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The Terminal Substituents of 7 α , 6-hexanyl Derivatives of Estradiol Determine their Selective Estrogen Receptor Modulator *versus* Agonist Activities

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Abstract

Pure antiestrogens were clinically developed as alternative therapies for estrogen receptor (ER) positive breast cancers. Unlike the selective estrogen receptor modulators (SERMs), these antiestrogens are devoid of tissue-specific ER agonist activity. Many of these compounds are steroidal in nature, containing an estradiol (E2) structural core with long alkyl side chains at the C-7 α position. Two novel 7 α -substituted E2 derivatives were evaluated that retain high binding affinity for ER. Compared to known pure antiestrogens, these compounds, referred to as compound 13 (C13) and C14, contain shorter 7 α alkyl side chains and differ only in their terminal substituent: a hydroxyl moiety *versus* a benzyloxy group, respectively. Herein we assessed the effects of these compounds on ER transcriptional activity and report that despite their similar overall structure, C13 and C14 produce distinct cell type-specific responses. Of note, C13 functions as a mixed agonist/antagonist in Hela cells, inducing only weak ER transcriptional activity while preventing coactivator recruitment and stabilizing ER expression. However, this compound effectively stimulates ER activity in MCF-7 cells, does not increase ER levels and promotes cell proliferation on par with E2. Conversely, C14 stimulates transcriptional activity in both cell types and enhances ER-coactivator interactions. The activities of both compounds were inhibited by the pure antiestrogen ICI 182,780. Taken together, these results reveal that C13 is a SERM while C14 is an ER agonist, and indicate that the terminal modification of the C-7 α hexanyl side chain of these estradiol derivatives is an important determinant of the biocharacter of these ER ligands.

Keywords

estrogen receptor; coactivator; SERM; antiestrogen; pharmacology

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Introduction

Steroid hormone receptors such as estrogen receptor- α (ER α) have long been associated with hormonally-responsive cancers. Breast cancer, the most commonly diagnosed malignancy in women in the United States [1], is not only clinically characterized by its ER α status but is often treated with drugs designed to disrupt ER activity. Under normal conditions ER α functions as a ligand-dependent transcription factor, mediating responses to estrogens such as 17 β -estradiol (E2). In contrast, antiestrogens, like those developed to treat breast cancer, competitively bind to ER α and prevent its ability to induce gene transcription.

Transcriptional activation through ER α is controlled by two regions: activation function-1 (AF-1) and AF-2. The former is found in the N-terminus while AF-2 is located in the C-terminal ligand binding domain (LBD). The LBD is composed of 12 α -helices that form a hydrophobic pocket with the final helix, helix 12 (H12), serving as a molecular switch whose orientation relative to the rest of the LBD is dependent upon the nature of the compound occupying the ligand binding pocket. In the presence of E2, H12 along with helices 3 and 5 is oriented to create a docking site used for binding to transcriptional coregulators [2,3]. However, in the presence of antiestrogens the position of H12 is shifted to shield these residues, thus diminishing coregulator-receptor interactions [2,3]. The inability of antiestrogen-bound ER α to recruit coactivators such as members of the steroid receptor coactivator (SRC) family to its LBD and hence to gene regulatory regions thereby inhibits target gene transcription [3,4].

Antiestrogens can be categorized as partial or pure. Partial antiestrogens, also referred to as selective estrogen receptor modulators (SERMs), exhibit properties of both ER agonists and antagonists, an effect which is cell type-specific. The ability of SERMs to retain agonist function is dependent upon the ER α AF-1 region, whose action is not impaired by ligand binding [5]. The SERM tamoxifen is currently used in the treatment of breast cancer due to its ability to antagonize ER in mammary tissue. However, tamoxifen exhibits pro-estrogenic activity in the uterus, inducing proliferation of the uterine epithelium [6]. Consequently, patients undergoing tamoxifen therapy are at increased risk for developing endometrial cancer [7]. Unlike SERMs, pure antiestrogens lack agonist activity and serve as an alternative treatment for ER α -positive breast cancers. ICI 162,780 (ICI), clinically known as fulvestrant, and ICI 164,384 are two pure antiestrogens. Interestingly, despite lacking estrogenic activity [8,9], these compounds contain an E2 structural core (Fig. 1). Nevertheless, they contain long side chains at the C-7 α position thought to ultimately block ER signaling by inducing ER α degradation via an ubiquitin-dependent mechanism and disrupting coactivator-receptor interactions [10-12]. Antiestrogen-induced degradation of the receptor is associated with ligand-mediated increases in the surface hydrophobicity of the receptor [13]. With respect to the latter mechanism, the C-7 α side chains of the ICI compounds limit the ability of H12 to cap the LBD pocket and prevent the conformational change necessary for binding to coactivators such as steroid receptor coactivator-3 (SRC-3) and its related family members [11]. Pure antiestrogens do not, however, block all interactions with transcriptional coregulators. A key residue in the LBD required for corepressor binding remains unobstructed in ICI-bound receptor [11], and ICI can promote receptor interactions with the NCoR corepressor [14]. It also promotes LBD binding to the CH3 domain of the cAMP response element-binding protein-binding protein (CBP) signaling integrator which is distinct from the N-terminal LXXLL motif that mediates interaction between CBP and agonist-bound nuclear receptors [15,16].

