Estrogen Receptor Covalent Antagonists:
The Best Is Yet to Come

Craig Furman1, Ming-Hong Hao2, Sudeep Prapagnat1, Dominic Reynolds1, Victoria Rimkunas1, Guo Z. Zheng1, Ping Zhu1, and Manav Korpal1

Abstract

The development of tamoxifen and subsequent estrogen receptor alpha (ERα) antagonists represents a tremendous therapeutic breakthrough in the treatment of breast cancer. Despite the ability of ERα antagonists to increase survival rates, resistance to these therapies is an all-too-common occurrence. The majority of resistant tumors, including those with hotspot mutations in the ligand-binding domain of ERα, remain dependent on ERα signaling, indicating that either a more potent or novel class of antagonist could have clinical benefit. With this thought in mind, we developed a novel ERα antagonist that exhibits enhanced potency due to its ability to covalently target a unique cysteine in ER. This review describes the design of this antagonist, H3B-5942, and discusses opportunities for future improvements, which could reduce the risk of escape mutations to this therapeutic modality.

Introduction

Breast cancer is the most common type of invasive cancer in women that, despite advances in detection and treatment, is responsible for over 500,000 deaths per year worldwide (1). A key driver of oncogenic growth in the majority of breast cancers is the transcription factor estrogen receptor alpha (ERα). In normal mammary tissue, ERα is tightly regulated through interaction with its ligand, estrogen. The binding of estrogen causes a conformational change in ERα, resulting in its association with chromatin and transcriptional activation of genes involved in mammary gland development and function. In the oncogenic setting, this pathway becomes dysregulated to support tumor growth. Because roughly 50% of ERα-positive breast cancers are dependent on estrogen/ERα signaling, therapies to hinder ERα signaling are widely employed in the clinic. These strategies include either blocking the synthesis of estrogen using luteinizing hormone releasing hormone analogs, or directly inhibiting ERα signaling using ERα antagonists. Tamoxifen, a selective ER modulator (SERM), was the first ERα antagonist to enter the clinic in the 1970’s (2), and it remains a standard-of-care (SOC) to this day. Despite the clinical success of tamoxifen, there are safety concerns over its ability to act as an ERα agonist in endometrial cells and, consequentially, an association with increased risk of developing endometrial cancer. The tamoxifen-induced agonism observed in the endometrial setting has fueled attempts to create new ERα antagonists that lack this liability. These efforts have led to the development of additional SERMs (3) that exhibit weaker agonist activity and selective ER degraders (SERD) such as the pure antagonist, fulvestrant (4). Although both classes of therapies have demonstrated preclinical and clinical benefits, the development of resistance remains a significant challenge.

Mutations in ERα Promote Constitutive Activity and Resistance to Endocrine Therapies

Several mechanisms of resistance to ERα antagonists have been identified including ERα/HER2 “cross-talk” (5), aberrant expression of ERα coactivators/corepressors (6), and most recently, recurrent mutations in ESR1, the gene that encodes ERα (7-9). In clinical studies, ERα mutations are rarely found in primary tumors; however, they are enriched in tumor biopsies collected from patients who progressed on the SOC endocrine therapies, supporting the idea that ERα mutations are a common mechanism of resistance across endocrine therapies (10, 11). Advances in noninvasive alternatives to tumor biopsies, such as droplet digital PCR (ddPCR) analysis of circulating free DNA (10, 12-19), have increased the detection rate of recurrent ESR1 mutations. Multiple studies using this approach have reported ESR1 mutations in approximately 25% to 40% of patients with ERα-positive metastatic breast cancer (MBC) previously treated with AI therapies (Table 1). Approximately 40% of ERα mutant patients harbor multiple ESR1 mutations, frequently with PI3KCA comutations.

