl acetate–hexanes (a solvent system that readily separates the estrone oxime **4a** from the bivalent estrogen **5b**), sprayed with 10% phosphomolybdic acid in ethanol, and heated to detect compounds as purple spots. The bivalent estrogen **5b** and none of the estrone oxime **4a** was detected in the samples from 1 well, 5 wells, 10 wells and 25 wells.

3. Results and discussion

3.1. Synthesis of estrogens

A prerequisite to steroid hormone genomic signaling is the ligand-induced dimerization of its intracellular receptor, and the concept of creating bivalent estrogens dates back to 1994 [26], when Bergmann et al. published the synthesis and characterization of bivalent hexestrols linked through polymethylene and polyethylene glycol spacers. One of these displayed partial agonism for the estrogen receptor while the remainder were characterized as antagonists. Since then several other groups have published bivalent or dimeric steroid syntheses using a variety of linkers [27–30], but these papers focused on design, synthesis and/or binding, with little to no characterization of relative efficacy, potency or specificity. More recently, the Katzenellenbogen laboratory, publishers of the original manuscript on the synthesis of bivalent hexestrols, extended these studies with bivalent estrogen designs that displayed variable estrogen receptor binding affinities that correlated with linker length [31]. These estrogen dimers were evaluated comprehensively with respect to linker composition, tether length and receptor binding, but did not evaluate biological activity.

We began our investigation of bivalent steroid synthesis using estrogen as a template for these studies. The estrogen molecule has any number of chemically accessible positions, but two stood out as logical targets for our initial efforts: the hydroxyl group at the C-3 position of β-estradiol or the carbonyl group at the C-17 position of estrone. We elected to modify the C-17 keto group of estrone (Fig. 1: compound **1**), estrone 3-methyl ether (Fig. 1: compound **2**), or equilenin (Fig. 1: compound **3**) as either oximes or semicarbazones in order to provide functionality to which a suitable tether could be attached. Reaction of hydroxylamine with estrone (compound **1**) or estrone 3-methyl ether (compound **2**) afforded good yields of the corresponding "monovalent" oximes (Fig. 1: compounds **4a** and **4b**). Coupling of these oximes to either 1,12-dodecanedioic diacid or 1,22-docosanedioic diacid using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole hydrate (HOBT) [32] afforded the tethered bivalent oxime esters compounds **5a**–**d** in modest yields after chromatography and recrystallization. No effort was made to optimize yields. The "monovalent" oxime ester compound **4c** was also prepared from a regioselective reaction of oxime compound **4b** and lauric anhydride.

At the turn of the last century, Girard developed water-soluble semicarbazone reagents P and T for the purpose of extracting and purifying ketone-containing steroids [33]. Reaction of Girard reagent P with estrone (Fig. 1: compound **1**), estrone 3-methyl ether (Fig. 1: compound **2**), and equilenin (Fig. 1: compound **3**) afforded good yields of the corresponding "monovalent" Girard reagents (Fig. 2: compounds **6a**, **6b**, and **7**). We anticipated that bis-Girard reagents would offer not only suitable tethers but also better water solubility than seen with the oxime esters. Preparation of bis-Girard reagents paralleled the published synthesis of Girard reagent P in so far as: (1) reaction of 4-aminopyridine and 1,12-dodecanoyl dichloride provided a bispyridylamide, and (2) treatment of the bispyridylamide with ethyl chloroacetate and hydrazine furnished the desired bis-Girard reagent [34]. Coupling of this bis-Girard reagent with estrone led to the bis-Girard derivative bivalent estrogen (Fig. 2: compound **8b**). Comparable procedures were used to synthesize compounds **8a** and **8c**.

