RESEARCH HIGHLIGHT

The function of membrane-associated molecules in acquired resistance to antiestrogens in breast cancer

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Long-term clinical adjuvant antihormone therapy for breast cancer has significantly improved survival of estrogen receptor (ER)-positive breast cancer patients, but acquired resistance to antiestrogens is a major challenge in clinic. The evolution of acquired resistance to selective estrogen receptor modulators (SERMs) is unique because the growth of resistant tumors is dependent on SERMs. Thus, it appears that acquired resistance to SERMs is initially able to utilize either estrogen (E_2) or a SERM as the growth stimulus in the ER-positive SERM-resistant breast tumors. However, no mechanism has been established to explain this paradox. Our newly established cell model MCF-7: PF, for the first time, replicates Phase I acquired resistance to SERMs *in vitro*. The cells are stimulated to grow robustly with E_2 and SERMs through the ER which is confirmed by the evidence that pure antiestrogen ICI 182,780 (ICI) completely blocks the stimulation induced by E_2 or SERMs. In contrast to E_2 that activates classical ER-target genes, SERMs continue to function as effective antiestrogens to inhibit classical ER-target genes, even at the time of growth stimulation. A significant alteration of ER function observed in SERM-resistant cells is the enhancement of the non-genomic pathway of ER and the activation of multiple membrane function-associated molecules including focal adhesion molecules and adapter proteins to further increase phosphorylation of insulin-like growth factor-1 receptor (IGF-1R). Inhibition of membrane-associated signaling, IGF-1R and focal adhesion kinase (FAK), completely abolishes 4-OHT-stimulated cell growth. Overall, the constant nuclear pressure causes broad activation of membrane-associated signaling to aid breast cancer cell survival during the selection process required for acquired SERM resistance. The targeting of these membrane function-associated pathways and seeking new unanticipated combination therapies may have further clinical potential to decipher and treat endocrine-resistant breast cancer.

Keywords: selective estrogen receptor modulator (SERM); resistance, estrogen receptor; membrane-associated molecules

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Introduction

The development of acquired resistance to SERMs treatment was discovered using MCF-7 tumors transplanted in athymic mice to mimic years of adjuvant treatment in patients [1-3]. Long-term therapy generates selection pressure for cell populations that evolve from acquired SERM resistance, ubiquitously observed in metastatic breast cancer, to eventually expose a vulnerability that is expressed as E_2-induced apoptosis [3-5]. Laboratory observations further show that three phases of acquired SERM-resistance exist (Figure 1), which depend on the length of SERMs exposure [4, 6, 7]. Tumors with phase I resistance are stimulated by E_2 and SERMs, but inhibited by aromatase inhibitors (AIs) and

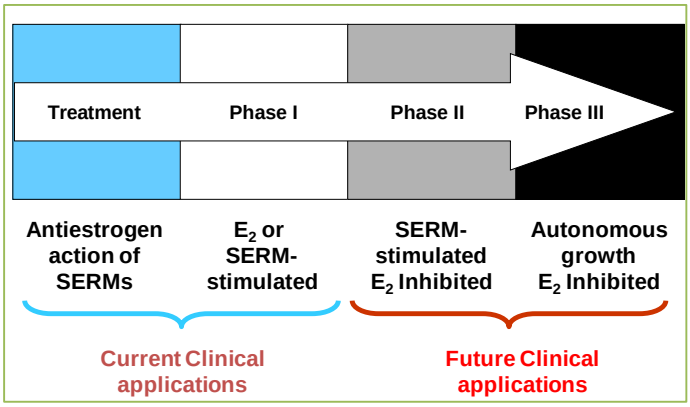

Figure 1. The development of acquired resistance to SERMs after long-term therapy. Phase I acquired resistance develops after a year or two of therapy of estrogen receptor (ER)-positive metastatic breast cancer. Phase II acquired resistance occurs after 5 years of SERM treatment in the laboratory or potentially as occult disease during 5 years adjuvant tamoxifen therapy. Phase III acquired resistance potentially develops after indefinite therapy for ER positive breast cancer [4, 7]. Reprint with permission [7].

