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Histone Deacetylase Inhibitors Equipped with Estrogen Receptor Modulation Activity

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Abstract

We described a set of novel histone deacetylase inhibitors (HDACi) equipped with either an antagonist or an agonist of the estrogen receptor (ER) to confer selective activity against breast cancers. These bifunctional compounds potentially inhibit HDAC at nanomolar concentrations, and either agonize or antagonize ER α and ER β . The ER antagonist activities of tamoxifen-HDACi conjugates (Tam-HDACi) are nearly identical to those of tamoxifen. Conversely, ethynyl-estradiol HDACi conjugates (EED-HDACi) have attenuated ER agonist activities relative to the parent ethynyl-estradiol. *In silico* docking analysis provides structural basis for the trends of ER agonism/antagonism and ER subtype selectivity. Excitingly, lead Tam-HDACi conjugates show anticancer activity that is selectively more potent against MCF-7 (ER α positive breast) compared to MDA-MB-231 (triple negative breast cancer), DU145 (prostate cancer) or Vero (non-cancerous cell line). This dual-targeting approach illustrates the utility of designing small molecules with an emphasis on cell-type selectivity, not merely improved potency, working towards a higher therapeutic index at the earliest stages of drug development.

Introduction

Histone deacetylases (HDAC) catalyze the removal of acetyl groups from histone and non-histone proteins for transcriptional regulation, cell cycle progression, and apoptosis. There are 18 human HDAC isoforms subdivided into four different classes (I-IV). Classes I, II, and IV (HDAC-1 through -11) require Zn²⁺ for enzymatic activity. The seven members of class III, called sirtuin (SIRT1 through 7), require NAD⁺ for activity, and do not have histones as their primary targets.¹ Class I HDACs are ubiquitously expressed and play essential roles in proliferation, whereas classes II and IV have tissue specific functions.² The maintenance of equilibrium between acetylation and deacetylation of histones and non-histone substrates is essential for normal cell growth. Aberrant HDAC activity can result in epigenetic imbalance³ and has been linked to cell proliferation in many cancers.¹⁻³ In particular, over-expression of HDAC1, HDAC6, and HDAC8 has been linked to breast tumors.⁴ As a result, the use of HDAC inhibitors (HDACi) as cancer therapeutics is an area of active investigation.²

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Supporting Information Available: ¹H NMR and ¹³C NMR spectral information, general methods, and molecular modeling outputs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Several HDACi are in various stages of clinical trials, with approximately 500 clinical trials initiated over the last decade,^{2b} thus far resulting in the FDA approval of SAHA (vorinostat)⁵ and FK228 (romidepsin).⁶ Nevertheless, a lack of solid tumor penetration and broad tissue distribution has resulted in clinical ineffectiveness⁷ and off target side effects, such as myelosuppression, fatigue, and cardiac toxicity.⁸ Selectively delivering HDACi into cells of interest could potentially decrease such unanticipated side effects and increase the potencies and efficacies of these drugs.⁹

The use of HDACi in combination with other anti-cancer agents has been found to increase the efficiencies of these agents.^{2c, 10} HDACi are more effective when used in combination with hormone therapy, as they potentiate the effects of tamoxifen in ER α positive cell lines.^{12, 13} Thus, an area of increasing interest is to combine hormone therapies with HDACi to target various stages of the cancer cell cycle and thus broadly inhibit tumor proliferation.^{12, 13} Indeed, a recent phase II study has shown that a combination of SAHA and tamoxifen is well tolerated with a 40% clinical benefit that positively correlates with histone hyperacetylation.¹¹

Furthermore, combining HDACi with estrogen modulators could target estrogen modulator resistant cancers. Many breast tumors which were once sensitive to estrogen modulators, such as tamoxifen and fulvestrant, acquire resistance over time.¹² The exact mechanisms of estrogen modulator acquired resistance are not completely understood. It is however clear that resistant tumors still maintained ER expression, either in the form of ER α (in more than 60% of the cases)¹² or by up regulation of the expression of ER β ,¹³ a closely related ER subtype. In other cases, resistance is acquired via epigenetic silencing of ER subtypes and HDACi restore tamoxifen sensitivity in such ER-negative breast cancers by inducing re-expression of ER α .¹⁴

ERs are initially bound to heat shock proteins in the cytoplasm, and translocate to the cell nucleus upon hormone binding. Small subsets of the population of both ERs, 5–10% for ER α , exist associated with the plasma membrane of target cells. These membrane receptors have been implicated in the rapid non-genomic signaling associated with estrogen modulating cell survival, motility, and proliferation.¹⁵ We hypothesized that cytoplasmic and nuclear ER could increase retention of ER targeted compounds, while surface expressed ER is anticipated to facilitate their tumor selective uptake.¹⁶ Regardless of mechanism, these bifunctional compounds could achieve higher concentrations in cells expressing ER.

In this study, we investigate the conjugation of a HDAC inhibition moiety to two estrogen modulator ligands, as an approach to selectively enhance HDACi concentration in hormone positive breast tumors. We investigated the biological effect of both agonist and antagonist scaffolds. Specifically, we have covalently linked aryl- and azido-hydroxamate HDACi to tamoxifen (an antagonist and selective ER modulator, or SERM) and 17 α -ethinylestradiol (an ER agonist) respectively. We anticipate that the interaction between the ER α and ER ligand-like portions of these conjugates will selectively sequester them into ER positive cells. Our results reveal that these dual acting molecules retain independent anti-HDAC and estrogen receptor binding activities. Tam-HDACi conjugates are generally less potent inhibitors of HDAC than EED-HDACi compounds, but exhibit greater anticancer activity across all cell lines. Particularly, the Tam-HDACi conjugates are selectively potent for MCF-7, less effective in MDA-MB-231 (ER α negative breast cancer cells), DU145 (prostate cancer cells) and VERO (healthy cells). These results suggest that conjugation of estrogen modulators to the HDACi moiety could facilitate a selective enrichment of HDACi in hormone positive tumors and possibly broaden the scope of ER ligand clinical use to ligand resistant breast tumors.

Results and Discussion

Design and synthesis of estrogen modulator HDACi conjugates

With the goal of creating novel HDACi that exhibit a therapeutic effect selectively more potent towards breast cancers, we designed HDACi that are covalently linked to either the ER agonist (17 α -ethinylestradiol, Figure 1B) or the selective ER modulator (SERM, tamoxifen, Fig 1B), named EED-HDACi and Tam-HDACi, respectively. To appropriately design the molecular connectivity in such a way that binding is retained to both the ER and HDAC, the crystal structures of each were analyzed. Crystal structures of several hydroxamic acid-derived HDACi (such as SAHA, Figure 1B) bound to various HDAC isoforms show a remarkable conservation of positioning, which has led to the recognition of a three part pharmacophore consisting of 1) a cap group that recognize the surface outside the binding pocket, 2) a linker that traverses the tunnel leading to the active site and 3) a zinc binding group (ZBG) for chelation to the Zn ion buried at the heart of the active site. In previous SAR studies we learned that the cap group preference is extremely flexible,¹⁷ as should be expected when considering the large number of structurally divergent protein substrates the HDAC enzymes act upon. Cap group variation also allows for selectivity among HDAC isoforms,¹⁸ Therefore, the ER modulators tamoxifen and EED were designed to function as cap groups in the HDACi pharmacophore, to be placed at the end of the linker distal to the ZBG. The size and shape of the linker and ZBG are consistently similar to their acetylated lysine substrate, explaining their more limited diversity as compared to the cap group. We designed all HDACi compounds with the same hydroxamic ZBG and varied alkyl linker lengths. We incorporated the triazole functionality at the junction between the ER targeting cap group and the alkyl linker, based on our previous observations that the triazole-ring not only enhanced the HDACi activity of SAHA-like hydroxamates but also facilitated the investigation of the linker length dependence of their activity.^{17d, 19}

For targeting ER, there are many examples of dual-function conjugates modified at various positions of the parent ER modulator.²⁰ We aimed here to explore the effect of both agonistic and antagonistic conjugates. Beginning with the steroidal agonist EED, the 17- α position was chosen as the point of tethering for compounds **1a–e** (Figure 1B). Added bulk at this location often results in a loss of binding affinity,²¹ but this is the only position where modification allows for retention of agonist activity (as seen in TFMPV-E2, Figure 1A). E2 derivatives with bulky modifications on the B/C rings have antagonistic activity, as the bulky group is able to extend towards and displace helix 12 in the same manner as tamoxifen (as seen in ICI164,384 and RU 39411, Supplemental Figure S1). As we envisioned the use of the ER as a route to enhanced nuclear localization, attenuation of binding affinity is a potential benefit, as the ER would be required to release the drug before its secondary activity (HDACi) would take effect. Furthermore, the linker length was varied to probe for optimal HDACi and ER activity.