3.2. Defining optimal linkage points for estrogens

In preliminary investigations of the impact of modifying estrogen at either C-3 or C-17, we created and evaluated the modified estrogens **4a**–**7** shown in Fig. 1. The "monovalent" and "bivalent" estrogens were analyzed for their ability to modulate estrogen genomic signaling as measured using an *in vitro* transcription assay. Estrogen genomic signaling was reconstituted in a human embryonic kidney (HEK) cell line by co-transfecting this cell line with a mammalian expression construct for human ERα along with an estrogen response element (ERE)-driven luciferase reporter plasmid. All transfections also included a mammalian expression construct for β-galactosidase (β-Gal), which was used to normalize for transfection efficiency. All compounds were analyzed at a concentration of 100 nM. Fig. 3 presents the results for "monovalent" C-17 oximes of estrone that possess either a hydroxyl group or a methoxy group at C-3. As seen here, the compounds **4a**, **4c**, **5a**, **5b**, **6a** and **7**, containing a C-3 hydroxyl, activated ERα-mediated reporter gene expression. Compounds **4b**, **5c**, **5d** and **6b** with the methoxy group at this position were inactive. This is not totally unexpected in that structural studies looking at estrogen binding of the ERα ligand binding domain demonstrated that the C-3 hydroxyl participates in crucial hydrogen-bonding with an Arg, Glu and water molecule within the binding site [8,34,35]. More recently, it has also been shown that the ERα ligand binding domain displays structural plasticity with respect to C-17 modifications [36]. These data led us quickly to the C-17 carbonyl group of estrone as a logical site on which to construct estrogen dimers.

3.3. Dose response analyses of "bivalent" estrogens

We further evaluated the bivalent oxime esters, **5a** and **5b** in Fig. 1, with either a $(CH_2)_{10}$ or a $(CH_2)_{20}$ spacer respectively, along with the monovalent estrogen **4c** containing a simple laurate ester, for their ability to activate ERα-mediated transcription in a dose-dependent manner (Fig. 4). As seen here, ERα was agonized by all three modified estrogens in a dose-dependent manner with an order of efficacy of estrogen > **5b** > **5a** > **4c**. These data support the

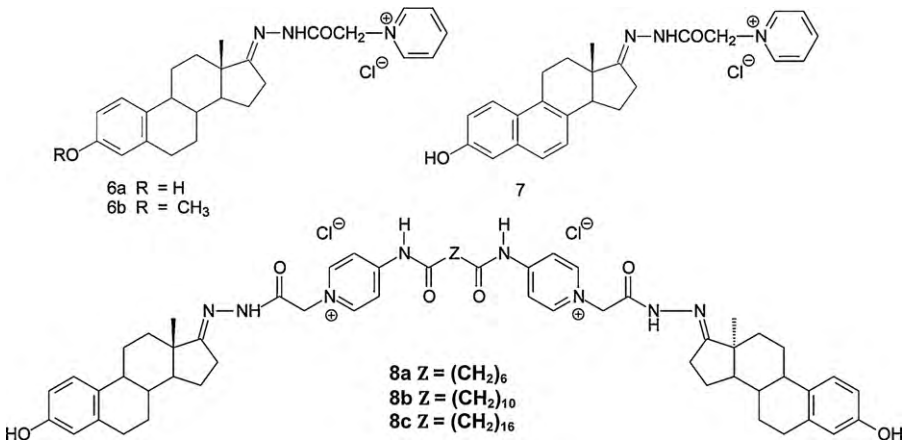

Fig. 2. Monovalent and bivalent Girard P-based oxime derivatives. Presented are the 2 monovalent (**6** and **7**) and 3 bivalent (**8a**, **8b**, **8c**) estrogens evaluated for their ability to modulate ERα-mediated gene expression events.

