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A mutant-selective anti-estrogen is a pure antagonist on EREs and AP-1 response elements

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Abstract

Estrogen Receptors (ERs) regulate gene transcription through classic estrogen response elements (EREs) as well as AP-1 responsive genes. The common SERMs Raloxifene, Tamoxifen and ICI164384 function as ER antagonists on EREs but as ER β agonists/partial agonists on AP-1 responsive genes. While developing a mutant selective analog of Raloxifene, that is an antagonist of ER α (E353A) we discovered an antagonist of wild-type ER α and ER β that is also an antagonist of ER β /AP1 response. The analog, DRL527, represses basal AP-1 gene expression and antagonizes Raloxifene stimulated AP-1 expression. Therefore DRL527 has a unique, previously unreported, ERE/AP1 activity profile.

Estrogens play an essential role in the growth, differentiation and homeostasis in a broad range of target tissues, including the male and female reproductive tracts, mammary glands, bone, brain, liver, and cardiovascular system.^{1–6} The biological effects of estrogen are mediated through two Estrogen Receptor (ER) subtypes, ER α and ER β , which belong to a superfamily of nuclear receptors that act as ligand dependent transcription factors.^{4–6} The classic mechanism of ER activation involves dimerization and nuclear translocation upon ligand binding. In the nucleus ERs can bind directly to estrogen response elements (EREs) located in the promoter region of estrogen receptor genes (ER/ERE pathway). ERs have also been shown to regulate gene expression in the absence of direct DNA-binding by associating with other DNA bound transcription factors. Through interactions with transcription factors c-Jun and c-Fos, ligand bound ERs can also regulate AP-1 dependent genes (ER/AP-1 pathway) in a manner that can be distinct from ER mediated responses on classic EREs.^{7–9} ER ligands that show differential responses within different tissues and have been more accurately described as Selective Estrogen Receptor Modulators (SERMs).¹⁰

The ability of Tamoxifen (Tam) to function as an ER antagonist in breast tissue, but an agonist in the uterus and bone or the ability of Raloxifene (Ral) to act as agonist in bone while an antagonist in breast and uterine tissues may likely result from differential activation of multiple estrogen responsive pathways.^{10,11} For the ER/AP-1 pathway SERMs such as Raloxifene, Tamoxifen and ICI164384 that were originally classified as ER/ERE antagonists have been found to be ER β -AP-1 agonists.^{7–9} Although the molecular details of the ER/ERE pathway are well characterized, our understanding of the molecular details of ER/AP-1 pathway, especially mechanisms of altered SERM pharmacology, is limited.^{7,9,12–15} In the

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process of exploring mutant selective ER antagonists we discovered a new SERM DRL527 that exhibits a unique ERE/AP-1 activity profile.

Design of a new mutant-selective antagonist

Previously our group developed mutant-specific analogs of estradiol which selectively activate ER α and ER β mutants, ER α (E353A) and ER β (E305A) that are otherwise not responsive to physiological concentrations of estradiol (< 1 nM). They were designed based on the principle of “polar-group exchange,” wherein a carboxylate modified ligand complemented Glu→Ala substitution at the E2/ER interface (Figure 1). The carboxylate-functionalized ligand (ES8) displayed a 95-fold and 400-fold preference for ER α (E353A) and ER β (E305A) over the wild type receptors.¹⁶ Such systems are being explored as a means to selectively regulate ER dependent transcription pathways independent of E2. Herein we explore the application of a similar design strategy applied to the ER-Ral complex and explore its ability to regulate ER dependent responses on ERE and AP-1 dependent genes.

Raloxifene binds the E2 binding pocket of ER with the 6-phenolic hydroxyl of the benzothiophene core mimicking the A-ring phenolic hydroxyl of E2 by binding in the polar pocket between helix-3 and helix-6.¹⁷ By analogy to the mutant selective ER agonist ES8, it was envisioned that a similar strategy could be employed to make a mutant selective ligand receptor pair from Raloxifene. The ability of the carboxylate functionalized Raloxifene, DRL527 (Figure 1), to selectively antagonize ER α (E353A) and ER β (E305A) over the wild-type ER's was evaluated in cellular reporter gene assays on both ERE and AP-1 response elements.

