(Z)-Endoxifen Maintains ERα Antagonist Function Against ESR1 Mutants via Inactive Conformation Stabilization and Reversal of Mutant ESR1-Associated Transcriptional Signatures

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INTRODUCTION

Mutations in the estrogen receptor gene (ESR1), particularly Y537S and D538G, are key drivers of resistance in estrogen receptor-positive (ER+) metastatic breast cancer (MBC) following aromatase inhibitor therapy. These mutations promote ligand-independent ER activation. In this in silico modeling study, we examined the biophysical behavior and downstream transcriptional impacts of (Z)-endoxifen, an active tamoxifen metabolite, a selective estrogen receptor modulator (SERM), across ESR1 variants to further elucidate its mechanism of action and therapeutic potential.

METHODS

Computational Studies

- Molecular Dynamics (MD) Simulations:
  - WT, D538G, Y537S, and dual mutants simulated
  - Assessed conformational flexibility and stability of antagonist-bound states.
- Enhanced Sampling (Metadynamics):
  - Energy landscape explored for antagonist-compatible states
- Alchemical Free Energy Calculations (NES):
  - Used Non-Equilibrium Switching (NES) simulations to directly compare binding affinities of (Z)-Endoxifen 4-Hydroxy-Tamoxifen (4-OHT)

Cell-Based Functional Assays

- Luciferase Reporter Assays:
  - Conducted in HEK293T and MCF-7 cells expressing ESR1-WT or ESR1-D538G.
  - Measured ligand-dependent transcriptional activity of ERα.
- 2D Growth Assays:
  - Quantified proliferative responses to drug treatment in WT vs. mutant backgrounds.

Transcriptomic Analyses

- Data Sources:
  - Integrated public RNA-seq and microarray datasets:
    - ESR1-mutant tumors (n=56)
    - ESR1-mutant MCF7 cells (n=166)
    - Drug-treated breast cancer cells
      - (Z)-Endoxifen (n=27)
      - Elacestrant (n=5)
- Differential Expression & Enrichment Analysis:
  - Identified gene sets, pathways, and transcription factors altered by ESR1 mutation and treatment.
  - Analyses performed using limma (for DE analysis) and gseapy (for enrichment).

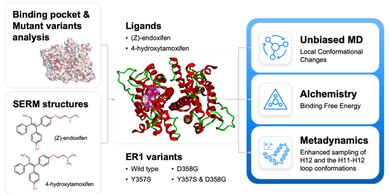


Figure 1. A computational framework for systematic analysis of ERα mutations and their impact on small molecule binding and receptor dynamics. The left panel highlights key components of the system under investigation: (a) structural analysis of the ERα binding pocket in its mutant variants, visually depicted as a molecular surface model, and (b) the small molecule selective estrogen receptor modulators (SERMs) under study, including (Z)-endoxifen and 4-hydroxytamoxifen, with their chemical structures shown. The central panel illustrates the ERα ligand-binding domain. The right panel details the methodological approach, consisting of three stages: (1) Unbiased molecular dynamics (MD) simulations to investigate local conformational changes in the mutant ligand-protein systems; (2) Alchemistry (a module of the AI-driven platform Chemistry42) to compute the relative binding free energy differences between (Z)-endoxifen and 4-hydroxytamoxifen in both wild-type and mutant ERα variants; and (3) Metadynamics simulations to enable enhanced sampling of large-scale conformational transitions, including the critical repositioning and stabilization of the H12 helix and the H11-H12 loop. These integrative computational strategies collectively provide deeper insights into the structural and thermodynamic impacts of ERα mutations on receptor function and antagonist binding.

RESULTS

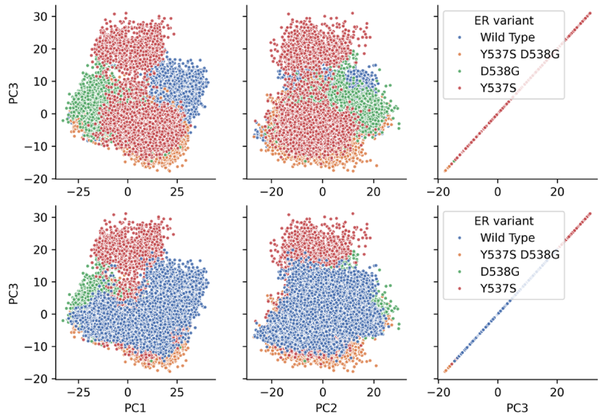


Figure 2. Principal Component Analysis (PCA) of (Z)-Endoxifen Dynamics. The PCA plots show how the conformations of the wild-type (WT) and mutant systems compare. In the top panels, WT conformations are shown over the mutant data, while in the bottom panels, mutant conformations are shown over the WT data—this highlights the overlap and similarities in the conformational space sampled. The figure focuses on relationships involving the third principal component (PC3): **PC1 vs. PC3**, **PC2 vs. PC3**, and **PC3 vs. PC1**