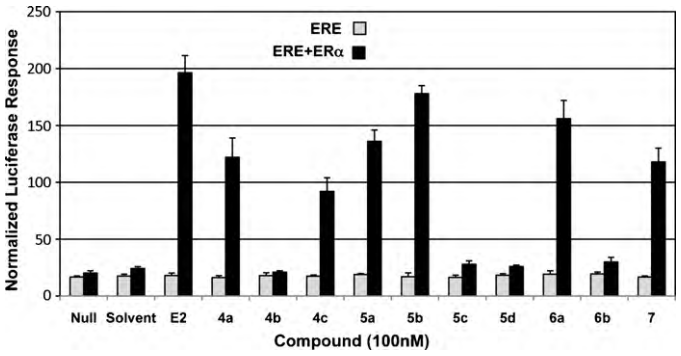

Fig. 3. Monovalent and bivalent estrogens modified at their C-3 and C17 moieties analyzed for their ERα agonist activities. Compound number identifiers are as designated in Fig. 1. ERα-mediated transcription was reconstituted in an HEK cell line through the co-transfection of an ERα mammalian expression plasmid and an ERE-driven luciferase reporter plasmid. As controls, parallel analyses were run to evaluate solvent impact as well as background attributable to the ERE reporter vector (ERE). ERα's cognate ligand 17β-estradiol (E2) along with a series of monovalent and bivalent estrogens modified at their C-3 and C-17 positions (A) were analyzed (B) in a 96-well plate format. For normalization of data, included in all transfections was a mammalian expression construct for β-galactosidase (β-Gal). All compounds were analyzed at 100 nM, and data from 6 wells for each ligand was averaged and divided by the average β-Gal rate for these wells. Error bars represent the SEM for the 6 wells.

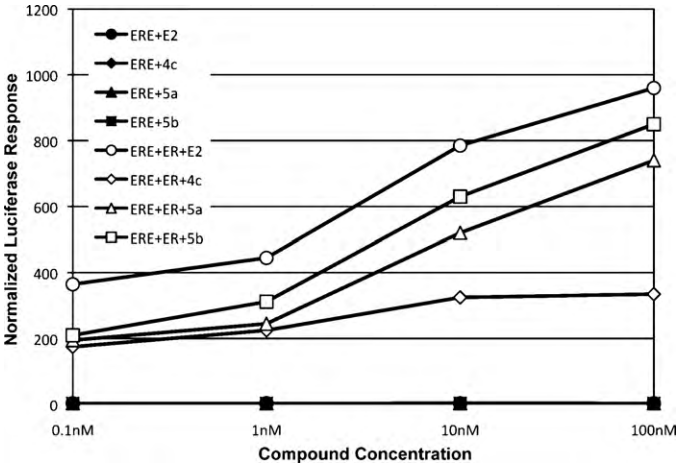

Fig. 4. Dose Response analysis of the bivalent estrogens **5a** and **5b**. Utilizing the ERα-specific HEK co-transfection analysis described in Fig. 3, the bivalent estrogens **5a** and **5b**, along with the monovalent estrogen **4c** and 17β-estradiol (E2), were analyzed at concentrations varying from 0.1 nM to 100 nM. Data are expressed as averaged reporter gene luciferase values for sextuplet wells divided by the average β-Gal rate for these wells.

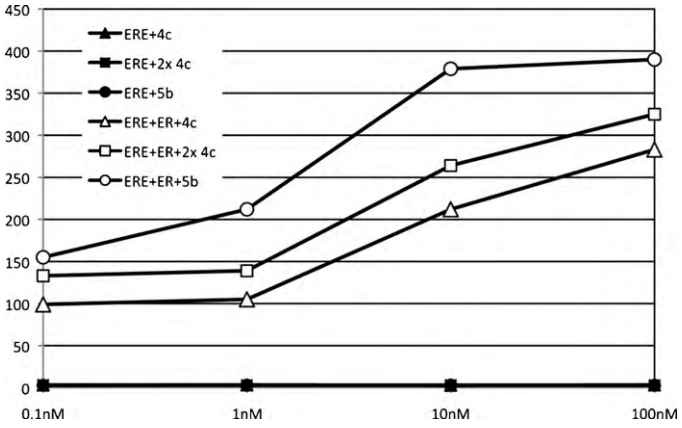

Fig. 5. Efficacy comparison of the bivalent estrogen **5b** to its monovalent oxime derivative **4c**. Utilizing the ERα-specific HEK co-transfection analysis described in Fig. 3, the bivalent estrogen **5b** and its monovalent oxime derivative **4c** were analyzed in molar equivalents of estrogen for their ability to stimulate ERα-specific reporter gene expression. Data are expressed as averaged reporter gene luciferase values for sextuplet wells divided by the average β-Gal rate for these wells.

hypothesis that estrogen dimers are able to enter the cell effectively and modulate ERα genomic signaling.