Several other antiestrogens able to destabilize ER expression, namely ZK-703 and ZK-253, resemble ICI compounds as they also are composed of an E2 core with long C-7 α side chains [17], and this behavior is consistent with the idea that side chain size and composition

are critical for their antagonist properties [18]. More recently [19], two novel E2 derivatives with extended C-7 α side chains, 7 α -(6-hydroxyhexanyl)-17 β -estradiol (compound 13; C13) and 7 α -(6-benzyloxyhexanyl)-17 β -estradiol (compound 14; C14), that differ only in the terminal side chain substituent were generated (Fig. 1). These compounds bind to ER α with an affinity similar to that of ICI [19,20], but their ability to regulate ER α transcriptional activity has not been tested. We hypothesized that both of these compounds would exhibit modest antagonist properties due to their short side chain length relative to the pure antiestrogens noted above. Surprisingly, our results indicate that C13 is a SERM with cell type specific activity, while C14 is an ER α agonist. Hence, the relative biological activity of these estradiol derivatives is primarily determined by the nature of the 7 α side chain terminal substituent, an effect that may be exploited in the design of novel antiestrogens.

Experimental

2.1 Chemicals

17 β -Estradiol and 4-hydroxytamoxifen (4HT) were purchased from Sigma Chemical Company. ICI 182,780 was obtained from Tocris. C13 and C14 were generated as previously described and were the kind gift of Dr. Bao Ting Zhu [19].

2.2 Cell culture and transfection

Hela and MCF-7 cell lines were purchased from ATCC and cultured in Dulbecco's Modified Eagle (DME) media containing 10% fetal bovine serum (FBS). For transfection, 200,000 cells/well were plated in a 6-well dish in phenol red-free DME media containing 5% charcoal-stripped FBS. The next day media was removed and reserved. Cells were washed with 1x PBS buffer and transfected with the indicated amounts of plasmids using Lipofectamine reagent (Invitrogen) in Opti-Mem (Invitrogen) following the manufacturer's instructions. Six to eight hours later, Opti-Mem was replaced with the reserved DME media. The following day, cells were treated with ligands at the specified concentrations.

2.3 Trans-activation assay for ER

HeLa and MCF-7 cells were transfected with 1 μ g ERE-E1b-Luc (luciferase) reporter plasmid [21]; HeLa cells also were transfected with 5 ng pCR3.1-ER α expression vector [22] as described above. Twenty-four hours after ligand treatment, cells were harvested using TEN buffer (40mM Tris, pH 8, 1mM EDTA and 150mM NaCl). Cell pellets were resuspended in Reporter Lysis Buffer (Promega) and lysed according to the manufacturer's instructions. Luciferase activity was determined using a Luminoskan Ascent Microplate Luminometer (Thermo Labsystems) with Luciferase Assay Reagent (Promega). Values were normalized to total protein concentration as determined with Bio-Rad Protein Assay reagent (Bio-Rad Laboratories).

2.4 Mammalian two-hybrid assay

Cells were transfected using the Checkmate Mammalian Two-hybrid System (Promega) with previously generated fusion plasmids [15]. Specifically, vectors containing the GAL4 DNA binding domain fused to SRC-1, CBP, or the CBP interaction domains CH3 and NID (100 ng) were co-transfected with the VP16-ER α LBD fusion plasmid (1 μ g) and the luciferase reporter pG5-Luc (1 μ g), which contains five GAL4 binding sites. Cells were treated with ligand for 24 h, and transcriptional activity was determined as described in section 2.3.