fulvestrant; tumors with phase II resistance are stimulated by SERMs, but are inhibited by E_2 due to apoptosis; tumors with phase III resistance grow autonomously regardless of SERMs, but are inhibited by E_2 with apoptosis [4, 6, 7]. The cell populations are clearly being modulated over years of therapy so that those cells that can adapt and grow in new environment [4, 6, 7]. Understanding this process provides an opportunity to save more lives. We will focus on the recent progress in the discovery of SERM-stimulated growth in phase I acquired resistance.

SERMs consistently inhibit the function of nuclear ER

The finding that the c-Src tyrosine kinase activity is increased in long-term E_2-deprived MCF-7:5C cells [8] and inhibition of c-Src in short-term (7 days) experiments will reversibly block E_2-induced apoptosis [8, 9], created an opportunity to determine what long-term inhibition of c-Src in the presence of E_2 would do to the biological properties of the cell populations [10]. A two month period of selection pressure was chosen, as this is the time period used clinically to evaluate tumor response to therapy [10]. The cell populations (MCF-7: PF) that grow out under the pressure of E_2 plus the c-Src inhibitor is particularly interesting as, for the first time, it replicates phase I acquired resistance to SERMs *in vitro* [10, 11]. Our recent publication "*A molecular model for the mechanism of acquired tamoxifen resistance in breast cancer*" [11] demonstrates that MCF-7: PF cells grow robustly with E_2 but also SERMs will stimulate growth *in vitro* based on their individual intrinsic estrogenic efficacy as partial agonists [11]. Further investigation suggests that ER is a major driver of growth utilized by both E_2 and SERMs in resistant models in vivo [1, 3] and *in vitro* [11]. The finding in laboratory model is consistent with the clinical observation that aromatase inhibitors (AIs) or fulvestrant (ICI) are equally effective in the treatment of acquired tamoxifen resistant patients [12, 13]. It therefore appears that ER remains fully functional in the acquired tamoxifen resistance. In support with these findings, global gene expression microarray in back-to-back article reveals a remarkable overlap in genes deregulated in the same direction by E_2 and 4-OHT in MCF-7: PF cells [14]. The deregulation of these genes by E_2 or 4-OHT is able to be blocked by the pure antiestrogen ICI [14]. In contrast to E_2 that activates classical ER-target genes, SERMs continue to act as effective antiestrogens to inhibit classical ER-target genes, even at the time of growth stimulation [11, 14]. This result is supported by our previous finding in vivo [15] that growth of tamoxifen or fulvestrant resistant tumors does not rely on classical ER transcriptional pathways, which is evidenced by suppression of E_2-responsive genes [15]. Other groups have reported similar observations with tamoxifen suppressing classical ERE-regulated genes despite acquired resistance in vitro [16] or in vivo [17].

SERMs increase the non-genomic activity of ER and activate membrane function-associated molecules

A significant alteration of ER function observed in SERM-resistant cells is the enhancement of the non-genomic pathway of ER [11] which results in the activation of multiple membrane function-associated molecules including focal adhesion molecules, adapter proteins, and growth factor receptor [11]. In addition, the well known focal adhesion molecules FAK and p130CAS are activated by 4-OHT which participate in the non-genomic pathway of ER [11, 18]. Our pathway enrichment analysis also reveals a significant enrichment in a variety of genes associated with actin cytoskeleton remodeling, adaptor proteins, and other membrane-related functions activated by 4-OHT or E_2 in MCF-7: PF cells [14]. For example, both E_2 and 4-OHT up-regulated EH-domain containing 2 (EHD2) [14, 19], four and a half LIM domains 2 (FHL2) [14, 20], homer homolog 3 (HOMER3) [14, 21], and Ras homolog family member F (RHOF) [14, 22]. EHD2 is found to regulate trafficking from the plasma membrane by controlling Rho GTPases activity [19]. EHD proteins also have the capacity to associate with phospholipids in plasma membrane [23, 24] and form complexes with IGF-1R to regulate downstream Akt pathway [25]. The LIM domain protein, FHL2, interacts with filamin A to remodel the cytoskeleton and acts as a novel coactivator in regulating target genes [20]. HOMER3 is a cytoplasmic scaffolding protein, which mediates protein-protein interactions [21]. RHOF, belonging to the Rho family GTPases, has a critical role in controlling the formation of filopodia [22]. It is unclear the biological