To determine the amenable point of chemical tethering for the tamoxifen cap group, we examined the co-crystal of 4-hydroxy tamoxifen (the active metabolite of tamoxifen) bound to the ligand binding domain of ER α (PDB: 3ERT, Figure 1A). The dimethylamino group of tamoxifen displaces helix-12 (H12, Figure 1A), an extension of steric bulk from the triphenylethylene core which if removed results in a switch to estrogenic activity.²² Analysis revealed that the methyl groups of the dimethylamino moiety are solvent exposed, allowing for modification without disruption of binding affinity. This structural information has been used to conjugate tamoxifen to cytotoxic agents in studies aimed at developing targeted breast cancer therapeutics.²³ We designed two Tam-HDACi conjugates using the dimethylamino moiety of tamoxifen as the conjugation locale (Figure 1B), with either a 1, 2, 3-triazole ring (compound **2**) or simple amide-bond linkage (compound **3**). The choice of the methylene linker length of compound **2** is based on our prior observation which suggests

that five methylene groups, separating the triazole from the hydroxamate moiety, are optimal for HDAC inhibition of aryltriazolylhydroxamates.^{17,19} Compound **3** on the other hand is a direct Tam-SAHA conjugate. We envisioned that this design approach would enable a facile unmasking of the minimal structural attributes for simultaneous HDAC inhibition and estrogen modulation within a single molecular template.

All designed compounds were synthesized as outlined in Schemes 1–3. Starting with alkyne containing EED and trityl-alkyl-azides **4a–e**, Cu (I) catalyzed azide-alkyne cycloaddition reaction (“click” chemistry) was employed to obtain protected precursors **5a–e** (Scheme 1).²⁴ Trifluoroacetic acid (TFA) facilitated removal of the trityl protecting groups furnished EED-HDACi conjugates (**1a–e**) of various linker lengths.

Tamoxifen conjugates **2** and **3** were derived from N-desmethyl tamoxifen **10**, obtained via demethylation of tamoxifen according to literature protocols.²⁵ Alkylation of **10** with mesylated compound **9**,^{17a} followed by KCN catalyzed reaction with NH₂OH gave the hydroxamic acid final compound **3**. The triazole containing conjugate was synthesized by first alkylating **10** with mesylated alkyne **12** to obtain tamoxifen alkyne **13**. Cu (I) catalyzed cycloaddition reaction between azide **9** and alkyne **13** was utilized to obtain triazole ester **15**. Treatment of **15** with NH₂OH and catalytic KCN gave the desired hydroxamate **2** (Scheme 3).

ER ligand-HDACi conjugates exhibit ER α agonist or antagonist activity

To evaluate whether our designed ER ligand-HDACi conjugates would retain their estrogenic or anti-estrogenic activity, we tested all conjugates for modulation of ER transcriptional activity in mammalian cell culture. Agonist assays test the ability of the conjugates to bind the ER ligand-binding domain and activate transcription of a luciferase reporter gene in HEK 293T cells. Antagonist assays test the ability of the conjugates to antagonize the transcriptional activation of 300 pM E2 acting on the ER ligand binding domain.

Both EED-HDACi and Tam-HDACi conjugates retain the ER α agonist or antagonist activity of their parent drugs EED and tamoxifen, respectively (Figure 2). All EED-HDACi conjugates have attenuated ER agonist activities relative to the parent ethinylestradiol. Of these EED-HDACi conjugates, **1b** and **1d** showed the most significant ER α agonist effect. As expected, Tam-HDACi conjugates have no detectable ER α agonist activity. Tamoxifen derived conjugates have significant ER α antagonist activity, with IC₅₀ values in the nanomolar range, but like tamoxifen they should also undergo transformation to much more potent hydroxylated metabolites *in vivo*.

Estrogen Receptor docking analysis

In an attempt to understand the structural basis for the observed ER α agonist and antagonist effects of EED-HDACi (**1a–e**) and Tam-HDACi (**2, 3**), *in silico* docking analysis was performed using Autodock Vina. This generates low-energy binding poses by evaluating the combined energetic contributions of torsion, steric repulsion, hydrogen bonding and hydrophobic interaction between the ligand and the protein binding pocket. SAR insights are greatly aided by molecular docking analysis, but must be taken as putative due to the rigid nature of the protein target and the potential for conformational bias.²⁶ The wealth of crystal structures for various ER agonist and antagonist scaffolds increases reliability of *in silico* results. Although direct comparisons are speculative, our docking outputs agree with the agonist and antagonist effects exhibited by all analogs of EED-HDACi and Tam-HDACi (Figure 3).

Using the crystal structures of ER α obtained in complex with weak agonists 17- α modified EED analogues TFMPV-E2 (PDB: 2P15, Figure 3A)^{21a} and EEu (PDB: 2YAT, Figure 3B),²⁷ we observed that EED-HDACi compounds adopt docked poses in which their EED moieties are invariant from the EED moieties of the original ligands. These structures show unusual bulging of the ER ligand binding domain (distal from helix-12 rather than through the helix-12 opening) caused by addition of bulk to the 17- α position of EED. Because similar modification is known to result in weak ER agonists^{33, 34} these docked structures may reflect the most probable mode of binding. This is also supported by our observation with EED-HDACi conjugates in ER-luciferase reporter assays.

Conversely, Tam-HDACi conjugates did not dock into the binding pockets of 2P15 and 2YAT, which may explain their inability to agonize ER. This is reflected in an average decrease in binding affinity (BA, kcal/mol) of for 2P15 and 2YAT (Figure 3D). Docking analysis on the 4-hydroxy metabolites of Tam-HDACi produced results very similar to those of the unmodified Tam-HDACi.

The opposite trend is seen when all conjugates are docked against the crystal structure of ER α complex with 4-hydroxy tamoxifen (PDB: 3ERT). In this structure tamoxifen prevents helix 12 from closing properly upon the binding pocket of the LBD (Figure 3C, helix 12 colored red). None of the EED-HDACi conjugates docked with the EED moiety in the binding pocket (Figure 3C, thin cyan sticks). Their bulky 17- α modification cannot go through the pocket opening while also allowing the EED moiety the crucial hydrogen bonding interactions with Arg-394/Glu-353 in the base. Tam-HDACi conjugates, however, like their parent antagonist, are able to fill the hydrophobic space, latch onto Arg-394/Glu-353 (for the hydroxylated derivatives), and extend their alkyl hydroxamate moieties through the opening left by the displaced helix-12 (Figure 3C). These structural factors result in superior *in silico* binding affinities for all Tam-HDACi relative to their EED-HDACi congeners (Figure 3D). A closer inspection of the docked structures of the EED derivatives **1a–e** reveals additional key interactions that may be responsible for their varying ER α agonist effect. All conjugates maintain the critical H-bonding interactions between the steroid's A-ring phenolic hydroxyl with Arg-394/Glu-353 and the D-ring 17- α alcohol with His-524.

Each of the EED-HDACi compounds of various linker lengths has two major binding modes: one with the HDACi moiety coils up inside the binding pocket (Figure 4A), and a second where the alkyl chain extends outward toward residues 414–417 of H7 (Figure 4B). In the 2P15 crystal structure, a small tunnel opposite helix-12 is opened by the bulkiness of the 17- α substituent on the TFMPV-E2 ligand used. Because the amino acid residues lining this induced-tunnel (Met-342, Met-343, Phe-425, Met-421, and Leu-410) are hydrophobic, they are amenable to the greasy alkyl chain linker of the EED-HDACi conjugates. In the docking conformations where the alkyl hydroxamate moiety extends through this tunnel toward the exterior, the hydroxamic acid group is within hydrogen bonding distance to the side chains of Cys-417 and Gln-414, and the amide backbone of Gln-414 and Gly-415 (Figure 4B). Potential correlations between *in vitro* ER agonist activity and *in silico* binding affinities, as well as an overlay of all compounds in both binding modes, can be seen in Supplemental Figure S2.