2.5 Western blot

Following transfection with the indicated plasmids, cells were treated with ligand for 24 h. Cells were collected using TEN buffer and resuspended in ER extraction buffer [50mM Tris, pH 8, 400mM NaCl, 5mM EDTA, 1% NP40, 0.2% Sarkosyl, and EDTA-free Complete Mini protease inhibitor (Roche)]. Cells were lysed on ice for 30 min after which total protein content was determined as above. Ten micrograms of protein was mixed with LDS Buffer containing Sample Reducing Agent (both Invitrogen), then heated to 75°C for 10 min. Proteins were separated using a 4-12% Bis-Tris gel and transferred in NuPage Transfer Buffer (Invitrogen) to a nitrocellulose membrane (GE Water & Process Technologies). The membrane was blocked in PBST (1X PBS plus 0.1% Tween-20) containing 5% dry milk powder (w/v). Primary antibodies were diluted in PBST with 1% milk powder and detected using HRP-conjugated ECL IgG secondary antibodies and the ECL Plus Western Blotting Detection System (both from GE Healthcare). Primary antibodies consisted of ER α (HC-20; Santa Cruz Biotechnology), Actin (Clone C4; Millipore), and SRC-3 (sc-7216; Santa Cruz Biotechnology). Densitometric quantification of western blots was performed using ImageJ software (version 1.44p) and normalized relative to actin expression.

2.6 Co-immunoprecipitation

Following plating, cells were cultured in phenol red-free DME media containing 10% charcoal stripped FBS for 16-24h. Cells were then treated with the indicated concentrations of ligand for 1 h and collected on ice in 1X PBS containing Complete Mini protease inhibitor (Roche). Cell pellets were lysed at 4°C for 20 min in IP lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 5% glycerol, 1mM NaVO₄, 1mM NaF, and Complete Mini protease inhibitor), and the protein content of the resulting lysates was determined as described above. To reduce non-specific background, 1 mg of total protein per sample was pre-cleared for 2 h at 4°C with pre-washed Protein A/G agarose beads (Santa Cruz Biotechnology) under constant rotation. After bead removal, lysates were incubated with 4 μ g ER α antibody overnight at 4°C, followed by the addition of Protein A/G agarose beads and incubation for 1-2 h at 4°C. Immune complexes were washed four times with lysis buffer, eluted using NuPage sample buffer, and analyzed by Western blot analysis as described in section 2.5.

2.7 Cell growth assay

Cells were plated in phenol red-free DME media containing 10% charcoal-stripped FBS, and ligand treatments were added the following day at the indicated concentrations. Cells were harvested 5-6 d later and counted using a Beckman Coulter Z1 Particle Counter. In parallel, an additional plate of cells was counted on day zero in order to assess cell proliferation over the length of the growth assay.

Results

The ability of C13 and C14 to inhibit ER activity was initially tested using a *trans*-activation assay in Hela cells co-transfected with ER α and a luciferase reporter plasmid containing an estrogen response element (ERE). Contrary to expectations and unlike the pure antiestrogen ICI, C13 and C14 were able to stimulate ER activity, an effect that was dose-dependent (Fig. 2A). At all concentrations tested, C14 elicited the stronger response, inducing ER activity to a level up to eight times greater than vehicle control. Co-treatment of cells with E2 and either C13 or C14 demonstrated that each of the latter was able to partially antagonize E2-induced luciferase expression (Fig. 2B). Moreover, ICI administration was able to block activity induced by either C13 or C14, demonstrating the ability of ICI to antagonize C13 and C14 stimulatory responses. Collectively these data indicate that C13 and C14 exhibit partial agonist and antagonist properties.

Next the effects of C13 and C14 on steady-state ER α protein expression were determined. Both E2 and ICI are known to reduce ER α levels by stimulating protein degradation via a proteasome-mediated pathway [12,22,23]. To avoid the potential impact of ligand on ER α mRNA expression [24,25], a CMV expression system was employed in which HeLa cells were transfected with exogenous ER α . As expected, experiments showed decreased levels of ER α in cells exposed to either E2 or ICI (Fig. 2C). However, only a limited reduction of cellular ER α was seen in the presence of C14. In contrast, an increase in the amount of ER α protein was detected for C13-treated cells, an effect similar to that seen with the SERM tamoxifen [12,26]. Since the exogenously expressed ER α mRNA is under the control of a constitutive promoter, this result indicated a potential protective effect of C13 on ER degradation. Moreover, in light of the increased amount of ER α present in C13-treated cells, the low level of C13-induced activity on an ERE-driven reporter (Fig. 2A) further emphasizes that this ligand is a poor agonist in HeLa cells.