The compound DRL527 was synthesized in 4 steps from Raloxifene by monobenzoylation of the 4'-phenolic hydroxyl group followed by triflate ester formation of the 6-phenolic hydroxyl group. This approach though cumbersome, proved more effective in our hands than a variety of strategies explored to selectively protect or differentially deprotect the two hydroxyls.^{18,19} The aryl triflate underwent Heck coupling with methyl acrylate to afford compound DRL527 after deprotection (Figure 2).

DRL527 was evaluated in cell based reporter gene assays for its ability to selectively antagonize wild-type ERs, ER α (wt), and ER β (wt) and the ES8-selective mutants hER α (E353A) and hER β (E305A) using an ERE-luciferase reporter in HEK293T cells. The mutant receptors ER α (E353A) and ER β (E305A) are not activated by physiological concentrations of E2 (EC₅₀=2.5 nM, 10.4 nM respectively) but are activated by ES8 (EC₅₀=0.24 nM, 0.07 nM respectively) at concentrations that do not activate ER(wt).¹⁶ As E2 is an inefficient agonist of hER α (E353A) and hER β (E305A), DRL527 was evaluated as an antagonist of these mutant ERs in competition with ES8. In order to practically compare the intrinsic ability of DRL527 to antagonize ER(wt)+E2 with ER(mutant)+ES8 the concentrations of E2 and ES8 were evaluated at their respective EC₉₀'s for each receptor. Therefore Raloxifene was evaluated as an antagonist with ER α (wt) by competition with 0.05 nM E2 (IC₅₀=0.3 nM), with ER α (E353A) with 3 nM ES8 (IC₅₀= 55.5 nM), ER β (wt) +0.1nM E2 (IC₅₀= 6.86 nM) and ER β (E305A)+0.3 nM ES8 (IC₅₀= 576.9 nM). Under these conditions, Raloxifene is 185-times more selective for the ER α (wt) than the ER α (E353A) and 84-times more selective for the ER β (wt) than the ER β (E305A) on an ERE response element in HEK293T cells (Figure 3a,b).

By contrast, under similar conditions, DRL527 was found to be an almost equipotent antagonist with both wild type and mutant ERs of both the alpha and beta subtypes (Figure 3b). Thus whereas the antagonist activity of DRL527 cannot be effectively evaluated directly due to the inherent E2 insensitivity of ER α (E353A) and ER β (E305A), DRL527 was

effective in negating the 84- to 185-fold selectivity bias of Raloxifene consistent with its complementary design (Table 1).

Raloxifene is known to be an ER α selective antagonist.²⁰ However, DRL527 while less potent than Raloxifene, was found to be a highly subtype selective ER antagonist with ($IC_{50\beta}/IC_{50\alpha}=300$) comparable to some of the most selective antagonists reported to date (Figure 3c).²¹ Modification of 4-hydroxyl of raloxifene may offer an additional means to impart subtype selectivity.

Whereas the interpretation of mutant-selective antagonist activity is complicated by the use of the corresponding mutant selective agonist ES8, we were intrigued by the possibility that DRL527 may potentially serve as a mutant selective agonist of ERs on AP-1 responsive genes because existing SERMS Raloxifene, Tamoxifen and ICI 164384 have all been shown to be ER β (wt) agonists on AP-1 promoters.^{8,12} DRL527 was tested for its regulatory effects on transcription from ER β (wt) and ER β (E305A) on an AP-1 based reporter in transiently transfected HeLa cells. The addition of DRL527 afforded no significant change in AP-1 activity with ER β (E305A) up to 10 μ M (data not shown). However, in contrast to Raloxifene which behaved as an agonist, DRL527 was found to be an hER β (wt)/AP-1 antagonist, repressing basal AP-1 transcription ($EC_{50}=150\pm43$ nM) (Figure 4a).

To further support the notion that DRL527 is indeed acting as an ER β -AP-1 antagonist, competition experiments were performed in the presence of 10 nM Raloxifene in transiently transfected HeLa cells. In these experiments, DRL527 exhibited competitive inhibition of Raloxifene induced expression to below basal levels; $IC_{50}=104\pm55$ nM (Figure 4b). The repression of ER β /AP-1 dependent expression is ER β specific as no change in basal AP-1 driven expression is observed with ER α . However, whereas we observe and have previously reported no ER α /AP-1 response with Tam,²² the ER α /AP-1 activities appear to be highly context dependent and others have concluded that Tam, Ral and ICI can act as ER α /AP-1 agonists.^{8,12,15} DRL527 is unique as the only reported ER β /AP-1 antagonist (Figures 4c and 4d).