ER α and ER β are differentially agonized by EED-HDACi

Selectivity for ER α or ER β is important for drugs targeting the estrogenic signaling axis, as the two subtypes play significantly divergent roles in disease progression,²⁸ and can determine response to treatments.²⁹ All of the EED-HDACi compounds with estrogenic activity, except **1b** exhibited a binding preference shift from ER α to ER β (Figure 5). The ER β preference of some EED-HDACi conjugates is a reverse of the binding preference of

the initial EED steroid scaffold. The ligand binding pockets are almost identical between the two ER subtypes. None of the critical hydrogen bonding amino acids differ, and only two amino acids are changed (Met-421→Ile-373 and Leu-384→Met-336) without significantly altering the overall pocket hydrophobicity. These amino acids occupy equivalent positions in the 3D structure of the ER subtypes. ER selectivity has therefore been attributed to changes in hydrophobic space-filling.³⁰ None of the conjugates is capable of fitting into the LBD of available ER β crystal structure (PDB: 3OLS, see Supplemental Figure S3) in the agonist conformation, so a homology model of the ER β subtype was constructed by mapping ER β AA sequence onto the 2P15 scaffold (see Experimental Section). Molecular docking analysis of **1a–e** with both ER α (crystal structure 2P15) and a homology model of ER β resulted in nearly identical positioning for the steroidal portion of all EED-HDACi compounds in both ER subtypes, with differences only in alkyl chain coiling. Of the two AA changes, the Met-421→Ile-373 substitution offers greater potential for subtype selectivity, as these amino acids line the tight hydrophobic space occupied by the coiled-up alkyl chain of the EED-HDACi conjugates (Figure 5C). Unless there is a structural reorganization to accommodate the bound ligand, the Met-421 of ER α may clash with the alkyl chains of some of the EED-HDACi conjugates due to distances as short as 3.0 Å between them (see Supplemental Figure S4). Ile-373 affords ER β an increased volume at this critical position, which we speculate to be responsible for the ability of longer linker chains **1c** and **1d** to preferentially bind ER β . Docking preference for ER subtype *in silico* (Supplemental Figure S5) closely matches the experimentally observed preference for **1b**, **1c** and **1d** (**1a** and **1e** are too weak *in vitro* to have an obvious preference). Tamoxifen and its Tam-HDACi derivatives show very little preference for either ER subtype (Supplemental Figure S6), owing to their alternative mode of binding that does not induce the same hydrophobic cavity (Supplemental Figure S7).

Dual-acting compounds exhibit potent HDAC inhibition activity

We tested all of the dual-acting ER ligand-HDACi conjugates for HDAC inhibition against three isoforms important in breast cancer progression, including HDAC1 (nuclear HDAC, responsible for silencing of genes encoding p21, ER α , via histone deacetylation and chromatin condensation), HDAC6 (cytoplasmic HDAC, controlling ER α stability by regulating the acetylation state of its chaperone HSP-90), and HDAC8 (both nuclear and cytoplasmic HDAC, responsible for transcriptional repression of tumor suppressor genes).²⁹ Recently, all three isoforms have been found to be critical in the invasion and migration of both ER positive (MCF-7) and negative (MDA-MB-231) cell lines.⁴

Experiments measuring the inhibition of these three HDAC isoforms reveal that attachment of ER targeting moieties, as the surface recognition cap group of a prototypical HDACi, does not abolish HDAC inhibition activity (Figure 6). In general, these conjugates strongly inhibit HDAC1 and HDAC6 and weakly inhibit the deacetylase activity of HDAC8. Almost across the board, the EED-HDACi conjugates have superior HDAC inhibition activity relative to the Tam-HDACi conjugates. Of the EED-HDACi, **1a–d** inhibit HDAC1 and HDAC6 with nanomolar range IC₅₀s. Conjugates **1a** and **1d** have better activity than the clinical HDACi SAHA, suggesting flexibility in the range of optimal linker-lengths that support HDAC inhibition activities of EED-HDACi. However, Tam-HDACi conjugates exclusively inhibit HDAC1 and HDAC6. Interestingly, conjugate **3** is selective for HDAC6 (Figure 6).

Dual-acting compounds have cell-type selective anticancer activity

The majority of HDACi tested clinically exhibit unselective cytotoxicity, off-target effects and on-target ineffectiveness.^{2b} Cell-type selective delivery of HDACi into targeted cancer cells could potentially increase potencies and decrease unanticipated side effects, thereby

improving drug therapeutic indices. In addition, this approach could lead to a new class of chemotherapy for targeted cancer therapy applications.

To evaluate cell-type selectivity and anticancer activity, all compounds were screened against two breast cancer cell-types – MCF-7 and MDA-MB-231, as well as two control lines – prostate cancer (DU145) and healthy kidney epithelial cells (VERO). MCF-7 is a commonly used representative of the almost 75% of breast cancer that express ER α , the majority of which initially respond to endocrine therapies such as tamoxifen.³¹ MDA-MB-231 represents the nearly 25% of breast cancers do not respond to endocrine therapy because ER α is epigenetically silenced by hyper-methylation and/or histone lysine hypo-acetylation.³² In this context, HDAC inhibition causes re-expression of ER α in these breast cancers by increasing acetylation of the chromatin scaffolding around the ER α gene,³³ and the cancers are thereby sensitized to drugs that target ER α (both *in vitro* and *in vivo*).¹¹

Overall, the Tam-HDACi are much more potent than EED-HDACi at reducing cancer cell proliferation (Figure 7A), despite the fact that Tam-HDACi are less potent HDACi (Figure 6). This observation suggests that targeting the ER goes beyond merely enabling improved cellular uptake/retention of the conjugates, and that antagonist/agonist activities more directly impact the anti-proliferative activities of these compounds.

The anti-proliferative activities of some EED-HDACi conjugates (**1a** against MCF-7/MDA-MB-231 and **1c–d** against MDA-MB-231/DU145) are comparable to those of the other clinically approved endocrine receptor targeting drugs for breast cancer (tamoxifen, 15–20 μ M) and prostate cancer (bicalutamide, 18–33 μ M).³⁴ The three EED-HDACi compounds - **1a**, **1c** and **1d** - which inhibit the growth of MDA-MB-231 are also the most potent HDACi. Compound **1d** stands out among these EED-HDACi because it shows both an order of magnitude greater HDAC8 inhibition and also the best anti-proliferative activity against MDA-MB-231. Recent studies reveal HDAC8 expression in MDA-MB-231 is much greater than in MCF-7 and is responsible for its significantly increased invasiveness.⁴ This may explain our observed enhanced cytotoxicity of **1d** against MDA-MB-231. Another interesting aspect of this data is that compounds **1c** and **1d**, the only analogs exhibiting both nM HDACi potency and significant ER β activation, are also the only EED-HDACi that show any evidence of DU145 cell growth inhibition. DU145 is a metastatic prostate cancer line that only expresses ER β .³⁵ Our result suggests that compounds with similar attributes as **1c** and **1d** may have potential benefit against ER β dependent cancers. Compound **1e** shows the least anti-proliferative activity against all cell lines studied, an observation which agrees with its poor HDAC inhibition profile. It is important to note here that EED, the starting scaffold of EED-HDACi, is devoid of anti-proliferative activity. Additionally, all EED-HDACi are non-toxic to the healthy VERO cell line (Figure 7A).

The Tam-HDACi conjugates, which inhibit HDAC and antagonize ER, have even more promising anti-proliferative activities. Conjugates **2** and **3** show enhanced potency against ER expressing (MCF-7) versus ER negative (MDA-MB-231) breast cancer cells (Figure 7), unlike SAHA which shows a similar dose response curve for both (Figure 7B). Specifically, **3** is equipotent to SAHA and 4-fold more potent than tamoxifen, while **2** is about 1.7-fold less potent than SAHA and 2-fold more potent than tamoxifen, against MCF-7. Interestingly, the trend of cytotoxicity to MCF-7 follows the ER α antagonist activity of these compounds. The enhancement of anti-proliferative activity relative to tamoxifen supports the contribution of the HDAC inhibition to the tumor cell growth inhibition activity of these conjugates. The MDA-MB-231 cell growth inhibition activity of Tam-HDACi could be due to HDAC inhibition which may lead to a reactivation of epigenetically silenced ER pathways which HDACi are known to induce.¹¹ There is however no clear evidence for

ER related trend in the sensitivity of DU145 to these conjugates. This suggests that the anti-proliferative activity of Tam-HDACi against DU145 derives mainly from HDAC inhibition.