Ligand-bound ER is able to regulate gene transcription in part through its interactions with coregulators such as CBP, p300 and the p160 SRC family of coactivators. Recruitment of these coactivators is ligand-specific and mediated by direct interaction with the ER LBD [27]. A mammalian two-hybrid assay was used to determine whether C13 and C14 could induce the recruitment of the p160 family member SRC-1 to the ER α LBD as indicated by an increase in luciferase reporter expression. As anticipated, a low level of basal activity was present when the GAL plasmid was co-transfected with either VP16 or the VP16-LBD fusion (Fig. 3A). A small increase in luciferase expression was seen when the GAL-SRC-1 fusion was transfected with VP16. However, co-transfection of the GAL-SRC-1 and VP16-LBD fusion proteins increased the level of luciferase activity, even in the absence of ligand. This increased activity is ER-dependent since treatment with ICI inhibited luciferase expression and E2 treatment significantly enhanced reporter gene expression (our data and [28]). Treatment with C14 increased luciferase activity above basal levels, indicating that this compound stimulated interactions between the ER α LBD and SRC-1 to an extent comparable to that seen for E2. Conversely, C13, which was a weak ER agonist in the luciferase reporter assay, inhibited the interaction between the LBD and SRC-1. This effect was similar to, although not as strong as, the inhibition seen in the presence of ICI.

Prior work has demonstrated that although ICI-bound ER α does not recruit SRC-1, it does induce a strong interaction between the ER α LBD and the CBP coregulator [15]. Therefore, a mammalian two-hybrid assay was used to ascertain whether other ER α ligands with extended 7 α side chains, particularly C13 which exerts greater antagonist activity, can promote receptor binding to CBP. Akin to the observation for SRC-1, an increase in basal luciferase activity was seen in cells transfected with expression vectors for both GAL-CBP and VP16-LBD fusion proteins, representing the basal interaction between CBP and ER α (Fig. 3B). As expected, a strong interaction between ICI-bound LBD and CBP was detected, with a relatively modest induction of luciferase activity observed for E2-treated cells. In contrast, C13 was unable to stimulate CBP recruitment above basal levels, and together with the SRC data above, these results suggest that C13's weak agonist activity is independent of SRC-1 and CBP coactivator interactions with the LBD. Consistent with its greater agonist activity, C14 treatment induced luciferase expression in the mammalian two-hybrid assay to a level comparable to E2, indicating an interaction between C14-bound LBD and CBP as well as SRC-1. Expression levels of VP16-LBD in C13- or C14-treated cells were equivalent (Fig. 3C), and therefore did not contribute to the observed ligand-dependent differences in coactivator interactions with the ER α LBD.

While both E2 and ICI induce strong interactions between the LBD and CBP, these responses are dependent on distinct CBP interaction domains [15]. The ICI-stimulated interaction is dependent on the third of three zinc finger motifs (CH3) located in the

coregulator's C-terminus, whereas the interaction in response to E2 results in binding of the ER α LBD to the CH3 domain as well as the N-terminal nuclear receptor interacting domain (NID). Thus, the ability of C13 and C14 to induce LBD interactions with these specific CBP domains was also examined by mammalian-two hybrid assay. Unsurprisingly, C13, which previously failed to stimulate an interaction between the LBD and full-length CBP, was unable to induce interactions with either of the CBP domains tested (Figs. 4A & B). On the other hand, increased luciferase activity was observed for cells expressing the LBD and the CBP NID domain after treatment with C14. This response was similar to but not as strong as that seen for E2. In contrast, no interaction was apparent between the LBD and the CH3 domain in the presence of C14, indicating that this compound only promotes LBD interactions with the NID region associated with binding to agonist-occupied ER α .

In order to test the potential agonist and antagonist effects of C13 and C14 in a cell line that expresses endogenous ER α , MCF-7 breast cancer cells were transfected with the same ERE-luciferase reporter plasmid used above. Similar to the effects seen in HeLa cells, both C13 and C14 induced ER activity over vehicle control in MCF-7 cells (Fig. 5A). Specifically, treatment with 100 nM C13 was $52 \pm 10\%$ as effective as 1 nM E2, while exposure to 100 nM C14 yielded ER α transcriptional activity nearly as great as the receptor's cognate ligand ($92 \pm 6\%$). Both of these effects were blocked in cells co-treated with the pure antiestrogen ICI. Consistent with their activity relative to E2, C13 antagonized E2-induced activity by $\sim 30\%$, while concurrent treatment of cells with E2 and C14 did not further change luciferase expression in comparison to each individual treatment. The ability of the C13 and C14 compounds to alter endogenous ER α expression also was tested (Fig. 5B). As expected, ICI greatly reduced ER α expression in MCF-7 cells. Treatment with C14 led to more modest, though not statistically significant, reductions in ER α levels. Interestingly, C13 administration, which increased ER α expression in HeLa cells, reduced expression of ER α in MCF-7 cells similar in extent to that seen for E2, suggesting a possible cell type-specific effect of C13 on ER α protein stability.

