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Original Research

A molecular model for the mechanism of acquired tamoxifen resistance in breast cancer

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Abstract ***Purpose:*** Oestrogen (E_2)-stimulated growth re-emerges after a c-Src inhibitor blocking E_2-induced apoptosis. A resulting cell line, MCF-7:PF, is selected with features of functional oestrogen receptor (ER) and over-expression of insulin-like growth factor-1 receptor beta (IGF-1Rβ). We addressed the question of whether the selective ER modulator (SERM), 4-hydroxytamoxifen (4-OHT) or other SERMs could target ER to prevent E_2-stimulated growth in MCF-7:PF cells.
Methods: Protein levels of receptors and signalling pathways were examined by immunoblotting. Expression of mRNA was measured through real-time RT-PCR. Recruitment of ER or nuclear receptor coactivator 3 (SRC3) to the promoter of ER-target gene was detected by chromatin-immunoprecipitation (ChIP).
Results: 4-OHT and other SERMs stimulated cell growth in an ER-dependent manner. However, unlike E_2, 4-OHT suppressed classical ER-target genes as does the pure antioestrogen ICI 182,780 (ICI). ChIP assay indicated that 4-OHT did not recruit ER or SRC3 to the promoter of ER-target gene, pS2. Paradoxically, 4-OHT reduced total IGF-1Rβ but increased phosphorylation of IGF-1Rβ. Mechanistic studies revealed that 4-OHT functioned as an agonist to enhance the non-genomic activity of ER and activate focal adhesion molecules to further increase phosphorylation of IGF-1Rβ. Disruption of membrane-associated signalling, IGF-1R and focal adhesion kinase (FAK), completely abolished 4-OHT-stimulated cell growth.

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Conclusions: This study is the first to recapitulate a cellular model *in vitro* of acquired tamoxifen resistance developed in athymic mice *in vivo*. Importantly, it provides a rationale that membrane-associated pathways may be valuable therapeutic targets for tamoxifen resistant patients in clinic.

1. Introduction

The clinical application of the laboratory strategy of long-term adjuvant antihormone therapy for the treatment of breast cancer [1] has significantly improved breast cancer survival. Selection of patients whose tumours express the oestrogen receptor (ER) are more likely to respond to long-term adjuvant tamoxifen (TAM) [2,3] or aromatase inhibitors (AIs) [4] than those without ER. However, acquired resistance to antihormone therapy remains a challenge as adjuvant therapy is extended [3,5].

The evolution of acquired resistance to TAM treatment was discovered using MCF-7 tumours transplanted in athymic mice to mimic years of adjuvant treatment in patients [6–8]. Long-term therapy generates selection pressure for cell populations that evolve from acquired TAM resistance, ubiquitously observed in metastatic breast cancer, to eventually expose a vulnerability that is expressed as oestrogen (E_2)-induced apoptosis [8–10]. Acquired resistance to TAM or other selective ER modulators (SERMs) is unique in that the growth of resistant tumours is dependent on SERMs [6–8]. Acquired TAM resistance during the treatment of metastatic breast cancer occurs within one or two years [11], consistent with the model of SERM resistance in athymic mice [6,8]. An AI (depleting E_2) or fulvestrant (ICI 182,780; a pure antioestrogen that destroys the ER) is effective as second-line therapy after TAM failure [12,13]. Thus, it appears that acquired resistance to SERMs is initially able to utilise either E_2 or a SERM as the growth stimulus in ER-positive TAM-resistant breast tumours. However, no mechanism has been established to explain this paradox.

We describe a new model of antihormone-resistant breast cancer *in vitro* that exhibits the characteristics of acquired TAM resistance *in vivo*. The MCF-7:5C cell line emerged unexpectedly from an established MCF-7 cell line after long-term E_2 deprivation, *i.e.* simulated AI resistance [14]. The E_2-deprived cell lines [14,15] created from MCF-7 cells have the unique ability to undergo E_2-induced apoptosis that has clinical significance for the treatment [16] and prevention of breast cancer [17]. We discovered that if a c-Src inhibitor is applied, E_2-induced apoptosis is initially blocked in MCF-7:5C cells [18], but with extended treatment, E_2-stimulated growth re-emerges [19]. A stable cell line, MCF-7:PF is established [19,20]. Unexpectedly, the derived cell line MCF-7:PF was found to mimic the SERM/E_2-stimulated models *in vivo* [6], thereby providing the opportunity to decipher the mechanism of SERM-stimulated growth. Here, we provide evidence that 4-hydroxytamoxifen (4-OHT)-stimulated growth of MCF-7:PF is ER-dependent despite suppression of classical ER-target genes. However, 4-OHT functions as an agonist to enhance the non-genomic activity of ER and activates focal adhesion molecules to further increase phosphorylation of insulin-like growth factor-1 receptor beta (IGF-1Rβ). All of these events promote 4-OHT-stimulated cell growth. Overall, the sustained inhibition of nuclear ER-signalling causes broad activation of membrane-associated signalling to aid breast cancer cell survival during the selection process required for acquired TAM resistance.

2. Materials and methods

2.1. Materials

Estradiol and focal adhesion kinase (FAK) inhibitor (PF573228) were purchased from Sigma–Aldrich (St. Louis, MO); ICI 182,780 (ICI) was purchased from Tocris (Park Ellisville, MO). SERMs: 4-hydroxytamoxifen (4-OHT) was purchased from Sigma–Aldrich (St. Louis, MO), raloxifene was a kind gift from Eli Lilly (Indianapolis, IN), endoxifen was gifted from Dr. James Ingle (Mayo Clinic, Rochester, MN), bazedoxifene (BZA) was gifted from Dr. Ronald Grigg (University of Leeds, United Kingdom (UK)), EM652 was gifted from AstraZeneca (UK). c-Src inhibitor, PP2 and IGF-1Rβ inhibitor, AG1024, were purchased from CalBiochem (San Diego, CA). Sources of antibodies for Western blot were as follows: ERα (sc-544), mouse IGF-1Rβ (sc-462) and rabbit IGF-1Rβ (sc-713) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Total MAPK (#9102), phosphorylated MAPK (#9101), total Akt (#9272), phosphorylated Akt (#9271), total STAT3 (#4903), phosphorylated STAT3 (#9131), total FAK (#3285), phosphorylated FAK (Y397, #3283), phosphorylated FAK (Y576/577, #3281), phosphorylated p130CAS (#4014), phosphorylated IGF-1Rβ (#3024) and phosphorylated c-Src (#2101) antibodies were from Cell Signaling Technology (Beverly, MA). Total c-Src (GD11) and anti-phosphotyrosine 4G10 antibodies were