All ER ligand-HDACi conjugates and the control compounds have different effects on the viability of the non-transformed VERO cells. Comparing their effects on VERO and MCF-7 cells, SAHA, the control HDACi, has the smallest *in vitro* therapeutic index³⁶ (IVTI) while ER ligand-HDACi such as **1a**, **2** and **3** have greater IVTI (Figure 7C). Using these metrics as surrogate for clinical therapeutic index, **2** is a promising lead among these ER ligand-HDACi compounds.

Conclusions

Interaction between ER α and estrogen plays a central role in pro-growth/ pro-survival signaling pathways in breast tumors and could stimulate tumorigenesis by inducing expression of genes involved in cell proliferation.³⁷ ER α modulators such as tamoxifen inhibit these processes by acting as estrogen antagonists in a tissue dependent manner and effectively inhibit ER α positive breast cancers through cell cycle arrest at the G0 and G1 phases.³⁸ Similarly, HDACi's promote anti-proliferation in cancer cells by arresting both G1/S,³⁹ as well as G2/M cell cycle phase progression⁴⁰ and by disrupting mitosis.⁴¹ A cooperative activity has been seen with tamoxifen and HDACi combination in ER α positive breast cancer cells.^{12, 13}

Combination therapy is a proven approach for overcoming drug induced resistance in many human diseases including breast cancer. However, a common liability of multiple drug therapies is the inherent pharmacokinetic disadvantage of two separate drugs. Other disadvantages of multiple drug combination include increase risk of drug-drug interactions and additive toxicities. Clinical trials of combination therapy of some FDA approved anticancer drugs have been halted due to unacceptable toxicities which were not presented when the drugs were administered individually.⁴² We demonstrate herein that conjugation of a HDACi moiety to representative ER ligands is a viable approach to generate dual acting agents which retain independent anti-HDAC and estrogen receptor binding activities, and have an improved *in vitro* therapeutic index.

Two types of ER ligands with divergent effects on ER activity – EED (ER agonist) and tamoxifen (ER antagonist) – are used in our design. Relative to the corresponding ER ligand, the EED-derived conjugates have attenuated ER agonist activity while the antagonist activity of the tamoxifen-derived conjugates remains essentially unchanged. A subset of these conjugates possesses tumor cell-type selective toxicity, potentially driven by their interaction with ER. Docking analyses provide insights on the molecular basis of the ER binding affinity and the ER subtype preferences of these ER ligand-HDACi compounds.

Although several of these compounds possess attributes that confer favorable pharmacokinetic profiles (logP < 5, total polar surface area (TPSA) < 140, molecular weight (MW) < 500, Supplemental Table 1),⁴³ we anticipate that their bioactivity will be significantly influenced by active cell uptake. It is conceivable that the ER ligand-like portions of these conjugates selectively sequester them into (or enhance their retention in) ER positive cells. In fact, we have previously observed that gold nanoparticle conjugated tamoxifen is capable of a selective uptake into MCF-7 cells in a mechanism that has all the signatures of cell surface receptor mediated endocytosis.⁴⁴ Making use of this phenomenon may prove beneficial as membrane ER has been found to be exclusively expressed in the tumor mass and not in surrounding healthy cells.⁴⁵ Agents that possess “combination chemotherapy” potential within a single molecule, such as the conjugates described herein, could ameliorate many of the shortcomings of the traditional combination therapy approach.

Experimental Section

Tamoxifen, 17- α Ethynylestradiol, 4-Ethynylbenzyl alcohol, (4-aminophenyl) methanol, methyl 8-chloro-8-oxooctanoate, ethyl 6-bromohexanoate, ethyl 7-bromoheptanoate were purchased from Sigma Aldrich. 17 β -estradiol was purchased from MP Biomedicals, (Salon, OH). Anhydrous solvents and other reagents were purchased and used without further purification. Analtech silica gel plates (60 F₂₅₄) were used for analytical TLC, and Analtech preparative TLC plates (UV 254, 2000 μ m) were used for purification. UV light was used to examine the spots. 200–400 Mesh silica gel was used in column chromatography. NMR spectra were recorded on a Varian-Gemini 400 magnetic resonance spectrometer. ¹H NMR spectra are recorded in parts per million (ppm) relative to the peak of CDCl₃, (7.24 ppm), CD₃OD (3.31 ppm), or DMSO-*d*₆ (2.49 ppm). ¹³C spectra were recorded relative to the central peak of the CDCl₃ triplet (77.0 ppm), CD₃OD (49.0 ppm), or the DMSO-*d*₆ septet (39.7 ppm), and were recorded with complete hetero-decoupling. Multiplicities are described using the abbreviation s, singlet; d, doublet, t, triplet; q, quartet; m, multiplet; and app, apparent. All biologically evaluated compounds were established to be >95% pure using HPLC. These HPLC analyses were done on a Beckman Coulter instrument with a Phenomenex RP C-18 column (250 mm $\times$ 4.6 mm), using 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), starting with 5% B for 4 minutes, then a gradient increase of 5% to 100% of B over 25 minutes. The flow rate was 1.0 mL/min and detection was at 254 nm. High-resolution mass spectra were recorded at the Georgia Institute of Technology mass spectrometry facility in Atlanta. 4-Ethynyl-benzyl methylsulfonate **12**, methyl 8-(4-(hydroxymethyl)phenylamino)-8-oxo-octanoate **8** and its mesylated analog **9**, and azidoalkyl esters were synthesized by adapting literature protocols.^{17a,19}

4-(4-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-(trityloxy)butanamide (**5a**)

17- α ethynylestradiol (280 mg, 0.95 mmol) was combined with 4-azido-O-trityl amide **4a** (352 mg, 0.95 mmol) and placed under argon. Dimethylsulfoxide (6.6 mL) was added and the solution was stirred. Argon was bubbled through the solution for 10 minutes. Diisopropylethylamine DIPEA (0.33 mL) was added via syringe. Copper iodide (90 mg, 0.47 mmol) was added and argon was bubbled through the solution from a cylinder for about 10 min. Argon line was replaced with an argon balloon and the reaction was allowed to proceed overnight. The solution was transferred to a separatory funnel using 2:1 EtOAc/Et₂O (100 mL) and 4:1 saturated NH₄Cl/NH₄OH was added (100 mL). The two layers were separated, the organic layer washed three times with 4:1 saturated NH₄Cl/NH₄OH (100 mL) and dried over Na₂SO₄. Solvent was evaporated *in vacuo* to give a white solid. The solid was triturated with hexane, filtered off and dried under vacuum at ~50 °C for several hours to obtain product **5a** (479 mg, 74.2%). ¹H NMR (400 MHz, CDCl₃) δ 7.85 (s, 1H), 7.78 – 7.05 (m, 15H), 7.00 (s, 1H), 6.56 (d, *J* = 12.8 Hz, 2H), 4.08 (t, *J* = 17.0 Hz, 2H), 2.76 (s, 2H), 2.32 (d, *J* = 14.0 Hz, 1H), 2.42 – 1.95 (m, 3H), 2.13 – 1.65 (m, 6H), 1.69 – 1.19 (m, 10H), 1.03 (s, 3H), 0.61 (t, 1H). HRMS (ESI) calculated for [C₄₃H₄₆N₄O₄ + H]⁺ 683.3592 found, 683.3590.