The comparative ability of C13 and C14 to influence the interaction of endogenous ER α with a transcriptional coactivator was tested by co-immunoprecipitation (co-IP). SRC-3 is a member of the p160 SRC coactivator family and is amplified in the MCF-7 cell line [29]. SRC-3 is also frequently overexpressed in breast cancer, even in the absence of gene amplification [29,30]. Co-immunoprecipitation assays revealed that ER α was able to bind to SRC-3 to a similar extent in cells treated with either C14 or E2, while only a very weak interaction was detected for C13-treated cells (Fig. 5C). Thus, the relative ability of C13 and C14 to promote an ER α -SRC-3 interaction was consistent with the relative agonist activity of these compounds measured in the *trans*-activation assay. As expected, an ER α -SRC-3 interaction was not seen with either the ICI or 4HT antiestrogens.

The SRC-3 coactivator is an oncogene required for proliferation and survival of MCF-7 breast cancer cells [31,32]. Based on the results of the luciferase and co-IP assays above, it was predicted that C14, but not C13, would strongly promote proliferation of MCF-7 cell cultures. However, measurements of cell growth following treatment of MCF-7 cells for 5-6 days revealed that both C13 and C14 stimulated cell proliferation relative to vehicle control, mimicking the E2 response (Fig. 6). Co-treatment of either compound along with E2 did not further enhance this effect. Conversely, simultaneous treatment of C13 or C14 with ICI inhibited cell growth below that seen in vehicle-treated control cells, indicating that the effects of the former two compounds on cell growth are ER α -dependent. Therefore, C13's modest induction of an ERE reporter gene in *trans*-activation assays and weak ability to induce ER α -SRC-3 interactions, but robust promotion of MCF-7 cell proliferation, suggests that this ligand utilizes other ER-dependent mechanisms to promote growth of this estrogen-dependent breast cancer cell line.

Discussion

The ICI pure antiestrogens are estradiol derivatives that contain long alkyl side chains at the C-7 α position [8,9,33]. These molecules retain high binding affinity for ER α while simultaneously inhibiting the receptor's transcriptional activity [8,33-35]. Investigations revealed that the antagonist properties were dependent on limiting the length of the side chain substituents to 16-18 atoms [18]. However, these studies focused on amidoalkyl substitutions and did not examine substituents with fewer than eight methylene units. Nonetheless, subsequent structural studies supported a role for long side chains in the displacement of H12 thereby preventing the formation of a high affinity coactivator docking site on the receptor's ligand binding domain and inhibiting AF-2 transcriptional activity [11]. In this report we evaluated the agonist and antagonist properties of two estradiol derivatives with C-7 α side chains featuring either a 6-hydroxyhexanyl (C13) or 6-benzyloxyhexanyl (C14) group. Surprisingly, the C13 compound with its relatively simple 7 α hydroxyhexanyl side chain exerted greater antagonistic activity than C14 in a cell-specific manner, indicating that this ER α ligand is a SERM. This observation also highlights the importance of the chemical composition of the side chain to the regulation of ER α stability and transcriptional activity.