In summary, DRL527 was designed as a mutant selective antagonist of ER α (E353A) and ER β (E305A) based on structural analogy to the mutant selective agonist, ES8. DRL527 functions in a mutant-selective manner towards repressing ES8/ER dependent transcriptional activation at EREs. Furthermore, DRL527 was found to be a highly subtype-selective inhibitor of ER α (wt) although with lower potency than Raloxifene. DRL527 did not alter ER α (E353A) and ER β (E305A) dependent AP-1 response but was rather found to be an antagonist of ER β (wt) dependent AP-1 activation capable of suppressing basal AP-1 activity and suppressing Raloxifene induced AP-1 expression whereas, classic ER antagonists, Tamoxifen, Raloxifene and ICI are agonists of ER β on AP-1 sites (Table 2). Modification of the 6-phenolic hydroxyl of benzothiophene core of Raloxifene provides a means to enhance ER subtype selectivity and provides a new SERM DRL527 that functions as a novel ER antagonist on AP-1 sites. To our knowledge DRL527 represents a unique example of an ER ligand that is an antagonist of ER α and ER β on EREs and an antagonist of ER β on AP-1. DRL527 or other ER ligands that possess unique ERE/AP-1 response profiles may serve as a unique tool to understand the role AP-1 signaling in SERM based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
ES8 and DRL527 design concept.

Figure 2.

Synthesis of DRL527. a. NaH, BzCl, DMF, 8% b. TEA, Tf₂O, CH₂Cl₂, -78°C to r.t., 38%
c. methyl acrylate, Pd(OAc)₂, DPPP, TEA, DMF, 95°C, 22% d. LiOH. THF-water, reflux, 37%

Figure 3. Cellular reporter gene activity of (A) RAL and (B) DRL527 in competition with hER α (wt) +0.05 nM E2 (■), ER α (E353A)+3 nM ES8 (□), hER β (wt)+0.1 nM E2 (●), and ER β (E305A) +0.3 nM ES8 (○), on an ERE response element in HEK293T cells. (C) DRL527 in competition with 0.1 nM E2 in wild type ER α (■) and ER β (●) respectively.

Figure 4. Cellular dose-response behavior of ER β /AP-1 driven expression in transiently transfected HeLa cells. A) Green; Raloxifene alone and Brown; DRL527 alone. B) Green; Raloxifene alone and Brown; variable concentrations of **DRL527** versus 10 nM RAL. C,D) Comparison of ligand-dependent responses on ER α (4C) and ER β (4D) at an AP-1-responsive promoter with 1 μ M E2, RAL and DRL527.

Table 1

Potencies (IC_{50}) of Raloxifene and DRL527 with wild-type and mutant ERs on ERE-reporters in cellular reporter gene assays in competition with E2 and ES8 concentrations as indicated. Error reported as std. dev. of triplicate run. Selectivity is defined as IC_{50} (mutant)/ IC_{50} (wild type).

	ER α +0.05 nM E2	ER α (E353A) +3 nM ES8	Mutant Selectivity	ER β +0.1 nM E2	ER β (E305A) +0.3 nM ES8	Mutant Selectivity
RAL	0.3 $\pm$.06 nM	56 $\pm$ 10 nM	185	6.9 $\pm$.8 nM	577 $\pm$ 51 nM*	84
DRL527	82 $\pm$ 18 nM	212 $\pm$ 32 nM	2.6	> 10 μ M	> 10 μ M	~1

* denotes estimated from incomplete saturation.

Table 2

A new SERM profile showing **DRL527** in comparison to Raloxifene and E2 at an ERE and AP-1 site on wild-type ER alpha and beta.

	ERα		ERβ	
	ERE	AP-1	ERE	AP-1
E2	+	+/ $\emptyset$ [*]	+	–
Tam ^L	–	+/ $\emptyset$ [*]	–	+
Ral	–	+/ $\emptyset$ [*]	–	+
ICI ^L	–	+	–	+
DRL527	–	$\emptyset$	–	–

‘+’ indicates an agonist, ‘–’ an antagonist, ‘ $\emptyset$ ’ indicates neither activation nor repression,

* literature results vary and show these compounds to be either agonists or non-agonists/non-antagonists,^{8,12,15,22}

^LBased on reported activities,^{8,12,15,22}