Bioorganic & Medicinal Chemistry 26 (2018) 4428–4440

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Incorporation of histone deacetylase inhibitory activity into the core of tamoxifen – A new hybrid design paradigm

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ARTICLE INFO

Keywords:
Hybrid molecules
Antiestrogens
Histone deactylase inhibitors
Breast cancer

ABSTRACT

Hybrid antiestrogen/histone deacetylase (HDAC) inhibitors were designed by appending zinc binding groups to the 4-hydroxystilbene core of 4-hydroxytamoxifen. The resulting hybrids were fully bifunctional, and displayed high nanomolar to low micromolar IC_{50} values against both the estrogen receptor α (ERα) and HDACs *in vitro* and in cell-based assays. The hybrids were antiproliferative against ER+ MCF-7 breast cancer cells, with hybrid **28b** possessing an improved activity profile compared to either 4-hydroxytamoxifen or SAHA. Hybrid **28b** displayed gene expression patterns that reflected both ERα and HDAC inhibition.

1. Introduction

Estrogens, mainly 17β-estradiol (E2, **1**, Fig. 1), are the primary hormones responsible for the development of female secondary sexual characteristics, including normal growth of the mammary gland.[1] E2 genomic signalling occurs mainly through estrogen receptor-α and –β (ERα and ERβ), members of the nuclear receptor superfamily of ligand-activated transcription factors.[2] Binding of E2 to ERs results in a conformational change that involves the folding of helix 12 (H12) over the ligand binding pocket (LBP), which induces receptor binding to DNA at estrogen response elements (EREs) located in the regulatory regions of target genes, release of transcriptional co-repressors, and the recruitment of co-activators and transcription machinery.

Expression of ERα, which is observed in about 70% of breast tumors, mediates the growth-stimulatory effects of estrogens on these tumors.[3–5] Efforts to inhibit E2-mediated tumor growth have led to the development of ER antagonists as therapeutic tools for ER+ breast cancer. The most commonly employed antiestrogen (AE) has been tamoxifen (**2**), which is classified as a selective estrogen receptor modulator (SERM) for its tissue-specific effects on estrogen signaling. In breast, it antagonizes estrogen-induced growth, while it has agonist activity for expression of estrogen target genes in uterine cells.[6–9] Tamoxifen itself has low affinity for ERs and acts mainly as a prodrug. It is oxidized *in vivo* to several active metabolites, including 4-hydroxytamoxifen (4-OHT, **3**) and endoxifen, which have potent antiproliferative activities in ER+ breast cancer cells *in vitro*.[10,11] Tamoxifen is used in first line endocrine therapy of all stages of ER+ breast tumors, especially in pre-menopausal women as aromatase inhibitors have demonstrated superior efficacy in the post-menopausal setting.[12] Tamoxifen has an overall clinical response rate of about 50%, although it is less effective in metastatic cases.[12–14] Unfortunately, relapse in patients with primary tumors can occur years after treatment, suggesting incomplete eradication of tumor cells and benefit from extension of hormonal therapy to 10 years instead of five.[15] A second class of antiestrogens called pure antiestrogens, or selective estrogen receptor down-regulators (SERDs), are devoid of the partial agonist activity of tamoxifen in the uterus and possess the ability to induce SUMOylation, ubiquitination, and degradation of ERα.[16–18] The SERD fulvestrant (**5**) has proven beneficial as a second line therapy for patients that have previously undergone hormonal treatment.[19,20]

Histone deacetylases (HDACs) function as transcriptional co-regulators, modulating in combination with histone acetyl transferases the acetylation state of histones and the accessibility of DNA in chromatin.[21] In addition, HDACs are also known to deacetylate non-genomic targets such as tubulin, HSP90, and p53.[22] HDACs are over-expressed in many cancers, including breast cancer.[23,24] Several HDAC

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https://doi.org/10.1016/j.bmc.2018.07.026
Received 23 March 2018; Received in revised form 5 July 2018; Accepted 14 July 2018
Available online 17 July 2018

