Bioorganic & Medicinal Chemistry Letters 20 (2010) 5258–5261

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

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ARTICLE INFO

Article history:
Received 15 April 2010
Revised 28 June 2010
Accepted 29 June 2010
Available online 24 July 2010

Keywords:
Estrogen receptor
Estrogen response element
AP-1
SERM

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 ERβ agonists/partial agonists on AP-1 responsive genes. While developing a mutant selective analog of Raloxifene, that is an antagonist of ERα(E353A), we discovered an antagonist of wild-type ERα and ERβ that is also an antagonist of ERβ/AP-1 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/AP-1 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, ERα and ERβ, 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 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 ERβ-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 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 ERα and ERβ mutants, ERα(E353A) and ERβ(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 (Fig. 1). The carboxylate-functionalized ligand (ES8) displayed a 95-fold and 400-fold preference for ERα(E353A) and ERβ(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 (Fig. 1), to selectively antagonize ERα(E353A) and ERβ(E305A) over the wild-type ER's was evaluated in cellular reporter gene assays on both ERE and AP-1 response elements.

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doi:10.1016/j.bmcl.2010.06.151

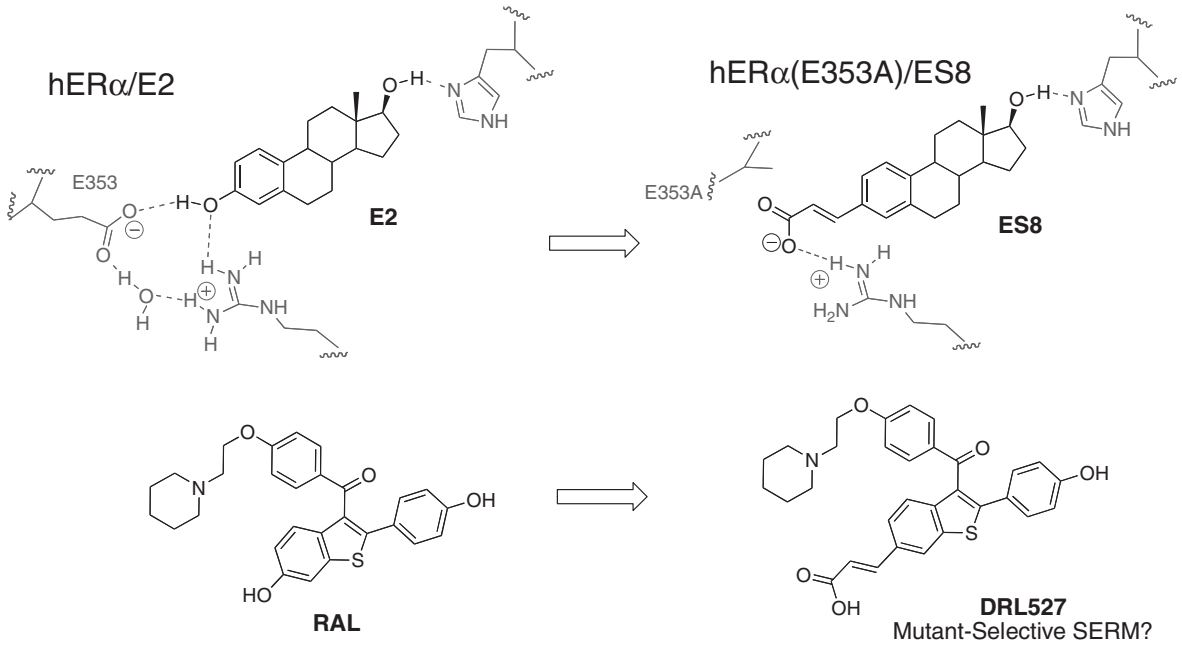

Figure 1. ES8 and DRL527 design concept.

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 (Fig. 2).

DRL527 was evaluated in cell based reporter gene assays for its ability to selectively antagonize wild-type ERs, ERα(wt),and ERβ(wt) and the ES8-selective mutants hERα(E353A) and hERβ(E305A) using an ERE-luciferase reporter in HEK293T cells. The mutant receptors ERα(E353A) and ERβ(E305A) are not activated by physiological concentrations of E2 (EC_{50} = 2.5 nM, 10.4 nM respectively) but are activated by ES8 (EC_{50} = 0.24 nM, 0.07 nM respectively) at concentrations that do not activate ER(wt).[16] As E2 is an inefficient agonist of hERα(E353A) and hERβ(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 EC_{90}'s for each receptor. Therefore Raloxifene was evaluated as an antagonist with ERα(wt) by competition with 0.05 nM E2 (IC_{50} = 0.3 nM), with ERα(E353A) with 3 nM ES8 (IC_{50} = 56 nM), ERβ(wt)+0.1 nM E2 (IC_{50} = 6.9 nM) and ERβ(E305A)+0.3 nM ES8 (IC_{50} = 577 nM). Under these conditions, Raloxifene is 185-times more selective for the ERα(wt) than the ERα(E353A) and 84-times more selective for the ERβ(wt) than the ERβ(E305A) on an ERE response element in HEK293T cells (Fig. 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 (Fig. 3b). Thus whereas the antagonist activity of DRL527 cannot be effectively evaluated directly due to the inherent E2 insensitivity of ERα(E353A) and ERβ(E305A), DRL527 was effective in negating the 84- to 185-fold selectivity bias of Raloxifene consistent with its complementary design (Table 1).