3.4. Stability of bivalent estrogens in the oxime ester family

We evaluated three concerns in the initial phases of developing these estrogen dimers: (1) potential hydrolytic liability of the oxime ester tether between the two estrone moieties; (2) the hydrophobicity of the aliphatic linker; and (3) the specificity of the modified steroid structure. To evaluate the issue of stability, transcriptional activation mediated by bivalent estrogen **5a** was compared to a monovalent estrogen, **4c**, containing a simple laurate ester (Fig. 5). A dose–response curve was analyzed for both compounds, and monovalent **4c** was assayed at a molar equivalent of estrogen concentration (2×) to that of the bivalent estrogen **5a**. The monovalent estrogen **4c** displayed substantially lower efficacy (~75% at the highest concentration tested) to that of bivalent estrogen **5a**. Although these results may involve differences in cellular uptake, differences in receptor binding, or differences in dimerization efficiency, we interpret these findings in support of the chemical stability of these compounds during the course of the 24-hour assay time-frame in which they were analyzed, and in support of the increase in dimerization efficiency incumbent in the bivalent estrogen **5b** structure.

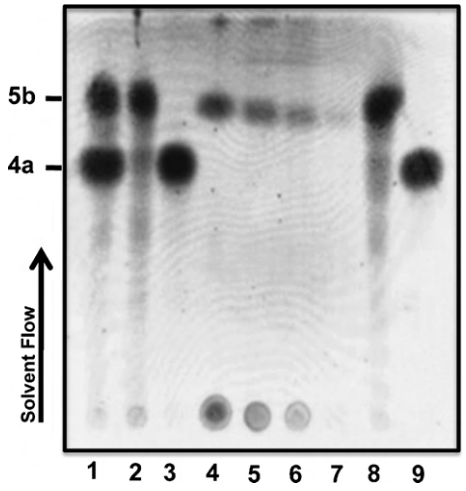

Fig. 6. Stability evaluation of the bivalent estrogen **5b**. HEK cells, aliquoted at 5×10^5 cells per well of a 96-well plate, were treated for 36 h in the presence and absence of 100 nM bivalent estrogen **5b** and analyzed by thin layer chromatography using 1:2 ethyl acetate–hexanes with 10% phosphomolybdic acid spray for detection. Lane 1: bivalent estrogen **5b** + oxime **4a**; lane 2: bivalent estrogen **5b**; lane 3: oxime **4a**; lane 4: extract from 25 wells; lane 5: extract from 10 wells; lane 6: extract from 5 wells; lane 7: extract from 1 well; lane 8: bivalent estrogen **5b**; lane 9: bivalent estrogen **5b** + oxime **4a**.

An analysis was performed that directly examined the chemical stability of the bivalent estrogen **5b**. HEK cells, aliquoted at 5×10^5 cells per well of a 96-well plate, were treated for 36 h in the presence and absence of 100 nM bivalent estrogen **5b**. The lipid components for cell populations from 1, 5, 10 and 25 wells were extracted into methanol and analyzed by thin layer chromatography for the presence of monovalent **4a** and bivalent estrogen **5b** components (Fig. 6). As seen here, only the bivalent estrogen **5b** was detected even in the sample where the contents of 25 cells were combined and analyzed. These results support the stability of the oxime ester bonds to the conditions of the assay, and exclude the possibility that adventitious esterases cleave the bivalent estrogen **5b** to produce **4a**. The stability of the bivalent Girard P derivatives was not an issue since these derivatives possess robust amide linkages.

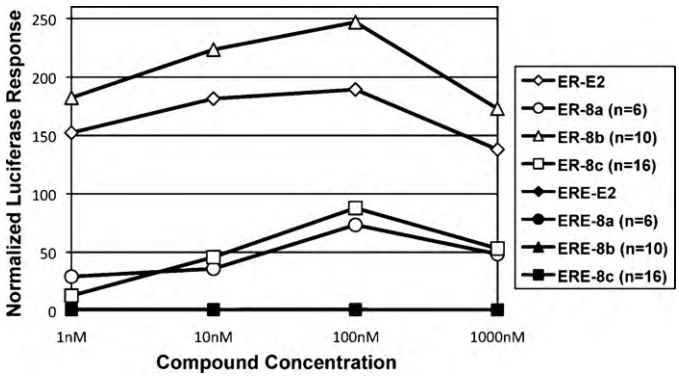

Fig. 7. Analysis of the Girard-based bivalent estrogens **8a**, **8b**, and **8c** for their ERα agonist activity. Utilizing the ERα-specific HEK co-transfection analysis described in Fig. 3, the Girard-based bivalent estrogens **8a**, **8b**, and **8c** along with 17β-estradiol (E_2), were analyzed at concentrations varying from 0.1 nM to 100 nM. Data are expressed as averaged reporter gene luciferase values for sextuplet wells divided by the average β-Gal rate for these wells.