5-(4-((13S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-(trityloxy)pentanamide (**5b**)

The reaction of 17- α ethynylestradiol (270 mg, 0.92 mmol), 5-azido-O-trityl amide **4b** (367 mg, 0.92 mmol), dimethylsulfoxide (6.4 mL), DIPEA (0.32 mL) and copper iodide (87 mg, 0.46 mmol) using the same procedure described for the synthesis of **5a** gave **5b** (580 mg, 90.8%) as white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.84 (s, 1H), 7.52 – 6.89 (m, 15H), 7.01 (d, *J* = 8.3 Hz, 1H), 6.76 – 6.40 (m, 2H), 4.17 (t, *J* = 12.5 Hz, 2H), 3.13 – 2.59 (m, 2H),

2.55 – 2.26 (m, 1H), 2.10 (d, J = 14.2 Hz, 2H), 1.91 (dd, J = 16.0, 5.3 Hz, 4H), 1.75 – 1.14 (m, 14H), 1.03 (s, 3H), 0.64 (t, J = 11.1 Hz, 1H). m/z : expected: 697.3, found: 697.3

6-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-(trityloxy)hexanamide (5c)

The reaction of 17- α ethynylestradiol (170 mg, 0.57 mmol), 6-azido-O-trityl amide **4c** (237.71 mg, 0.57 mmol), dimethylsulfoxide (4.0 mL), DIPEA (0.19 mL) and copper iodide (55 mg, 0.46 mmol) using the same procedure described for the synthesis of **5a** gave **5c** (422 mg, quantitative) as white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.78 (s, 1H), 7.66 – 7.11 (m, 15H), 7.01 (d, J = 8.6 Hz, 1H), 6.71 – 6.31 (m, 2H), 4.25 (t, J = 5.3 Hz, 2H), 2.78 (d, J = 17.1 Hz, 2H), 2.38 (d, J = 9.9 Hz, 1H), 2.24 – 1.99 (m, 2H), 2.00 – 1.79 (m, 5H), 1.75 (s, 3H), 1.69 – 1.18 (m, 12H), 1.03 (s, 3H), 0.65 (t, J = 13.4 Hz, 1H). m/z : expected: 711.4, found: 711.4

7-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-(trityloxy)heptanamide (5d)

The reaction of 17- α ethynylestradiol (255.9 mg, 0.8633 mmol), 7-azido-O-trityl amide **4d** (365 mg, 0.86 mmol), dimethylsulfoxide (6.0 mL), DIPEA (0.3 mL) and copper iodide (82 mg, 0.43 mmol) using the same procedure described for the synthesis of **5a** gave **5d** (517 mg, 82.6%) as white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.78 (s, 1H), 7.57 – 7.26 (m, 15H), 7.01 (d, J = 8.4 Hz, 1H), 6.61 – 6.51 (m, 2H), 4.28 (t, J = 6.9 Hz, 2H), 2.89 – 2.64 (m, 2H), 2.49 – 2.27 (m, 1H), 2.15 – 2.03 (m, 2H), 1.97 – 1.74 (m, 6H), 1.69 – 1.11 (m, 12H), 1.03 (s, 3H), 0.65 (td, J = 12.7, 3.9 Hz, 1H).

8-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-(trityloxy)octanamide (5e)

The reaction of 17- α ethynylestradiol (250 mg, 0.84 mmol), 8-azido-O-trityl amide **4e** (372 mg, 0.84 mmol), dimethylsulfoxide (6.0 mL), DIPEA (0.29 mL) and copper iodide (80 mg, 0.42 mmol) using the same procedure described for the synthesis of **5a** gave **5d** (549 mg, 88.3%) as white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.71 (s, 1H), 7.29 (d, J = 28.0 Hz, 17H), 7.04 (d, J = 8.3 Hz, 1H), 6.65 – 6.47 (m, 2H), 4.32 (t, J = 7.2 Hz, 2H), 2.80 (s, 2H), 2.39 (s, 1H), 2.11 (s, 2H), 1.90 (d, J = 13.0 Hz, 5H), 1.67–1.13 (m, 13H), 1.04 (s, 4H), 0.66 (t, J = 10.6 Hz, 1H).

4-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-hydroxybutanamide (1a)

EED-Triazolyl-O-trityl **5a** (200 mg, 0.29 mmol) was dissolved in of dichloromethane (7 mL), triisopropylsilane, TIPS, (0.35 mL) was added via syringe and the resulting mixture was stirred at room temperature for 10 min. Trifluoroacetic acid (0.35 mL) was added dropwise and the reaction was allowed to proceed for 10 min. The product was precipitated out of solution using hexane: petroleum ether: diethylether 10:10:1 (50 mL). The solid was filtered off and dried under vacuum at $\sim 45^\circ\text{C}$ to obtain **1a** (78 mg, 60.4%) as a white solid. HPLC retention time 16.12 min. ^1H NMR (400 MHz, CD_3OD) δ 7.84 (s, 1H), 6.97 (d, J = 8.4 Hz, 1H), 6.62 – 6.34 (m, 2H), 4.45 (t, J = 6.6 Hz, 2H), 2.74 (s, 2H), 2.55 – 2.35 (m, 1H), 2.32 – 2.02 (m, 6H), 2.01 – 1.79 (m, 3H), 1.75 – 1.21 (m, 7H), 1.03 (s, 3H), 0.68 (t, J = 11.2 Hz, 1H). HRMS (ESI) calculated for $[\text{C}_{24}\text{H}_{32}\text{N}_4\text{O}_4 + \text{H}]^+$ 441.2496 found, 441.2494.

5-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-hydroxypentanamide (1b)

The reaction of EED-Triazolyl-O-trityl **5b** (102 mg, 0.147 mmol), TIPS (0.35 mL) and TFA (0.35 mL) in dichloromethane (7 mL) as described for the synthesis of **1a** gave **1b** (35.5 mg, 53.4%) as white solid. HPLC retention time 16.32 min. ¹H NMR (400 MHz, CD₃OD) δ 7.82 (s, 1H), 6.97 (d, *J* = 8.2 Hz, 1H), 6.69 – 6.32 (m, 2H), 4.41 (t, *J* = 6.2 Hz, 2H), 2.73 (s, 2H), 2.44 (s, 1H), 2.31 – 2.01 (m, 4H), 1.88 (d, *J* = 37.9 Hz, 6H), 1.61 (d, *J* = 7.7 Hz, 6H), 1.48 – 1.16 (m, 3H), 1.02 (s, 3H), 0.66 (t, *J* = 11.1 Hz, 1H). HRMS (ESI) calculated for [C₂₅H₃₄N₄O₄ + H]⁺ 455.2653 found, 455.2649.

6-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-hydroxyhexanamide (1c)

The reaction of EED-Triazolyl-O-trityl **5c** (118 mg, 0.167 mmol), TIPS (0.25 mL) and TFA (0.25 mL) in dichloromethane (5 mL), as described for the synthesis of **1a** gave **1c** (50.9 mg, 65%) as white solid. HPLC retention time 16.68 min. ¹H NMR (400 MHz, CD₃OD) δ 7.82 (s, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.69 – 6.27 (m, 2H), 4.40 (t, *J* = 7.0 Hz, 2H), 2.75 (s, 2H), 2.57 – 2.30 (m, 2H), 2.32 – 2.01 (m, 4H), 1.92 (dt, *J* = 31.0, 11.7 Hz, 5H), 1.81 – 1.48 (m, 5H), 1.52 – 1.18 (m, 5H), 1.03 (s, 3H), 0.66 (t, *J* = 10.9 Hz, 1H). HRMS (ESI) calculated for [C₂₆H₃₆N₄O₄ + H]⁺ 469.2809 found, 469.2805.

7-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-hydroxyheptanamide (1d)

The reaction of EED-Triazolyl-O-trityl **5d** (160 mg, 0.22 mmol), TIPS (0.30 mL) and TFA (0.30 mL) in dichloromethane (6 mL), as described for the synthesis of **1a** gave **1d** (84.6 mg, 79.2%) as yellow-tinted white solid. HPLC retention time 17.12 min. ¹H NMR (400 MHz, CD₃OD) δ 7.79 (s, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.58 – 6.37 (m, 2H), 4.39 (t, *J* = 7.0 Hz, 2H), 2.93 – 2.67 (m, 2H), 2.45 (ddd, *J* = 14.6, 9.5, 5.3 Hz, 1H), 2.27 – 2.02 (m, 4H), 2.00 – 1.80 (m, 6H), 1.72 – 1.47 (m, 5H), 1.46 – 1.21 (m, 7H), 1.03 (s, 3H), 0.67 (t, *J* = 10.7 Hz, 1H). HRMS (ESI) calculated for [C₂₇H₃₈N₄O₄ + H]⁺ 483.2966 found, 483.2956.