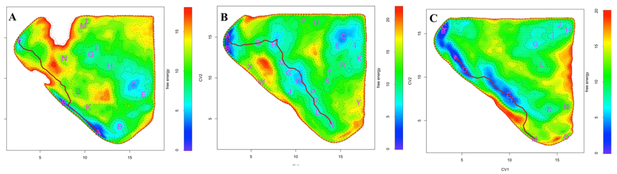


Figure 3. Free Energy Surfaces (FES) for (A) WT, (B) Y537S, and (C) D538G Simulations. The two axes, CV1 and CV2, represent the root-mean-square deviation (RMSD) of the H12 helix and the H11/H12 loop compared to their active and inactive conformations. In each plot, the lowest energy region (population A) shows the most stable structure. For the WT (A), this minimum corresponds to the inactive conformation, while for Y537S (B) and D538G (C), it corresponds to the active conformation.

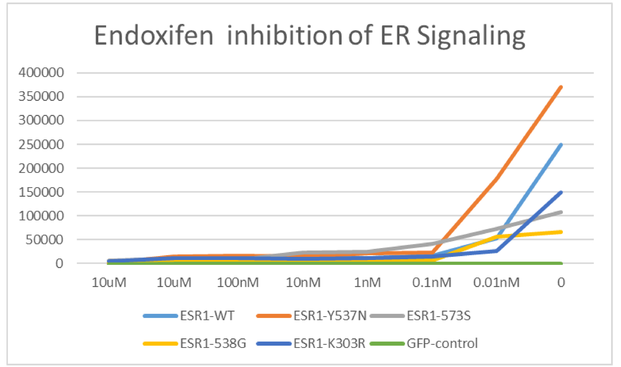


Figure 4. Endoxifen potently suppresses ER signaling. 293T cells were transfected with ER-Luc reporters and ESR1 expressing plasmids. Transfection were performed in the presence of phenol-red non-charcoal stripped media allowing ESR1-WT signaling. Luciferase measurements were performed 24hours post-transfection in presence of Endoxifen.

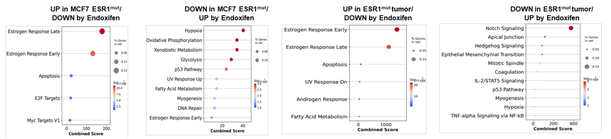


Figure 5. Pathway enrichment analysis of genes in the intersection between (Z)-Endoxifen and ESR1^{mut} breast cancer signatures. Pathway enrichment analysis of both ESR1^{mut} patient (two right-side figures) and cell line (two left-side figures) data revealed key signaling pathways affected by both disease and treatment. Notably, several pathways were significantly enriched (FDR corrected p-value < 0.05) with genes upregulated in ESR1 mutant tumors/cells and downregulated by (Z)-endoxifen (including estrogen response, E2F target, and Myc target pathways), or vice versa downregulated in ESR1 mutant samples and upregulated by (Z)-endoxifen (including the oxidative phosphorylation and p53 signaling pathways).

RESULTS CONT.

Type	ΔΔG (Endoxifen -> 4-OHT), kcal/mol
WT	0.09 ± 0.62
Y357S	-0.13 ± 0.63
D358G	1.30 ± 0.35
Y357S & D358G	-0.84 ± 0.58

ΔΔG values are reported as the mean of three replicates ± standard deviation (STD)

Non-equilibrium switching calculations using Chemistry42 Alchemistry module show minimal differences in binding free energy (ΔΔG) between 4-OHT and Z-Endox across all receptor variants.

CONCLUSION

- **MD Simulations:**
  - Mutations do not displace antagonists or substantially alter receptor conformational space during simulation (except minor sampling in Y537S; not statistically significant – see red points, figure 2, y-axis between 15–30).
- **Alchemical Results:**
  - Mutations have similar effects on binding affinity for both ligands.
  - No direct ligand/mutated residue interactions or major pocket rearrangement observed.
  - Differences between calculated ΔΔG and experimental Ki likely reflect indirect effects influencing apparent rather than intrinsic affinity.
- **Metadynamics:**
  - Mutations partially shift the receptor towards active states, but a fraction of antagonist-compatible conformations persists, explaining the sustained efficacy of Z-endoxifen even in mutants.
- Endoxifen strongly inhibits ER signaling in 293T cells expressing ESR1 variants.
- Effective under estrogen-rich conditions, showing robust suppression even in phenol red–containing media.
- Key signaling pathways affected in ESR1-mutant tumors/cells may be reversed by endoxifen treatment.
- Highlights Endoxifen's potential as a potent ER antagonist for treating ER-positive and ESR1-mutant cancers.

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