3.5. Analysis of an estrogen dimer with increased hydrophilicity

These promising preliminary studies clearly suggested that estrone dimers linked through their C-17 position function as specific agonists of ERα, but with a slightly lower efficacy relative to that of estrogen. These results propelled our interest in a second generation of C-17 linked estrogen dimers in which we manipulated the composition of the linker and its attachment to the estrone moieties. These modifications also provided us with an opportunity to augment the solubility of the dimer and evaluate its impact on efficacy. To achieve this goal, we explored the development of "Girard-based" estrogen dimers, as described above (Fig. 2). Girard-based estrogen dimers **8a**, **8b**, and **8c** along with 17β-estradiol, were analyzed in the ERα *in vitro* co-transfection assay (Fig. 7). As seen in Fig. 7, the Girard-based estrogen dimer **8b** displayed a potency equivalent to estrogen and an efficacy that was approximately 125% that of estrogen at its optimal 100 nM concentration. Dimers **8a** and **8b** had reduced efficacy in these assays, potentially reflecting an inability to span the estrogen binding sites in the case of **8a**, or too much linker-

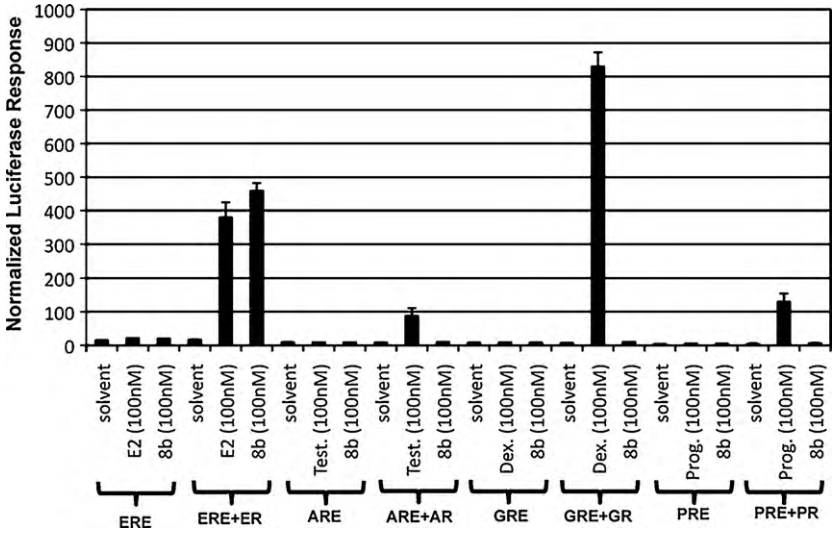

Fig. 8. Specificity analysis of the Girard-based bivalent estrogen **8b**. HEK cells were reconstituted with receptor/reporter systems for ERα, human progesterone receptor beta (PR), human androgen receptor (AR), and human glucocorticoid receptor (GR) through co-transfection of specific receptor and reporter plasmids as described in Fig. 3. Assays were performed in the presence of solvent, **8b** and receptor-specific ligands [ER: 17β-estradiol (E_2); AR: dihydrotestosterone (Test.); GR: dexamethasone (Dex); PR: progesterone (Prog.)]. For normalization of data, included in all transfections was a mammalian expression construct for β-Gal. All compounds were analyzed at 100 nM, and data from 6 wells for each ligand was averaged and divided by the average β-Gal rate for these wells. Error bars represent the SEM for the 6 wells.

associated flexibility leading to inefficient dimerization in the case of **8c**. It is anticipated that futures studies investigating the receptor binding specificities of these compounds may help define the mechanism by which linker length is influencing receptor dimerization and activity. Cumulatively, these data suggest that manipulation of the dimer length, linkage, and hydrophobicity may indeed serve as a mechanism for optimizing dimer efficacy and potency.