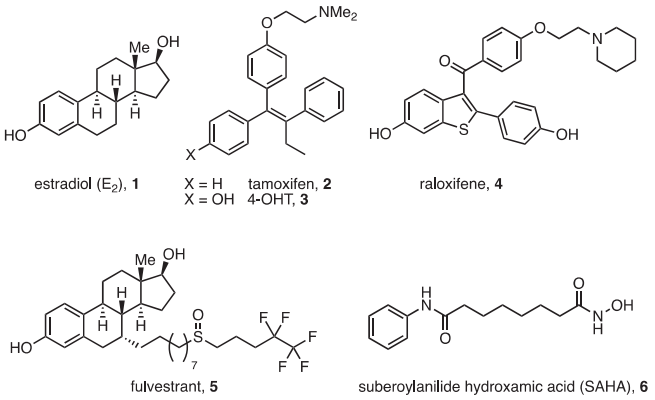

Fig. 1. Structures of antiestrogens and HDAC inhibitors.

inhibitors (HDACi's) are clinically approved for blood cancer indications and have been investigated in combination with other agents for use in solid tumors, including breast cancer.[25–27] The prototype of this class is suberoylanilide hydroxamic acid (SAHA, **6**, Fig. 1), which has been approved for treatment of cutaneous T-cell lymphoma.[28]

Several studies have shown a combinatorial effect of HDACi's and antiestrogens in breast cancer. Tamoxifen exhibited cooperativity with several HDACi's to inhibit growth of ER+ MCF-7 breast cancer *in vitro* and *in vivo*.[29,30] Other studies have shown combinatorial effects of antiestrogens and HDACi's in both ER+ and ER− breast cancer cell lines.[31–33] Moreover, the combination of tamoxifen and SAHA was shown in a phase II study to have a 40% clinical benefit for patients with ER+ tumors that had progressed during endocrine therapy.[34]

Based on the synergy between antiestrogens and HDACi's, several groups including ours have investigated hybrid structures that combine both biochemical activities in a single molecule.[35–39] Our previous work incorporated HDACi function in the side-chain of fulvestrant (**7**, Fig. 2).[35] Other hybrids have also incorporated HDACi function in the side-chains of raloxifene (**8**) and tamoxifen (**9**).[37–39] While all these hybrids possessed antiproliferative activity, they were generally less potent than standard monotherapies. For example, fulvestrant hybrid **7** displayed antiproliferative activity in both ER+ MCF-7 cells and in ER− MDA-MB-231 cells, but was less potent than 4-OHT (in MCF-7) or SAHA (in MDA-MB-231).[35]

The side-chains of fulvestrant, 4-OHT, and raloxifene are responsible for their antagonist action by preventing the proper folding of H12 over the LBP and thus interfering with the recruitment of transcription cofactors. In SERDs such as fulvestrant, the long hydrophobic side chain can interact with the coactivator binding groove,[40] a capacity that correlates with induction of ERα modifications and complete transcriptional suppression.[17] Thus, the incorporation of polar zinc

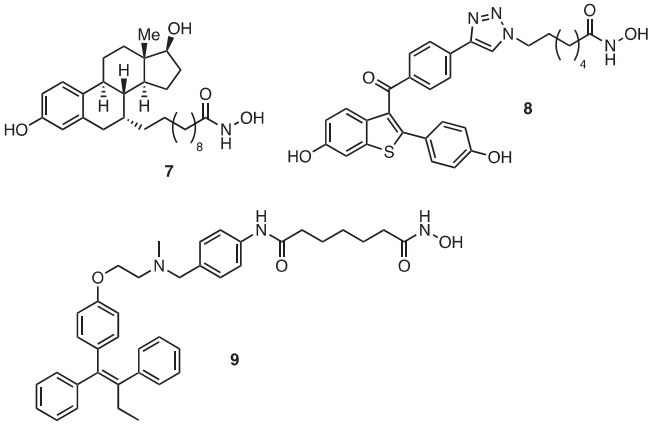

Fig. 2. Structures of antiestrogen/HDACi hybrids.

binding groups at the end of the side-chain might alter the ability of SERDs to induce ERα degradation.