ERα hotspot mutations in the ligand-binding domain (LBD) have been experimentally shown to support ligand-independent activation of the ERα pathway under estrogen deprivation conditions by favoring the agonist conformation in ERα (8, 9, 20-24). By adopting the agonist state, hotspot mutations not only enforce constitutive activity and resistance to AI therapies but also reduce the affinity and potency of existing antiestrogen therapies, thereby promoting some level of resistance to all existing endocrine therapies. Furthermore, ESR1 mutations also correlate with more aggressive disease and shorter overall survival relative to...
Development of the Next-Generation SERCA

Table 1. Summary of studies reporting ESR1 mutations in advanced breast cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Patient population</th>
<th>ESR1mut Detection method; sample</th>
<th>% ESR1mut (%/total)</th>
<th>Clinical implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>O’Leary (17)</td>
<td>2018</td>
<td>Paloma-3 Phase III study; ER+/HER2+ advanced breast cancer</td>
<td>Multiplex ddPCR; ctDNA</td>
<td>25.6% (114/445)</td>
<td>Patients with ESR1 mutations have worse outcome on fulvestrant</td>
</tr>
<tr>
<td>Paoletti (19)</td>
<td>2018</td>
<td>AZD9496 Phase I study; ER+/HER2− metastatic or locoregionally recurrent disease, which had progressed after &gt;6 months of ET</td>
<td>Multiplex ddPCR; ctDNA</td>
<td>31% (14/45)</td>
<td>N/A</td>
</tr>
<tr>
<td>Fribbens (14)</td>
<td>2018</td>
<td>Plasma DNA AI study; patients who progressed on first-line AI therapy for MBC</td>
<td>Multiplex ddPCR; ctDNA</td>
<td>56.4% (22/39)</td>
<td>ESR1 mutations are detectable prior to clinical progression</td>
</tr>
<tr>
<td>Chandarlapaty (23)</td>
<td>2016</td>
<td>BOLERO-2 Phase II study; postmenopausal women with MBC with prior AI exposure</td>
<td>ddPCR; ctDNA</td>
<td>28.8% (156/541)</td>
<td>ESR1 mutations are associated with shorter overall survival and do not predict benefit to everolimus plus exemestane</td>
</tr>
<tr>
<td>Spoerke (10)</td>
<td>2016</td>
<td>FERGI Phase II study; ER+/HER2− AI resistant locally advanced or MBC</td>
<td>ddPCR; ctDNA</td>
<td>37.3% (57/153)</td>
<td>ESR1 mutation did not show differential PFS in either fulvestrant or fulvestrant plus picitlisisib arm</td>
</tr>
<tr>
<td>Fribbens (15)</td>
<td>2016</td>
<td>SoFEA Phase III study; postmenopausal women with advanced, ER− breast cancer who had demonstrated prior sensitivity to Al or C0</td>
<td>ddPCR; ctDNA</td>
<td>39.3% (63/161)</td>
<td>Patients with ESR1 mutations had improved PFS after taking fulvestrant compared with exemestane</td>
</tr>
<tr>
<td>Fribbens (15)</td>
<td>2016</td>
<td>Paloma-3 Phase III study; ER+/HER2− advanced breast cancer</td>
<td>ddPCR; ctDNA</td>
<td>25.3% (91/360)</td>
<td>ESR1 mutations do not predict the outcomes of either fulvestrant alone or fulvestrant plus palbociclib</td>
</tr>
<tr>
<td>Clatot (13)</td>
<td>2016</td>
<td>Patients with ER+/HER2− MBC who had failed first-line AI treatment</td>
<td>ddPCR; ctDNA</td>
<td>30.6% (44/144)</td>
<td>ESR1 mutations are an independent risk factor for poor outcome after AI failure and are detectable before clinical progression</td>
</tr>
<tr>
<td>Schiavon (18)</td>
<td>2015</td>
<td>Patients with ER+/HER2− advanced breast cancer who had recently relapsed or progressed after AI</td>
<td>ddPCR; ctDNA</td>
<td>14.8% (9/62)</td>
<td>Patients with ESR1 mutations had shorter PFS on subsequent AI-based therapy</td>
</tr>
</tbody>
</table>

Abbreviations: ctDNA, circulating tumor DNA; NGS, next-generation sequencing.

wild-type ERα (23), collectively cementing the clinical importance of ERα mutations in endocrine-resistant breast cancer.