3.6. Evaluation of the ERα specificity of the bivalent estrogen **8b**

To evaluate the specificity of the bivalent estrogens, the bivalent estrogen **8b** was analyzed for its ability to modulate transcription events mediated by three other homodimerizing steroid hormone receptors; the progesterone receptor (PR), the androgen receptor (AR), and the glucocorticoid receptor (GR) (Fig. 8). As described above for ERα, a receptor-specific *in vitro* transcription system was reconstituted in HEK cells for each receptor through the co-transfection of human AR, GR or PRβ receptor mammalian expression plasmids, along with a corresponding receptor-specific response element-driven luciferase reporter plasmid. Transfected cells were analyzed in the presence of ligand solvent, in the presence of 100 nM cognate ligand, and in the presence of 100 nM of the bivalent estrogen **8b**. As seen in Fig. 8, the bivalent estrogen **8b** was able to stimulate reporter gene expression only with the ERα reconstituted transcription system.

In summary, we evaluated the hypothesis that bivalent steroid estrogens can be created capable of facilitating ER dimerization and consequently ER efficacy. This is an initial attempt to mimic the ER dimerization event using a synthetic bivalent ligand. As discussed above, this is not a novel idea, but separating itself from previous publications are both the synthetic approach and the functional characterizations employed. The chemistry required to achieve these results harkens back to the early days of the steroid literature, although this is the first time that bis-Girard reagents have been used to create bivalent steroids. More to the point, the chemistry developed here is straightforward and provides access to an array of other modified tethers or even unsymmetrical dimers between different steroids. The identification of C-17 as a readily modified position on the estrogen backbone that does not grossly impact its activity opens the door to a variety of alternative structures that might be developed. This might include bivalent steroid linkers capable of targeting bivalent hormone agonists or antagonists to any number of extracellular or intracellular molecules, such as: bone hydroxylapatite, cell surface proteins, proteasomal complexes, lysosomes, transcription coregulatory complexes, cell-specific proteases or perhaps even in the creation of novel transcription factor heterodimers that target specific gene promoters. Two issues not addressed in this study, but currently under investigation, are the estrogen receptor binding affinity for these steroids and the impact they have on estrogen non-genomic signaling pathways. Finally, the basic concept applied here to estrogen dimers would conceptually appear feasible for any of these steroid/nuclear receptor ligands.