The partial agonist/antagonist effects of SERMs are cell type dependent and stem from the relative contributions made by the receptor AF-1 and AF-2 domains [5]. While maximal ER activity is achieved when the two AFs synergize, each domain can function independently in a cell type-specific manner [5,36-38]. Consequently, even as SERMs fail to stimulate AF-2 transcriptional activity, the constitutively active AF-1 domain remains unimpaired by ligand binding [5,39]. The C13 ligand exhibits a limited ability to induce ER activity in HeLa cells and blocked greater than 50% of the reporter gene expression induced by E2, indicating that it acts predominantly as an antagonist in this cell environment. Like the SERM 4HT, C13 also was unable to recruit the SRC-1 coactivator to the ER LBD in HeLa cells [28]. The nature of the coregulators that support the limited C13 agonist activity in this environment are unknown. However, SRC-1 and other coactivators can interact with the AF-1 domain, and contribute to the partial agonist activity of SERMs in permissive cell environments [40,41]. Thus, the weak C13 agonist activity may reflect recruitment of coactivators with receptor regions outside the AF-2. Alternatively, C13 may induce the AF2 recruitment of coactivators not tested in these experiments. In MCF-7 cells, greater C13 agonist activity was measured by luciferase reporter assay, and C13 stimulation of MCF-7 cell proliferation was comparable to that induced by E2. Moreover, C13 promoted a modest ER α interaction with the SRC-3 coactivator, potentially through the AF1 domain [42]. As SRC-3 is a rate-limiting coactivator for the growth of MCF-7 cells [32], the binding of SRC-3 to ER α likely contributes to C13-induced cell proliferation. Collectively, these results indicate that C13's agonist activity is cell type-dependent, and this ligand is therefore a SERM.

The agonist activity of C14 was greater than that of C13 in HeLa cells. As this environment does not robustly support AF-1 function [5,37], the transcriptional activity is likely derived from the AF-2 rather than the AF-1 domain. This conclusion is supported by the ability of C14 to recruit SRC-1 to the ER α LBD in this cell line. This contrasts to the activities seen for SERMs such as 4HT which are unable to recruit these coregulators or stimulate ER transcriptional activity in HeLa cells [28]. Exposure of MCF-7 cells to C14 alone yielded reporter gene expression comparable to that achieved by E2, and C14 was unable to antagonize E2-induced activity. Moreover, both E2 and C14 recruited SRC-3 to ER α with much greater effect than C13 in MCF-7 cells, and this was consistent with their greater stimulation of ER α transcriptional activity in this cell line. Together, these results demonstrate the potent agonist activity of C14 and its preferential ability to induce interactions of coregulators with ER α in comparison to C13, and implicate the presence of

the benzyloxy group at the end of the C7 alkyl side chain as critical for this effect. In contrast, the presence of a hydroxyl group at the end of C13's six carbon 7 α side chain yields a ligand with SERM properties.

In addition to examining interactions of the ER α LBD with full length SRC-1, the ability of C13 and C14 to promote interactions with full-length CBP was tested. Consistent with the SRC-1 results, C14 but not C13 was able to promote binding of the ER α LBD to CBP. CBP possesses two ER interaction domains; the N-terminally located NID domain contains a LXXLL motif which is commonly used by coregulators to interact with agonist-occupied nuclear receptors, whereas the C-terminal CH3 domain does not [43]. Estradiol-bound LBD can interact with both of these domains, while ICI-bound LBD binds only to the CH3 region, and this latter interaction is more robust than for E2 [15]. Based on C14's ability to stimulate ER α activity, we anticipated this ligand would induce interactions similar to E2 but found that it significantly stimulated only LBD-NID binding. A small yet insignificant increase in ER binding to the CH3 domain raised the possibility of a weak interaction that is below the sensitivity of this assay. Nonetheless, it is concluded that the 7 α side chain of C14 alters the LBD conformation in such a manner that NID rather than CH3 interactions are preferentially maintained, and this emphasizes the agonist properties of C14.

The crystal structure of the LBD of rat ER β bound to ICI 164,384 reveals the 7 α N-(n-butyl)-N-methyl-undecanamide side chain of ICI exits the ligand binding pocket and bends at the fifth carbon so that the remainder of the side chain lies along the surface of the LBD in the groove located between the H3 and H5 helices, thus preventing H12 interaction with this region [11]. By analogy to this ICI structure, it is likely that the sixth carbon and its terminal modification group of the C7 α side chains of C13 and C14, although considerably shorter than for ICI, also protrude from the ligand binding pocket. As the only difference in composition of C13 and C14 is the terminal modification of the C7 α side chain, and C13 produced no interaction between the ER LBD and either SRC-1 or CBP, our data would argue that the terminal polar hydroxyl group present at the surface of the LBD is preventing H12 from adopting an orientation found for an agonist-bound LBD in which it packs against helices 3, 5/6 and 11 and contributes to the formation of the hydrophobic groove employed by coactivators to interact with AF2. However, while it seems certain that H12 does not assume an agonist conformation for C13-bound ER α , the likely position of the helix is unknown. Based on structural alignments of ICI 164,384 and raloxifene [2,11], the C-7 α side chain of C13 likely only extends 50% of the distance of raloxifene's side chain and it is therefore unlikely that C13 is able to push H12 to adopt an antagonist position. Indeed, the ability of C13 to robustly stimulate ER activity in MCF-7 cells suggests that this ligand is incapable of inducing an antagonist-like LBD conformation in which H12 occludes the coactivator binding groove. In contrast, the terminal benzyloxy group of C14 is hydrophobic and would be expected to pack against the groove located between helices H3 and H5, and potentially support the interaction of H12 with this region. Indeed, the relatively good interactions between C14-bound LBD and CBP or SRC-1 (in comparison to C13-bound LBD) suggest that this ligand enables H12 to adopt a more agonist-like conformation than does C13. Resolution of crystal structures for C13-bound *versus* C14-bound ER α LBDs would be required to resolve these questions.