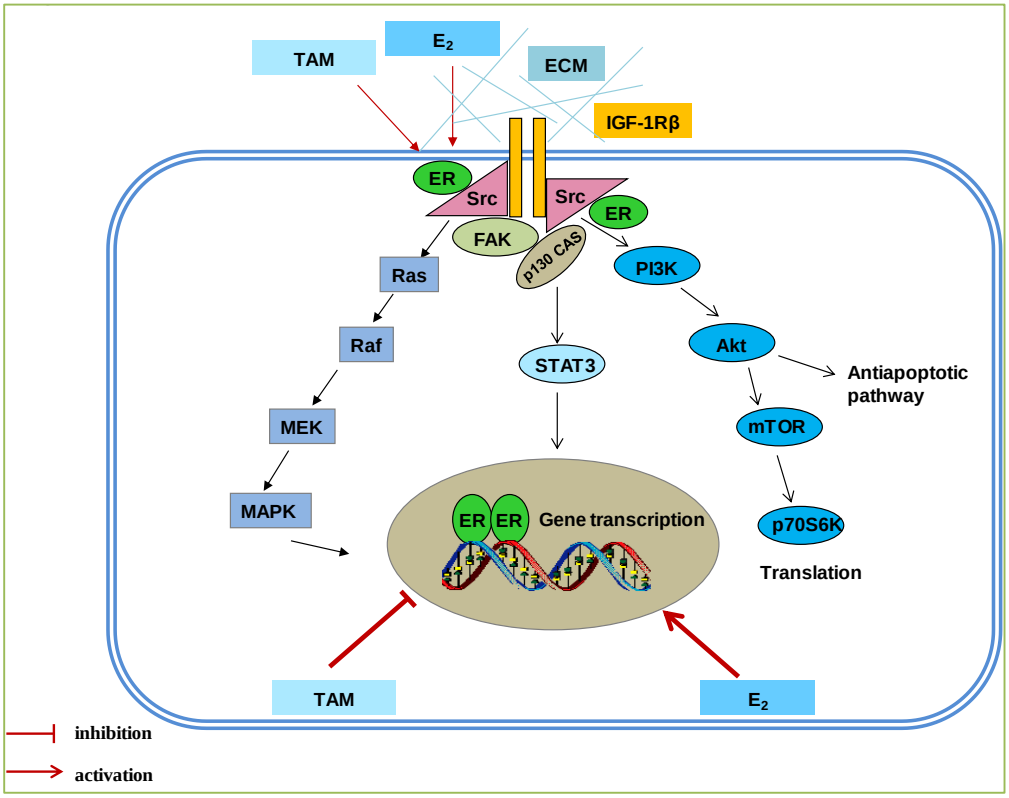

Figure 2. Signal transduction pathways in tamoxifen-resistant model. Estrogen (E_2) and tamoxifen (TAM) have differential functions on nuclear estrogen receptor (ER). E_2 activates classical ER-target genes but TAM functions to block gene activation. Both E_2 and TAM increase the non-genomic activity of ER through membrane-associated molecules, such as extracellular matrix (ECM), c-Src, insulin-like growth factor-1 receptor beta (IGF-1Rβ), and focal adhesion kinase (FAK) to enhance downstream signaling cascades. Reprint with permission [11].

functions of these molecules in SERM-resistant model. Inhibition of tyrosine kinase activities of IGF-1R or c-Src is able to modulation the expression of these molecules [14], demonstrating functional interactions exist among these membrane function-associated molecules and growth pathways [14]. Quite interestingly, in contrast to the overlapping gene regulation by E_2 or 4-OHT in MCF-7:PF cells [14], the gene regulation pattern of 4-OHT is distinct from that of E_2 in wild-type MCF-7 cells [14], in which 4-OHT acts primarily to antagonize these membrane function-associated molecules [14]. These findings suggest that the constant nuclear pressure by SERMs causes broad activation of membrane-associated signaling to aid breast cancer cell survival and creates new surviving cell populations during the selection process required for acquired SERM resistance.