2. Hybrid design and synthesis

The steroidal, 4-hydroxystilbene, or 2-arylbenzothiophene cores of antiestrogens mainly provide affinity for the LBP. We have observed with vitamin D/HDACi hybrids that groups that provide HDACi function can be accommodated by the LBP of the vitamin D receptor (VDR).[41–44] Given the similarity between nuclear receptor binding pockets we therefore postulated that it might be possible to incorporate HDACi function into the core of an antiestrogen without significantly affecting affinity for ERα, allowing the antiestrogenic side-chain to remain unmodified and retain full functionality.[45]

The phenol of 4-OHT mimics the A-ring phenol of E2, forming hydrogen bonds to Glu353 and Arg394.[46] While E2 possesses a second hydroxyl group in the D-ring that engages in a hydrogen bond with His524,[47] the remaining aromatic ring in 4-OHT remains unoxidized and thus appeared to be a potential position to incorporate polar functionality – indeed raloxifene places a second phenolic OH in this vicinity. Additionally, while many residues lining the ERα binding pocket show little positional variation among X-ray crystal structures of various estrogens and antiestrogens, His524 is mobile and can accommodate different positioning of hydroxyl groups, as in raloxifene,[48] and bulkier groups, as in 2-arylindole antagonists.[49] We sought to exploit this flexibility by developing hybrids which attach HDACi function to the B-ring of 4-OHT. The potential advantage of this design is that it would not require alteration of the side-chain that is essential for antiestrogen function. Moreover, metabolic inactivation of the HDACi unit would not be expected to alter the antiestrogenic character of the molecules.

The hybrids were prepared using two separate routes. Hybrid BMW-275 (**16**) was prepared using a McMurry cross-coupling strategy (see Scheme 1).[50] Mono-alkylation of symmetrical benzophenone **10** followed by acylation with pivaloyl chloride provided ketone **12** in 50% yield. McMurry cross-coupling with 4′-hydroxypropiophenone provided alkene **13** as a 7:1 *E/Z* mixture. Triflation under standard conditions and then palladium-catalyzed carboxylation afforded **15** in 55% yield over 2 steps. Finally, treatment of the methyl ester with hydroxylamine and KOH afforded hydroxamic acid **16** in 45% yield.

The remaining hybrids were prepared via a three-component, nickel-catalyzed alkyne/Grignard/halide coupling (see Scheme 2).[51] Treatment of aryl butyne **18** with an appropriately substituted aryl Grignard and aryl iodide in the presence of $NiCl_2{\cdot}6H_2O$ afforded alkene **19** as a single alkene stereoisomer. Unfortunately, unlike tamoxifen, the alkene in **19,** and its derivatives, is highly prone to isomerization, particularly under acidic conditions including purification by silica gel chromatography. For instance, simple removal of the TBS group in **19** with NaOH in methanol followed by workup and silica gel chromatography afforded **20** as a 1:1 *E/Z* mixture. This propensity to isomerize presumably arises from the additional electron donating groups on the aryl rings not present in the parent tamoxifen.[52] We thus proceeded with the 1:1 mixture and separated alkene isomers by HPLC upon completion of the syntheses. Treatment of **20** with NaH and methyl 5-bromopentanoate followed by hydrogenolytic cleavage of the benzyl protecting group and hydroxamate formation, as above, afforded AFP-277 (**23**) in 21% yield over three steps.

Alternatively, triflation of **20** followed by Suzuki-Miyaura cross-coupling afforded styrene **25** in excellent yield. Cross metathesis with either methyl acrylate or methyl 4-pentenoate using Grubbs' second-generation catalyst proceeded cleanly to afford alkenes **26a/b** in good yield. Subsequent treatment with H_2/Pd-C resulted in alkene hydrogenation and hydrogenolysis of the benzyl protecting group. Finally, treatment with hydroxylamine afforded hybrids AFP-345 (**28a**) and AFP-477 (**28b**) in 35% and 21% yield, respectively, over three steps. Finally, hybrid AFP-458 (**29**) bearing a cinnamate unit could be