Development of Next-Generation Oral SERDs

Because the existing endocrine therapies are only partially effective in the ERα-mutant setting and a significant proportion of endocrine therapy–resistant metastases remain dependent on ERα signaling, improved ERα antagonists that can overcome residual ERα activity are needed. There is currently significant interest in the development of SERDs with better pharmacokinetic (PK) properties than fulvestrant to permit oral administration. Indeed, several oral SERDs, such as GDC-0810, AZD9496, and Rad1901 (Elacestrant), have demonstrated significant preclinical antitumor activity across multiple ERα wild-type and mutant breast cancer models (25–27). Elacestrant specifically has exhibited clinical activity with an overall response rate of 23% noted at the recommended phase II dose. Notably, oral SERDs GDC-0810 and AZD9496 present mild estrogenic activity in the uterus (25, 26). As the scientific community continues to improve the SERD modality through improving PK, increasing potency, and tuning down estrogenic activity, there remains an opportunity to develop novel modes of ERα antagonism that could also effectively overcome resistance to the current SOC endocrine therapies.

Ligand-Induced Changes in ERα Conformation Differentially Impacts Activity

Previous studies using affinity-selected peptides demonstrated that estrogen and SERM/SERD ligands induce different conformational changes in ERα that expose unique binding surfaces that are distinct from each other. These altered binding surfaces may influence interactions with different proteins, including transcriptional coregulators, and change the overall biological response (25, 28). Interestingly, even if the ER ligand conformation is conserved across tissues, the biological output may differ depending on the prevalence and type of cofactors present, as is the case for tamoxifen in breast versus endometrial tissues (29).

In addition to altering cofactor associations, ligand-induced conformational changes in ERα may also influence the stability, mobilization, and cellular localization of the complex, indirectly impacting the interaction with other proteins. For example, although estradiol and 4-hydroxytamoxifen (4-OHT) induce a conformational change resulting in the association of ERα with chromatin, fulvestrant causes ERα to become trapped in the nuclear matrix through interactions with cytokeratins 8 and 18, leading to rapid degradation by the ubiquitin–proteasome pathway (30). Cumulatively, with the knowledge that ERα ligands can significantly alter ERα conformation and hence downstream
biological activity, understanding the relationship between structural changes and biological activity may improve our ability to develop the next-generation ERα-directed antagonists with the desired properties.

**Development of Covalent ERα-Directed Ligands**

In light of the limitations of the existing classes of ERα-directed therapies, we aimed to identify a novel mode of ERα antagonism that would demonstrate improved potency over SOCs. With this objective in mind, we noted a positionally nonconserved cysteine at amino acid 530 (C530) in the ligand-binding pocket of ERα that could be covalently modified. Covalent targeting of ERα has previously been accomplished by others using estrogenic (keto-noestrol aziridine) and antiestrogenic (tamoxifen aziridine) affinity labels as tools to map the hormone-binding domain (31).

These studies demonstrated that of the four cysteines in the LBD (amino acids 381, 417, 447, and 530), C530 was preferentially labeled by tamoxifen aziridine and ketoestrol aziridine (31), however, C381 could also be labeled if C530 was mutated (32, 33). Indeed, analysis of the x-ray crystal structure of ERα LBD that became available after these studies were published confirmed that C530 and C381 could be engaged by an electrophile attached to the ligand bound in the ligand-binding pocket (Fig. 1A). However, of the four cysteine residues in the ERα LBD, only C530 is directly associated with the ligand-dependent activation function 2 domain. We observed that when ERα transitions from the agonist to the antagonist state, the position of C530 moves by more than 6Å, reorienting from facing outward to toward the ligand-binding pocket (Fig. 1B). We focused on C530 as the optimal target cysteine for converting an ERα-labeling agent into a pharmacologically useful antagonist and explored whether a covalent bond between C530 and a ligand bound in the LBD could potentially fix ERα in the antagonist state. This exploration resulted in the development of novel covalent ERα antagonists through the molecular incorporation of electrophiles that can covalently bind the target. Our structure-based medicinal chemistry efforts explored multiple analogs having different sidechains and electrophiles and identified a flexible sidechain containing an internally positioned beta-substituted acrylamide that provided the desired potency and selectivity.