SERMs increase the crosstalk between ER and membrane function-associated molecules including growth factor receptors

As mentioned above, SERMs widely activate membrane-associated molecules for the adaption to the nuclear ER suppression to promote cell growth. It is well known that cytoplasmic adapter proteins have the capacity to provide docking sites for the redistribution of a low percentage of ER [26] that functionally associates with the cell membrane, and facilitate the crosstalk between the ER and growth factor receptors [26, 27]. Accumulating evidence has suggested that growth factor receptors, such as epidermal growth factor receptor (EGFR) [27], IGF-1R [11, 28, 29], and HER-2 [30] are implicated in acquired SERM resistance, but its precise contributions are not well understood. In our SERM-resistant cell model, levels of IGF-1R are increased by E_2 in an ER-dependent manner [10, 11]. In other aspect, IGF-1R cross talks with membrane-associated ER [11] and regulates cell growth signaling pathways resulting in progressive tumor growth [31]. In contrast, 4-OHT functions as an antiestrogen to suppress the gene expression of IGF-1R to reduce the amount of receptor [11]. Interestingly, 4-OHT is able to simultaneously enhance the non-genomic activity of ER and activates focal adhesion molecules to increase phosphorylation of IGF-1R [11, 32]. Our observations precisely define the mechanisms underlying the paradoxical finding *in vivo* that acquired resistance to tamoxifen is associated with lower levels of total IGF-1R [11, 17, 28, 33] but keep [33] or gain

of phosphorylated IGF-1R [11, 17, 28]. Together, these results demonstrate that 4-OHT exerts distinct functions on nuclear ER and membrane-associated ER, which differentially affects the function of IGF-1R. Cross-talk between membrane-associated ER and IGF-1R appears to reinforce one another to stimulate breast cancer cell growth [11]. Similar observations have shown that the non-genomic activity of ER is enhanced by tamoxifen which facilitates the association with growth factor receptors, HER2 or EGFR to promote cell growth [27, 30]. The ER and growth factor receptors are two major growth pathways to promote breast cancer cell growth through their own receptors or via cross talk with each other [27, 30, 34]. Evidence has demonstrated that adapter protein c-Src is a crucial molecule to mediate the crosstalk between ER and growth factor receptors [27]. More data have shown that c-Src is activated and promotes cellular capacity of invasion and motility in acquired tamoxifen resistant models [35, 36]. These observations demonstrate that multiple membrane function-associated molecules including IGF-1R and membrane-associated ER are tightly linked to subvert long-term nuclear suppression by SERMs (Figure 2).

Challenges and conclusions

All together, over the past four decades, long-term tamoxifen treatment has significantly improved the survivorship of the ER-positive breast cancer patients [37, 38]. Although extensive studies have advanced the understanding of the mechanisms underlying acquired SERM-resistance, acquired resistance to SERMs is not one-dimensional with a simple solution. Resistant cell populations are in constant evolution depending upon selection pressure and the availability of growth stimuli that enhances population plasticity and survival of new clones [7]. Breast cancer cells have the potential to integrally modulate a variety of membrane-associated molecules to subvert long-term nuclear pressure exerted by SERMs (Figure 2). This results in the promotion of cell growth. These functional alterations lead to acquired SERM resistance. As a result, the strategic definition of molecular mechanisms driving the development of endocrine resistance is an important and proactive first step to improve therapy. How to prioritize and advance individualized treatment is another challenge to improve the therapeutic efficacy of antihormone therapy [39].

Conflicting interests

The authors have declared that no competing interests exist.

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