8-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-N-hydroxyoctanamide (1e)

The reaction of EED-Triazolyl-O-trityl **5e** (124 mg, 0.16 mmol), TIPS (0.25 mL) and TFA (0.25 mL) in dichloromethane (5 mL) as described for the synthesis of **1a** gave **1e** (64.4 mg, 79.6%) as white solid. HPLC retention time 17.68 min. ¹H NMR (400 MHz, CD₃OD) δ 7.82 (s, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.56 – 6.42 (m, 2H), 4.38 (t, *J* = 6.9 Hz, 2H), 2.88 – 2.62 (m, 2H), 2.45 (td, *J* = 9.8, 5.1 Hz, 1H), 2.20 – 1.76 (m, 10H), 1.67 – 1.49 (m, 5H), 1.46 – 1.20 (m, 10H), 1.06 (s, 3H), 0.66 (t, *J* = 10.9 Hz, 1H). HRMS (ESI) calculated for [C₂₈H₄₀N₄O₄ + H]⁺ 497.3122 found, 497.3113.

N-Desmethyltamoxifen (10)—The synthetic procedure was adapted from Olofson et al (S1). Briefly, to a solution of tamoxifen (860 mg, 2.32 mmol) in 1, 2-dichloroethane (15 mL) at 0 °C was added 1-chloroethyl-chloroformate (332 mg, 2.32 mmol). Stirring continued at 0 °C for 15 min, followed by heating under reflux for 24h. TLC revealed a near quantitative conversion to a higher R_f intermediate. Solvent was evaporated off and the oily residue was dissolved in methanol and the mixture was refluxed for 3 hr during which a re-conversion to a lower R_f product was seen. Solvent was evaporated off to give 753 mg

(91%) of white solid. ^1H NMR (CDCl_3 , 400 MHz) δ 0.89 (3H, t, $J = 7.2$ Hz), 2.43 (2H, q, $J = 14.8, 7.6$ Hz), 2.56 (3H, s), 3.12 (2H, t, $J = 4.0$ Hz), 4.08 (2H, t, $J = 4.8$ Hz), 6.57 (2H, d, $J = 8.8$ Hz), 6.76 (2H, d, $J = 8.8$ Hz), 7.03–7.31 (10H, m), 9.56 (1H, s). HRMS (FAB, mba) calculated for $[\text{C}_{25}\text{H}_{27}\text{NO} + \text{H}]^+$ 358.2171, found 358.2198.

Tamoxifen-SAHA Conjugate (3)—To a stirring mixture of N-desmethyltamoxifen **10** (230 mg, 0.58 mmol) and mesylated compound **9** (296 mg, 0.80 mmol) in DMSO (7 mL), was added Hunig's base (0.7 mL, 7.31 mmol). After 4 h of stirring at 80 °C, reaction mixture was cooled down to room temperature and partitioned between EtOAc (40 mL) and saturated NaHCO_3 (35 mL). Organic layer was washed successively with NaHCO_3 (35 mL), brine (30 mL) and dried over Na_2SO_4 . Solvent evaporated and the residue was purified by prep-TLC eluting with 12:1:0.1 $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{Cl}$ to give 158 mg (43 %) of ester **11** as a colorless oil. HRMS (FAB, mba) calcd for $[\text{C}_{41}\text{H}_{49}\text{N}_2\text{O}_4 + \text{H}]^+$ 633.3692, found 633.3724.

To a solution of ester **11** (98 mg, 0.11 mmol) in 1:1 THF (5 mL) and methanol (5 mL) was added 50% aqueous hydroxylamine (0.1 mL, 1.51 mmol) and KCN (4 mg, 0.06 mmol), and stirring continued for 2 days. The reaction was diluted with 10% methanol in EtOAc (30 mL), washed with saturated NaHCO_3 (2×30 mL) and saturated brine (30 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give 65 mg (66%) of Tamoxifen-SAHA Conjugate **3** as a white solid. HPLC retention time 22.88 min. ^1H -NMR (CD_3OD , 400MHz) δ 0.86–0.92 (3H, m), 1.26–1.36 (6H, m), 1.54–1.67 (4H, m), 2.27–2.34 (5H, m), 2.42 (2H, q, $J = 7.2$ Hz), 2.81–2.84 (2H, m), 3.61–3.66 (2H, m), 3.94–3.97 (2H, m), 6.52 (2H, d, $J = 8.8$ Hz), 6.74 (2H, d, $J = 8.8$ Hz), 7.05–7.13 (5H, m), 7.17–7.32 (7H, m), 7.52–7.54 (2H, m); ^{13}C NMR (CD_3OD , 400 MHz) δ 14.0, 26.5, 26.7, 29.9, 30.8, 37.8, 42.4, 54.7, 56.1, 62.3, 65.9, 114.2, 120.8, 126.9, 127.4, 128.9, 130.2, 130.6, 131.2, 131.3, 132.5, 132.7, 136.8, 139.3, 139.5, 142.3, 143.3, 144.7, 157.6, 174.2. HRMS (FAB, mba) calculated for $[\text{C}_{40}\text{H}_{48}\text{N}_3\text{O}_4 + \text{H}]^+$ 634.3645, found 634.3676.

4-(4-Ethynylbenzyl)-tamoxifen (13)—To a stirring solution of N-desmethyltamoxifen **10** (358 mg, 0.9 mmol) and 4-ethynylbenzyl methanesulfonate **12** (250 mg, 1.2 mmol) in DMSO (5 mL), was added Hunig's base (0.5 mL, 2.78 mmol). The reaction mixture was heated at 80 °C for 2h and partitioned between ethyl acetate (40 mL) and saturated NaHCO_3 (30 mL). The two layers were separated and organic layer was washed with saturated brine (1×30 mL) and dried over Na_2SO_4 . Solvent was evaporated off and crude was purified by prep-TLC, eluting with 4:1:0.1 Hexanes/EtOAc/ Et_3N to yield 329 mg (78 %) of **13** as a white-brown solid. ^1H NMR (CDCl_3 , 400 MHz) δ 0.98 (3H, t, $J = 7.2$ Hz), 2.28 (3H, s), 2.51 (2H, q, $J = 7.2$ Hz), 2.75 (2H, t, $J = 5.6$ Hz), 3.06 (1H, s), 3.57 (2H, s), 3.96 (2H, t, $J = 6$), 6.57 (2H, d, $J = 8.8$ Hz), 6.83 (2H, d, $J = 8.4$ Hz), 7.10–7.22 (6H, m), 7.27–7.30 (4H, m), 7.35–7.39 (2H, m), 7.45 (2H, d, $J = 8.0$ Hz). ^{13}C -NMR (CDCl_3 , 400MHz) δ 11.4, 28.9, 42.7, 55.6, 62.1, 65.7, 76.9, 83.4, 113.0, 120.4, 125.7, 126.2, 127.6, 127.8, 128.5, 129.1, 129.3, 131.5, 131.7, 135.1, 137.9, 139.5, 140.9, 142.0, 143.4, 156.3. HRMS (ESI) calculated for $[\text{C}_{34}\text{H}_{33}\text{NO} + \text{H}]^+$ 472.2640, found 472.2566

Triazolyl ethyl ester (15)—Ethyl 6-azidohexanoate **14** (129 mg, 0.70 mmol) and 4-(4-Ethynylbenzyl)-tamoxifen **13** (126 mg, 0.27 mmol) were dissolved in anhydrous THF (10 mL) and stirred under argon at room temperature. Copper iodide (11 mg, 0.07 mmol) and DIPEA (0.1 mL, 0.57 mmol) were then added to the reaction mixture, and stirring continued for 4h. The reaction mixture was diluted with dichloromethane (20 mL) and washed with 1:4 NH_4OH /saturated NH_4Cl (3×30 mL) and saturated NH_4Cl (30 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by prep-TLC, eluting with 10:1:0.1 Hexanes/Acetone/ Et_3N to give 127 mg (71 %) of **15** as a colorless

oil. $^1\text{H-NMR}$ (CDCl_3 , 400MHz) δ 0.89–0.93 (3H, t, $J = 7.2$ Hz), 1.32–1.35 (3H, m), 1.57–1.62 (2H, m), 1.90–1.94 (2H, m), 2.27–2.30 (3H, m), 2.44 (2H, q, $J = 7.6$ Hz), 2.76 (2H, t, $J = 5.6$ Hz), 3.59–3.63 (5H, m), 3.95 (2H, t, $J = 5.6$ Hz), 4.35 (2H, t, $J = 7.2$ Hz). $^{13}\text{C-NMR}$ (CDCl_3 , 400MHz) δ 13.6, 14.2, 24.1, 25.8, 28.9, 29.9, 33.8, 42.7, 49.8, 55.6, 60.1, 62.1, 65.7, 113.0, 119.1, 125.2, 125.7, 126.2, 127.5, 127.7, 129.1, 129.2, 129.3, 131.5, 135.1, 137.8, 138.4, 140.8, 142.0, 143.4, 147.2, 156.3, 172.9. HRMS (FAB, thioglycerol) calculated for $[\text{C}_{42}\text{H}_{48}\text{N}_4\text{O}_3 + \text{H}]^+$ 657.3805, found 657.3804.