Additional modifications in the core scaffold aimed at improving stability led to the discovery of H3B-5942, a first-in-class orally available selective ER covalent antagonist (SERCA). As intended, H3B-5942 showed exquisite selectivity for C530 (34) and demonstrated stability against other nucleophiles, such as following glutathione addition.

In addition to enhancing the residence time and significantly increasing efficacy in the ERαY537S/WT mutant model relative to SOCs, engagement with C530 also promoted a unique conformation in both wild-type and mutant ERα relative to the known ER conformations induced by SOC and experimental agents. The conformation of ERα induced by H3B-5942 resulted in differentiated biology as little/no agonist activity was noted in the endometrial carcinoma cell line under the specific assay conditions applied (34). It is currently unclear why SERCA H3B-5942 lacks the agonist activity in the endometrial carcinoma setting that 4-OHT exhibits. One hypothesis is that covalent engagement with the cysteine loop of ERα may stabilize this region of the protein, resulting in a different pattern of coregulator recruitment compared with SERMs. Assessment of coregulator interactions using two independent methods, however, did not yield significant differences in global coregulator recruitment between covalent and noncovalent antiestrogens (34). The major limitation of the analysis was that interaction of the ERα–ligand complex with various coregulator peptides was monitored under nonphysiological conditions. To definitively test this working hypothesis, the impact of ligands on endogenous ERα–coregulator complexes within cell lysates could be assessed using Rapid

Figure 1.

X-ray crystal structures of ERα LBD. **A**, ERα in complex with an antagonist in green and cysteine residues highlighted in magenta (PDB: 1XPC). **B**, Comparison of ERα LBD in the agonistic state (cyan cartoon model, PDB: 1ERE) and antagonistic state (yellow cartoon model, PDB: 3ERT) where the antagonist 4-OHT is shown in magenta.
Immunoprecipitation Mass spectrometry of Endogenous proteins (RIME), a technique previously used to identify ERα-interacting proteins such as GREB1 (35).

Independent of the potential modulation of canonical signaling, it is also conceivable that alterations in ERα conformation following engagement with SERCAs might alter nongenomic signaling, that is, signaling pathways initiated by receptor tyrosine kinases following direct interactions with ERα-ligand complexes. Additional studies are needed to understand the potential differences between SERCAs and existing SERMs/SERDs on canonical and nongenomic signaling pathways.

Potential Resistance Mechanisms to SERCA H3B-5942

It is currently unclear which mechanisms of resistance may emerge following SERCA treatment, although general mechanisms such as ERα/HER2 cross-talk, aberrant expression of coregulators, or more specific resistance mechanisms such as ERα mutations and nongenomic signaling, are possible. Whereas the well-characterized constitutively activating mutations described above are unlikely to arise following antiestrogen therapy, it is conceivable that antagonist-to-agonist switch mutations, often noted in androgen receptor in prostate cancer (36–38), may arise depending on the antiestrogen being administered. Indeed, despite the limited deep-sequencing data for ESR1 in the relapsed tumors following antiestrogen treatment alone (39), a recent analysis of clinical databases identified one patient who, following 5 years of tamoxifen-only therapy (39), presented with a metastasis harboring the L540Q mutation in ERα, an antagonist-to-agonist switch mutation previously identified in mutagenesis studies that may confer resistance to multiple antiestrogens (40–44). In addition to the L540Q mutation, D351Y (45–47) and G400V (48, 49) mutations were also noted in the preclinical setting that convey a more agonistic response to some SERMs.