Acknowledgments

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References

[1] Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: how do they signal and what are their targets. Physiol Rev 2007;87:905–31.
[2] Katzenellenbogen BS, Choi I, Delage-Mourroux R, Ediger TR, Martini PG, Montano M, et al. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol 2000;74:279–85.
[3] Levin ER. Cellular functions of plasma membrane estrogen receptors. Steroids 2002;67:471–5.
[4] Levin ER. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005;19:1951–9.
[5] Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol 2008;70:165–90.
[6] Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 2005;19:833–42.
[7] Ordonez-Moran P, Munoz A. Nuclear receptors: genomic and non-genomic effects converge. Cell Cycle 2009;8:1675–80.
[8] Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753–8.
[9] Katzenellenbogen BS, Sun J, Harrington WR, Kraichely DM, Ganessunker D, Katzenellenbogen JA. Structure–function relationships in estrogen receptors and the characterization of novel selective estrogen receptor modulators with unique pharmacological profiles. Ann N Y Acad Sci 2001;949:6–15.
[10] Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and nonreproductive functions? Biochem Biophys Res Commun 2000;270:1–10.
[11] Gee AC, Katzenellenbogen JA. Probing conformational changes in the estrogen receptor: evidence for a partially unfolded intermediate facilitating ligand binding and release. Mol Endocrinol 2001;15:421–8.
[12] Tamrazi A, Carlson KE, Katzenellenbogen JA. Molecular sensors of estrogen receptor conformations and dynamics. Mol Endocrinol 2003;17:2593–602.
[13] Ascenzi P, Bocedi A, Marino M. Structure–function relationship of estrogen receptor alpha and beta: impact on human health. Mol Aspects Med 2006;27:299–402.
[14] Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL. Estrogen and progesterone receptors: from molecular structures to clinical targets. Cell Mol Life Sci 2009;66:2405–26.
[15] O'Malley BW, Kumar R. Nuclear receptor coregulators in cancer biology. Cancer Res 2009;69:8217–22.
[16] Xu J, Wu RC, O'Malley BW. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 2009;9:615–30.
[17] Biglia N, Mariani L, Sgro L, Mininanni P, Moggio G, Sismondi P. Increased incidence of lobular breast cancer in women treated with hormone replacement therapy: implications for diagnosis, surgical and medical treatment. Endocr Relat Cancer 2007;14:549–67.
[18] Billeci AM, Paciaroni M, Caso V, Agnelli G. Hormone replacement therapy and stroke. Curr Vasc Pharmacol 2008;6:112–23.
[19] Jeanes H, Newby D, Gray GA. Cardiovascular risk in women: the impact of hormone replacement therapy and prospects for new therapeutic approaches. Expert Opin Pharmacother 2007;8:279–88.
[20] Peters RH, Crowe DF, Avery MA, Chong WK, Tanabe M. 17-Desoxy estrogen analogues. J Med Chem 1989;32:1642–52.
[21] Regan BM, Hayes FN. 17- and 17a-Aza-D-homosteroids. J Am Chem Soc 1956;78:639–43.
[22] Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature 1988;331:91–4.
[23] Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985;318:635–41.
[24] Weinberger C, Hollenberg SM, Ong ES, Harmon JM, Brower ST, Cidlowski J, et al. Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. Science 1985;228:740–2.
[25] Noonan DJ, Henry K, Twaroski ML. A high-throughput mammalian cell-based transient transfection assay. Methods Mol Biol 2004;284:51–65.
[26] Bergmann KE, Wooge CH, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA. Bivalent ligands as probes of estrogen receptor action. J Steroid Biochem Mol Biol 1994;49:139–52.
[27] Berube G, Rabouin D, Perron V, N'Zemba B, Gaudreault RC, Parent S, et al. Synthesis of unique 17beta-estradiol homo-dimers, estrogen receptors binding affinity evaluation and cytocidal activity on breast, intestinal and skin cancer cell lines. Steroids 2006;71:911–21.
[28] Groleau S, Nault J, Lepage M, Couture M, Dallaire N, Berube G, et al. Synthesis and preliminary in vitro cytotoxic activity of new triphenylethylene dimers. Bioorg Chem 1999;27:383–94.
[29] Osella D, Dutto G, Nervi C, McGlinchey MJ, Vessieres A, Jaouen G. Ferrole–estradiol complex as a test for receptor dimerization. J Organomet Chem 1997;533:97–102.
[30] Rabouin D, Perron V, N'Zemba BRCG, Berube G. A facile synthesis of C(2)-symmetric 17 beta-estradiol dimers. Bioorg Med Chem Lett 2003;13:557–60.

[31] LaFrate AL, Carlson KE, Katzenellenbogen JA. Steroidal bivalent ligands for the estrogen receptor: design, synthesis, characterization and binding affinities. Bioorg Med Chem 2009;17:3528–35.
[32] Dagan A, Wang C, Fibach E, Gatt S. Synthetic, non-natural sphingolipid analogs inhibit the biosynthesis of cellular sphingolipids, elevate ceramide and induce apoptotic cell death. Biochim Biophys Acta 2003;1633:161–9.
[33] Girard A, Sandulesco G. Sur une nouvelle série de réactifs du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la caractérisation microchimique des aldéhydes et cétones. Helv Chim Acta 1936;19:1095–107.
[34] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927–37.
[35] Pike AC, Brzozowski AM, Walton J, Hubbard RE, Bonn T, Gustafsson JA, et al. Structural aspects of agonism and antagonism in the oestrogen receptor. Biochem Soc Trans 2000;28:396–400.
[36] Nettles KW, Bruning JB, Gil G, O'Neill EE, Nowak J, Hughs A, et al. Structural plasticity in the oestrogen receptor ligand-binding domain. EMBO Rep 2007;8:563–8.