Tamoxifen-Triazolylhydroxamic acid (2)—To a solution of **15** (83 mg, 0.11 mmol) in 1:1 THF (1.5 mL) and methanol (1.5 mL) was added 50% aqueous hydroxylamine (0.1 mL, 1.51 mmol) and KCN (4 mg, 0.06 mmol), and the stirring continued for 2 days. The reaction was diluted with 10% methanol in EtOAc (30 mL) and washed with saturated NaHCO_3 (2×30 mL) and saturated brine (30 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give 72 mg (quantitative) of **2** as a white solid. HPLC retention time 22.68 min. 0.89 (3H, t, $J = 7.2$ Hz), 1.14–1.131 (8H, m), 1.53–1.59 (2H, m), 1.77–1.86 (2H, m), 2.05 (1H, t, $J = 6.8$ Hz), 2.21 (1H, s), 2.39–2.45 (5H, m), 2.90 (2H, t, $J = 5.2$ Hz), 3.64 (2H, s), 3.92 (2H, t, $J = 5.2$ Hz), 4.24–4.30 (2H, m), 6.43 (2H, d, $J = 8.8$ Hz), 6.74 (2H, d, $J = 8.4$ Hz), 7.06–7.14 (5H, m), 7.19–7.24 (3H, m), 7.28–7.32 (3H, m), 7.65 (2H, d, $J = 7.6$ Hz), 7.82 (1H, s). $^{13}\text{C-NMR}$ (CDCl_3 , 400MHz) δ 13.3, 24.4, 25.5, 25.7, 28.8, 29.2, 29.5, 29.6, 29.7, 32.2, 41.7, 49.9, 50.0, 54.9, 61.3, 64.6, 112.9, 120.0, 125.3, 125.6, 126.1, 127.4, 127.7, 129.0, 129.2, 129.4, 129.9, 131.5, 135.5, 137.7, 141.0, 141.9, 143.2, 146.9, 155.8, 170.3, 180.1. HRMS (ESI) calcd for $[\text{C}_{40}\text{H}_{45}\text{N}_5\text{O}_3 + \text{H}]^+$ 644.3595, found 644.3647.

HDAC Activity Assay—*In vitro* HDAC inhibition was assayed through a contract agreement with BPS Bioscience (San Diego, USA; www.bpsbioscience.com). All of the compounds were dissolved in DMSO. A series of dilutions of the compounds were prepared with 10% DMSO in HDAC assay buffer and 5 μL of the dilution was added to a 50 μL reaction so that the final concentration of DMSO is 1% in all of reactions. The enzymatic reactions were conducted in duplicate at 37 $^\circ\text{C}$ for 30 minutes in a 50 μL mixture containing HDAC assay buffer, 5 μg BSA, HDAC substrate, HDAC enzyme (human recombinant HDAC1, HDAC6 or HDAC8) and various concentrations of each compound. After enzymatic reactions, 50 μL of 2 $\times$ HDAC Developer was added to each well and the plate was incubated at room temperature for an additional 15 minutes. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a Biotek Synergy microplate reader.

HDAC Activity Data Analysis—The fluorescent intensity data were analyzed using the computer software, Graphpad Prism. In the absence of the compound, the fluorescent intensity (F_t) in each data set was defined as 100% activity. In the absence of HDAC, the fluorescent intensity (F_b) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: %activity = $(F - F_b)/(F_t - F_b)$, where F = the fluorescent intensity in the presence of the compound. The values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation $Y = B + (T - B)/1 + 10^{((\text{LogEC}_{50} - X) \times \text{Hill Slope})}$, where Y = percent activity, B = minimum percent activity, T = maximum percent activity, X = logarithm of compound and Hill Slope = slope factor or Hill coefficient. The IC_{50} value was determined by the concentration causing a half-maximal percent activity.

Luciferase assay cell lines and plasmids—The human embryonic kidney cell line, HEK 293T, was purchased from ATCC (Manassas, VA) and routinely cultured in DMEM with 10% fetal bovine serum (FBS). The pCMXGBDhER α vector was previously

constructed in the lab and contains a CMV promoter expressing the yeast Gal4 DNA binding domain fused to the human ER α ligand binding domain (GBD:hER α LBD). To construct the pCMXGBDhER β plasmid, the human ER β LBD was first amplified from pReciever-M01 ESR2 (GeneCopoeia, Rackville, MD) with primers having a BmtI site (5'-aagctagcctgagccccgagcag-3') and a SpeI site (5'-aaactagttcactgagactgtgggttc-3'). The amplified gene was first cloned into the pGBD plasmid creating the GBD:hER β LBD fusion. The resulting fusion was amplified with primers having the SacII site (5'-tccccgcggatgaagctactgtcttctatcgaacaag-3') and the NotI site (5'-aagcgccgctcactgagactgtgggttc-3') and subsequently cloned into pCMX. The p17*4TATALuc vector, containing firefly luciferase downstream of Gal4 response elements, was used as a reporter. pCMX β Gal was used to express β -galactosidase as an internal control, and to assess transfection efficiency.

Transfection and Luciferase Assay for ER α and ER β activity—Cells were cultured in phenol red-free DMEM (MediaTech, Manassas, VA) with 10% charcoal stripped FBS to an approximate confluence of 60–70% in 48-well plates. Cells were then transiently transfected as previously described.⁴⁶ Briefly, for each well, 20 ng of ER expression vector (expressing residues 301 to 595 of ER α or residues 263 to 530 of ER β fused to the Gal4 DNA binding domain), 40 ng of p17*4TATALuc reporter vector, and 40 ng of pCMX β Gal vector (used as an internal standard) were mixed with 0.3 μ L of Lipofectamine 2000 in 40 μ L of Opti-MEM (Invitrogen, Grand Island, NY). After 45 min. incubation at room temperature, the mixture was diluted with 160 μ L Opti-MEM. Growth media was aspirated and transfection mixture was added to the cells. After incubation for 8 hours at 37 °C, transfection mixture was aspirated and cells were dosed with phenol-red free DMEM with 10% charcoal stripped FBS medium with varied ligand concentrations. For antagonism assays, 300 pM E2 was added to all wells in addition to antagonist ligand. After 40 hours (20 hours for antagonists), cells were lysed, and assayed for both luciferase and β -galactosidase activities.

Luciferase assays were performed on a Dynex MLX microtiter luminometer by addition of 100 μ L of firefly luciferin (0.311 g/mL in 0.1 M potassium phosphate pH = 7.8) to a mixture of 100 μ L of luciferase assay buffer (0.1 M potassium phosphate pH = 7.8, 21.5 mM MgCl₂, 3.7 mM ATP) and 20 μ L of cell lysate. β -galactosidase activity was determined by addition of 125 μ L of β -gal assay buffer (80 mM sodium phosphate pH = 7.4, 8.0 mM KCl, 0.8 mM MgCl₂, 40 mM mercaptoethanol, 0.4 mg/mL ortho-Nitrophenyl- β -D-galactopyranoside, 5 mM PIPES) to 40 μ L of cell lysate. Mixture was incubated at 37 °C for 2 to 3 minutes and 50 μ L of stop buffer (1 M sodium carbonate, 0.8 μ L/mL Antifoam A concentrate) was added and absorbance readings were taken at 405 nm with a Dynex OpsysMR plate reader. Data reported represent the average of three sets luciferase assays normalized to β -galactosidase activity.