Raloxifene is a known to be an ERα selective antagonist.[20] However, DRL527 while less potent that Raloxifene, was found to a highly subtype-selective ER antagonist with ($IC_{50}\beta/IC_{50}\alpha$ = 300) comparable to the some of most selective antagonists reported to date (Fig. 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

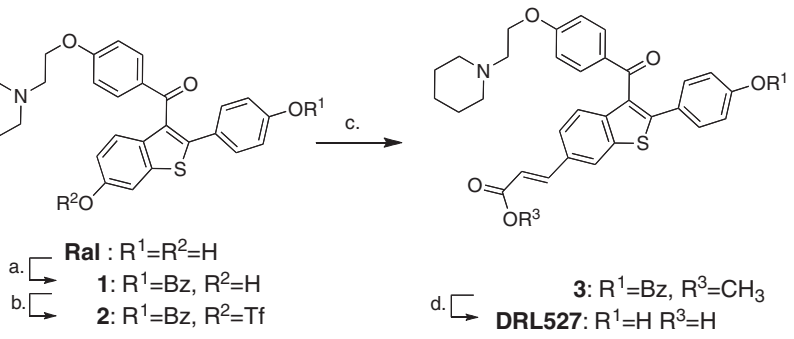

Figure 2. Synthesis of DRL527. (a) NaH, BzCl, DMF, 8%; (b) TEA, Tf_2O, CH_2Cl_2, −78 °C to rt, 38%; (c) methyl acrylate, $Pd(OAc)_2$, DPPP, TEA, DMF, 95 °C, 22%; (d) LiOH. THF–water, reflux, 37%.

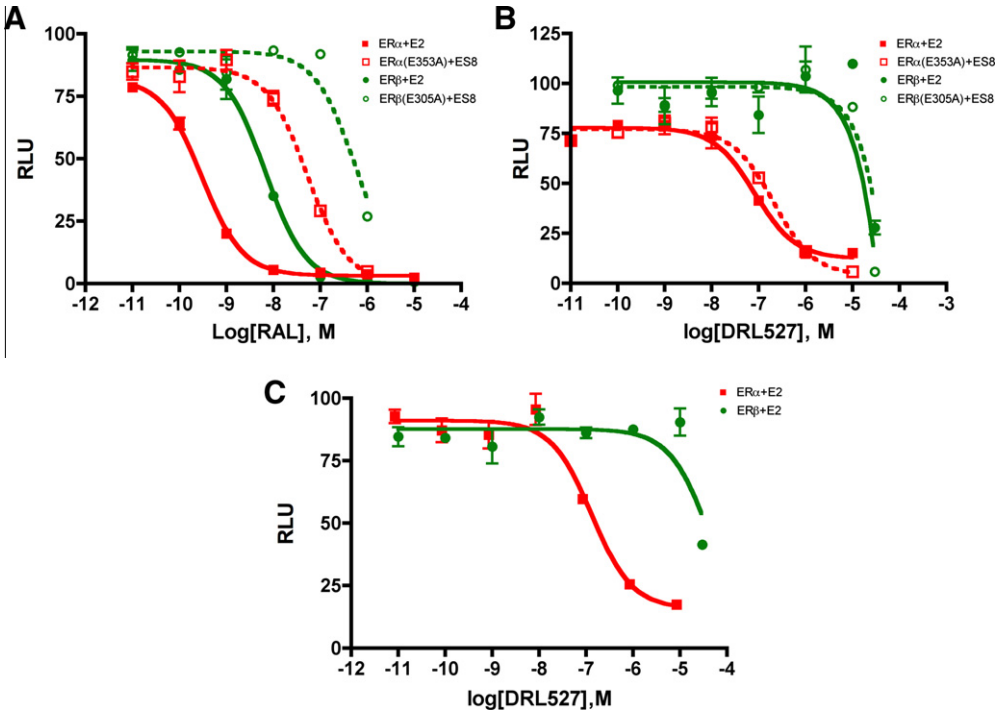

Figure 3. Cellular reporter gene activity of (A) RAL and (B) DRL527 in competition with hERα(wt)+0.05 nM E2 (■), ERα(E353A)+3 nM ES8 (□), hERβ(wt)+0.1 nM E2 (●), and ERβ(E305A)+0.3 nM ES8 (○), on an ERE response element in HEK293T cells. (C) DRL527 in competition with 0.1 nM E2 in wild-type ERα(■) and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