Previous studies have shown an inverse relationship between agonist-driven ER activity and protein stability [12]. When ER transcriptional activity increases, the protein becomes progressively more ubiquitinated, targeting it for degradation by the proteasome [12,22,23,44]. As a result, E2 treatment reduces ER levels. Treatment with ICI also leads to a reduction in ER protein, although this is associated with increased hydrophobicity of the receptor and hyperubiquitination rather than increased transcriptional activity [12,13]. In the present study C14 treatment led to reductions in ER α protein in both HeLa and MCF-7 cells,

an outcome consistent with its ability to induce ER activity in the two cell lines and promote interactions with SRC-3, a coactivator associated with ligand-dependent receptor down regulation [45]. Conversely, treatment with C13 increased ER α levels in Hela cells but decreased them in MCF-7 cells, effects consistent with C13's relative ability to stimulate ER transcriptional activity and interactions with SRC coactivators in the two cell lines, and the SERM-like activity of this compound.

In this paper we evaluated the antagonism of two estradiol derivatives with short C7 α side chains. Despite structural similarities to pure antiestrogens, we hypothesized that these compounds would exhibit weaker antagonist behavior. We show that C13 but not C14 exhibits SERM-like activity. Since the structure of these ER ligands differs only at the terminal modification of the C7 α side chains, our data indicate that the chemical properties of the side chain protruding from the ligand binding pocket is a critical determinant of the compound's agonist *versus* antagonist bioactivity. Although the stronger agonist properties of C14 may seem counterintuitive, its C7 α side chain is terminated with a benzyloxy group, which is less polar than the hydroxyl moiety found in C13. As such, the side chain of C14 may experience stronger interactions with the hydrophobic surface exterior to ER's ligand binding pocket and favor agonist-like interactions of H12 with this surface. Interestingly, a previous study using the non-steroidal SERM idoxifene found that increasing side chain length did not improve functional antagonism [46], which further supports our contention that the length of a ligand's side chain is less important than the nature of its functional groups.

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Abbreviations

4HT	4-hydroxytamoxifen
AF-1	activation function-1
AF-2	activation function-2
C13	7 α -(6-hydroxyhexanyl)-17 β -estradiol
C14	7 α -(6-benzyloxyhexanyl)-17 β -estradiol
CBP	cAMP response element-binding protein-binding protein
DME	Dulbecco's Modified Eagle Medium
E2	17 β -estradiol
ERα	estrogen receptor-alpha
ERE	estrogen response element
FBS	fetal bovine serum
H12	helix 12
ICI	ICI 182,780
LBD	ligand binding domain
Luc	luciferase
NCoR	nuclear receptor corepressor
NID	nuclear receptor interaction domain
PBS	phosphate buffered saline
SERM	selective estrogen receptor modulator
SRC	steroid receptor coactivator

Highlights

- E2 derivatives with short C-7 α alkyl side chains regulate ER α activity
- Side chain terminal modifications impact ER α stability and coactivator binding
- SERM or agonist activity of derivatives depends on side chain terminal substituent

Figure 1.
Chemical structures of E2, ICI 182,780, and the novel compounds C13 and C14. ICI 182,780, C13, and C14 all contain an E2 backbone with unique side chains at the C-7 α position.

Figure 2.