Molecular Docking Analysis—In silico docking was performed using Autodock Vina⁴⁷ run through PyRx to manage the workflow and PyMol to visualize the results. Ligands were prepared by first generating an energy minimized 3D structure in ChemBioDraw3D, followed by processing with Autodock Tools 1.5.4 to assign Gasteiger charges, merging non-polar hydrogens and set torsional bonds. Initial docking runs were performed within a 25–30 Å cubic search space surrounding the binding pocket, with solutions found using an exhaustiveness of 8, with output modes were ranked according to binding affinity (BA). For detailed comparisons, multiple runs with reduced search space were run with an increased exhaustiveness of 16 and 32. Autodock Vina finds ligand poses with the best fit and strongest BA (global minimums) by a stochastic algorithm to explore surfaces/pockets of the rigid macromolecule, through an iterative series of local optimizations evaluating both

intermolecular (hydrophobic interactions, repulsions, hydrogen bonding, etc.) and intramolecular (torsion, rotational torque) factors.

Crystal Structures—For ER α , bulky agonist crystal structures 2P15 (bound to trifluoromethyl-substituted phenylvinyl estradiol, TFMPV-E2), 2YAT (bound to estradiol-pyridine tetra acetate europium, EEu) and antagonist structure 3ERT (bound to 4-hydroxy tamoxifen) were employed. These structures are of the ER α ligand binding domain (LBD) only. They were prepared for molecular docking analysis in Autodock Tools 1.5.4 by removing the ligand from the binding pocket, merging non-polar hydrogens, adding polar hydrogens, and rendering Gasteiger charges to each atom. In the base of the binding pocket, ER ligands are held in place by hydrogen bonding with Arg-394, Glu-353 and a critical water molecule. Routine docking analysis ignores water molecules by removing them from the rigid macromolecular structure, so we selectively kept the critical water molecule. The WHAT-CHECK web-based modeling program was used to add polar hydrogens to the crystal structure prior to processing, and kept for docking analysis.⁴⁸

Homology Models of ER β : There are no available crystal-structures of ER β solved with a bulky 17- α modified EED agonist. None of the conjugates fit into the ER β structure solved with estradiol (PDB: 3OLS, Supplemental Figure S3). Therefore, we generated a homology model using the SWISS-MODEL server running in automated mode.⁴⁹ The protein sequence of the human ER β (accession: AAC05985.1) was submitted with the ER α 2P15 structure as the template. Models of both the A and B chains from 2P15 were generated in this manner. The LBD of ER β has 60% sequence homology with the LBD of ER α .⁵⁰ Residues 262–500 of ER β were built into the model for chain A and residues 262–501 for chain B.

Cell Culture and Cell Viability Assay—The human breast cancer cell line MCF-7 was a generous gift from Dr. Al Merrill's laboratory (Georgia Institute of Technology, GA). MDA-MB-231, DU145 and VERO cells were obtained from ATCC (Manassas, VA). Cells were routinely cultured in EMEM (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS) (Hycone, Logan, UT) with and without antibiotics. All cell cultures were incubated at 37 °C under a 5% CO₂ atmosphere. For all experiments, cells were grown in 96-well cell culture treated microtiter plates (Techno Plastic Products AG, Trasadingen, Switzerland) with the appropriate ligand in duplicate for 72 hours. MTS and MTT colorimetric tests (CellTiter 96 Aqueous One Solution and CellTiter 96 Non-Radioactive Cell Proliferation Assays, Promega, Madison, WI) were employed to determine cell viability per manufacturer instructions. Logit plot analysis was used to determine the IC₅₀ values for each compound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(HDAC) Histone deacetylases

(SERMs)	selective estrogen receptor modulators
(HDACi)	histone deacetylase inhibitor
(ER)	estrogen receptor
(ERα)	estrogen receptor alpha
(E2)	estradiol
(EED)	ethynyl-estradiol
(EED-HDACi)	ethynyl-estradiol HDACi conjugates
(Tam-HDACi)	tamoxifen-HDACi conjugates
(SAHA)	suberoylanilide hydroxamic acid
(ZBG)	zinc binding group
(H12)	helix-12
(BA)	binding affinity
(SAR)	structure-activity relationship
(TPSA)	total polar surface area
(LBD)	ligand binding domain

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^aReagents and conditions: (a) CuI , DIPEA, DMSO, Argon, rt, 18 h; (b) TFA, TIPS, DCM, rt, 15 min.

Scheme 1.
Synthesis of EED based conjugates (**1a–e**)^a

^aReagents and conditions: (a) TMSCl, Pyridine, DMAP; (b) MsCl, DCM, TEA; (c) DIPEA, DMSO; (d) aq. NH₂OH, KCN, THF, MeOH.

Scheme 2.
Synthesis of Tam-HDACi (3)^a

Scheme 3.
Synthesis of triazole containing Tam-HDACi (2)^a

Figure 1. Design of Dual-Acting ER ligand-HDACi conjugates

A) Crystal structures of antagonist Tamoxifen (PDB: 3ERT, blue) and agonist TFMPV-E2 (PDB: 2P15, teal) bound to ER α , and molecular determinants of agonism or antagonism for E2 and tamoxifen scaffolds. B) EED/Tamoxifen-HDACi conjugates, with SAHA bound to HDAC (PDB: 1ZZ1, gold) showing the solvent exposed cap group for chemical modification.

Figure 2. HDACi conjugates exhibit either ER α agonist or antagonist activity

Agonist activity (fold activation, relative to control no drug) was measured at 10 μ M.

Antagonist activity was measured by competition with 300 pM estradiol. Dose response curves for antagonist effect are shown for Tam-HDACi only, as EED-HDACi showed no antagonist activity at any concentration. All cells were grown in charcoal stripped serum. Antagonist dose response inset is a representative single experiment.

Figure 3. Crystal structures agonist bound ERα 2P15 (A), 2YAT (B) and antagonist bound 3ERT (C) were used in docking analysis of dual ER modulator/HDACi compounds. Their original ligands are shown in dark blue-purple, and helix-12 in red. (D) Binding affinities (BA) calculated for **1a–e** (EED-HDACi, cyan), **2, 3** (Tam-HDACi, orange) and their hydroxylated metabolites (**2-OH** and **3-OH**). Only the highest ranked poses with strongest BA were selected. Poses where the EED or tamoxifen moiety is in the ligand binding domain are shown as thicker molecules, and poses where they are outside the pocket are shown as thin lines.

Figure 4.

In silico docking results of coiled (A, magenta) and extended (B, pink) conformations of the HDACi alkyl chain, inside the ligand binding pocket of ERα (PDB: 2P15). Critical hydrogen bonding residues are labeled. Helix-12 (H12) is highlighted in red.

Figure 5. ER α and ER β agonist activity measured in HEK-luciferase assay

A) EED-HDACi, E2 (estradiol) and EED (ethinylestradiol) show varying degrees of agonist activity. Grey dotted line indicates basal level threshold. Doses are 10 μ M. B) The ratio of ER α to ER β agonist activity. C) Compound **1c** docked into both ER α (PDB: 2P15, purple) and ER β (2P15 derived homology model, blue), with arrows indicating Met-421 (ER α) substitution with Ile-373 (ER β) and the resulting expansion of hydrophobic space.

Figure 6. EED-HDACi, Tam-HDACi and SAHA tested for inhibition of HDAC isoforms 1, 6 and 8. ND, Not Determinable, up to maximum concentrations tested (100 μM).

Figure 7.

Cell-type selective antiproliferative activity of ER ligand-HDACi. A) Whole cell antiproliferative activity (IC_{50} , μM) measured 72 hours after drug dosing, across ER α / β + (MCF-7) and ER α -/ β - (MDA-MB-231) breast cancer, metastatic prostate cancer (DU-145) and healthy kidney epithelial cells (VERO). B) Dose response curves for antiproliferative activity of SAHA and Tam-HDACi compound **3** against ER+ (MCF-7) and ER- (MDA-MB-231) breast cancer cells. C) In vitro therapeutic index of EED-HDACi **1a**, Tam-HDACi **2** and **3**, SAHA and tamoxifen. IC_{50} values are an average of at least 2 independent experiments, $\pm$ standard deviation. ^aNot determinable, IC_{50} greater than 40 μM or ^bgreater than 20 μM .