Besides these antagonist-to-agonist switch mutations in ERα, another mechanism by which antiestrogens might paradoxically stimulate the growth of ERα-positive breast cancer is by activating nongenomic signaling. A recent study showed that 4-OHT could directly stimulate growth of ERα-positive tumor cells in an ERα-dependent manner by promoting activity of focal adhesion molecules to further increase phosphorylation of IGF1Rβ, suggesting that membrane-associated pathways may also be critical in promoting resistance to antiestrogens (50). It is currently unclear whether H3B-5942 may also trigger such mechanisms of resistance; however, given the low prevalence of such mechanisms, the potential is likely to be low.

In contrast to the weak potential for constitutively activating mutations and/or antagonist-to-agonist switch mutations in ERα to emerge following H3B-5942 treatment, there remains a distinct possibility that the cysteine targeted by H3B-5942 could become mutated. Indeed, cysteine mutations have previously been identified as acquired resistant mechanisms to multiple irreversible covalent inhibitors (51, 52). Consistent with this.
possibility, site-specific mutation of C530 to either C530A or C530S results in a considerable loss of sensitivity to H3B-5942 (34). This finding in itself may pose a challenge for this class of small-molecule antagonists of ERα because we (34) and others (32) have shown C530A/S mutations are functionally tolerated and exhibit no compromise in estradiol-stimulated transcriptional activation profiles relative to wild-type ERα.

Development of the Next-Generation SERCA

Given the possibility of C530 mutations, one could envision a SERCA that maintains considerable potency in the advent of cysteine mutation. The potency of a covalent antagonist is dependent on both the ability of the compound to engage a suitably reactive residue within the receptor (k_on) and the reversible binding properties of the compound to the receptor (k.). Having extensively optimized the capacity of the ligand to present an appropriately disposed electrophile to engage C530, one could turn their attention to noncovalent interactions utilized by the SERCA scaffold. Computational examination of the crystal structure of H3B-5942 bound to ERα (Fig. 2) using fast Fourier transform (FFT) mapping of molecular fragment probes highlights the potential areas for expansion into adjacent hydrophobic pockets and opportunities to pick up additional hydrogen bond interactions. This analysis revealed three fragment clusters proximal to the H3B-5942 core that provides reasonable vectors for substitutions that could gain beneficial space-filling interactions. In addition, there are two discrete polar residues, threonine 347 and histidine 524, located in the core-binding region of the LBD that could be engaged by an appropriately positioned functionality. For example, the side chain of histidine 524 is known to form hydrogen bonds with small-molecule ER ligands (53). In addition to being beneficial to binding, such modifications should also help balance the physiochemical properties of the ligand.

We hypothesize that exploration of both these observations provides the opportunity to develop second-generation SERCA compounds with improved noncovalent binding interactions and potentially a lower reliance on covalency-driven potency.

Future Perspectives

Since the identification of recurrent mutations in ERα, there has been a resurgence in efforts to develop potent ERα antagonists that could effectively block both wild-type and mutant ERα activity. Presently, there is a focus on interrogating the functional relevance of the most clinically frequent ERα mutations. However, it is now clear that several lower frequency mutations, such as histidine 524, are also enriched in the metastatic setting (naïve or treatment-related), for which the function is unclear (54). Some of these low-frequency mutations may be important for disease progression or resistance to antagonists. It is conceivable that a subset of low-frequency mutations could share a common mechanism by inducing similar conformational changes in ERα, increasing the clinical relevance of targeting these less prevalent mutations.

The next generation of compounds should overcome not only the activity of existing ERα mutants but also the emergence of secondary mutations in ERα that resist antagonist activity. As discussed in this review, increasing the number and strength of the interactions between antagonist and receptor can achieve both of these goals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank our colleagues at H3 Biomedicine Inc., for the critical review of this article.

References


31. Harlow KW, Smith DN, Katzenellenbogen JA, Greene GL, Chan-