Agonist and antagonist activity of C13 and C14 in HeLa cells. Cells were transfected with the expression vector pCR3.1-ER α and the reporter plasmid ERE-Elb-Luc. A) Luciferase activity in cells treated with vehicle (0.1% ethanol, lane 1), 1 nM E2, 100 nM ICI, 1-100 nM C13 or 1-100 nM C14. * $p < 0.05$ compared to vehicle. B) Luciferase activity in cells treated with vehicle (0.1% ethanol, lane 1), 1 nM E2, 100 nM ICI, 100 nM C13 or 100 nM C14 as indicated. For (A) and (B), values are normalized to those obtained for E2 treatment. Bars represent the average $\pm$ SEM of at least 3 experiments. # $p < 0.05$ compared to E2; ° $p < 0.05$ compared to the respective E2, C13, or C14 individual treatment group. C) A representative Western blot assessing ER α expression in HeLa cells treated for 24 h with vehicle (0.1% ethanol), 1 nM E2, 100 nM ICI, 100 nM C13 or 100 nM C14 (left). Results from 4 experiments were quantitated by scanning densitometry and are presented as the average $\pm$ SEM (right). * $p < 0.05$ compared to vehicle.

Figure 3.

Mammalian two hybrid examining interactions between the ER α LBD and SRC-1 or CBP in HeLa cells treated with C13 or C14. A) Luciferase activity of cells transfected with expression vectors for the GAL DBD alone or GAL-SRC-1 in the presence of either expression vector VP16 or VP16-ER α -LBD, along with the reporter plasmid pG5-Luc. Cells were treated with vehicle (0.1% ethanol), 1 nM E2, 100 nM ICI, 100 nM C13, or 100 nM C14. Values are normalized to those obtained for VP16-ER α -LBD and GAL-SRC-1 in the presence of E2. Bars represent the average $\pm$ SEM of four experiments. B) Similar experiment as in (A) except using GAL-CBP in place of GAL-SRC-1. * $p < 0.05$ compared to vehicle in LBD and SRC-1 or CBP co-transfected cells; # $p < 0.05$ compared to E2 in same. C) Representative western blot assessment of VP16-LBD in cells treated with ligand as indicated above (*left*). Blots from 5 experiments were quantitated by scanning densitometry, and bars represent the average $\pm$ SEM (*right*). ° $p < 0.05$ compared to vehicle.

Figure 4.

Mammalian two hybrid examining interactions between the ER α LBD and the CBP domains NID or CH3 in HeLa cells treated with C13 and C14. A) Luciferase activity of cells transfected with expression vectors for the GAL DBD alone or GAL-CBP-NID in the presence of either expression vector VP16 or VP16-ER α -LBD, along with the reporter plasmid pG5-Luc. Cells were treated with vehicle (0.1% ethanol), 1 nM E2, 100 nM ICI, 100 nM C13, or 100 nM C14. Values are normalized to those obtained for VP16-LBD and GAL-CBP-NID in the presence of E2. B) Luciferase activity of cells transfected with either the GAL DBD alone or GAL-CBP-CH3 in the presence of VP16-ER α -LBD and pG5-Luc. Cells were treated as in (A), with values normalized to those obtained for VP16-LBD and GAL-CBP-CH3 in the presence of E2. Bars represent the average $\pm$ SEM of at least three experiments.

* $p < 0.05$ compared to vehicle in LBD and NID or CH3 co-transfected cells; # $p < 0.05$ compared to E2 in same.

Figure 5.

Effect of C13 and C14 on ER α activity in MCF-7 cells. A) Luciferase activity of cells transfected with the reporter plasmid ERE-Elb-Luc. Cells were treated with either vehicle (0.1% ethanol, lane 1), 1 nM E2, 100 nM ICI, 100 nM C13 or 100 nM C14 as indicated. Values are normalized to those obtained for E2 treatment. Bars represent the average $\pm$ SEM of at least three experiments. * $p < 0.05$ compared to vehicle; # $p < 0.05$ compared to E2. B) Western blot of ER α expression in cells treated for 24 h with either vehicle (0.1% ethanol), 1 nM E2, 100 nM ICI, 100 nM C13 or 100 nM C14 (*left*). Blots from 3 experiments were quantitated by scanning densitometry, and bars represent the average $\pm$ SEM (*right*). * $p < 0.05$ compared to vehicle. C) Coimmunoprecipitation of ER α and SRC-3 in cells treated with either vehicle (0.1% ethanol), 100nM E2, 1 μ M ICI, 1 μ M 4HT, 1 μ M C13, or 1 μ M C14. Blots are representative of three experiments.

Figure 6.

MCF-7 cell growth during exposure to C13 and C14. Cells were treated with vehicle (0.1% ethanol, lane 1), 1 nM E2, 100 nM ICI, 100 nM C13 or 100 nM C14 as indicated. Values were determined by cell count and are normalized to those obtained for E2 treatment. Bars represent the average $\pm$ SEM of at least three experiments. * $p < 0.05$ compared to vehicle.