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

	ERα+0.05 nM E2	ERα(E353A) +3 nM ES8	Mutant Selectivity	ERβ +0.1 nM E2	ERβ(E305A) +0.3 nm ES8	Mutant Selectivity
RAL	0.3 ± 0.06 nM	56 ± 10 nM	185	6.9 ± .8 nM	577 ± 51 nM*	84
DRL527	82 ± 18 nM	212 ± 32 nM	2.6	>10 μM	>10 μM	~1

Error reported as SD of triplicate run. Selectivity is defined as IC_{50} (mutant)/IC_{50} (wild-type). *Denotes estimated from incomplete saturation.

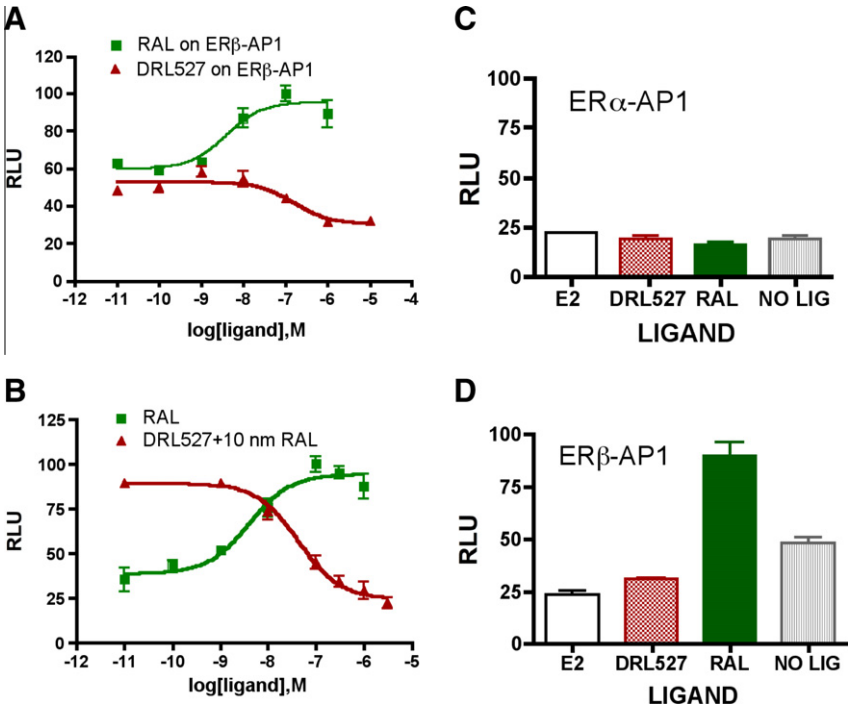

Figure 4. Cellular dose–response behavior of ERβ/AP-1 driven expression in transiently transfected HeLa cells. (A) Green; Raloxifene alone and brown; DRL527 alone. (B) Green; Raloxifene alone and brown; variable concentrations of DRL527 versus 10 nM RAL. (C,D) Comparison of ligand-dependent responses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

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	+	+/∅*	+	–
Tam$^{\perp}$	–	+/∅*	–	+
Ral	–	+/∅*	–	+
ICI$^{\perp}$	–	+	–	+
DRL527	–	∅	–	–

'+' indicates an agonist, '–' an antagonist, '∅' 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] $^{\perp}$based on reported activites.[8,12,15,22]

DRL527 may potentially serve as a mutant selective agonist of ERs on AP-1 responsive genes because existing SERMS Raloxifene, Tamoxifen and ICI164384 have all been shown to be ERβ(wt) agonists on AP-1 promoters.[8,12] DRL527 was tested for its regulatory effects on transcription from ERβ(wt) and ERβ(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 ERβ(E305A) up to 10 μM (data not shown). However, in contrast to Raloxifene which behaved as an agonist, DRL527 was found to be an hERβ(wt)/AP-1 antagonist, repressing basal AP-1 transcription (EC_{50} = 150 ± 43 nM) (Fig. 4a).

To further support the notion that DRL527 is indeed acting as an ERβ-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 ± 55 nM (Fig. 4b). The repression of ERβ/AP-1 dependent expression is ERβ specific as no change in basal AP-1 driven expression is observed with ERα. However, whereas we observe and have previously reported no ERα/AP-1 response with Tam,[22] the ERα/AP-1 activities appear to be highly context dependent and others have concluded that Tam, Ral and ICI can act as ERα/AP-1 agonists.[8,12,15] DRL527 is unique as the only reported ERβ/AP-1 antagonist (Fig. 4c and d).

In summary, DRL527 was designed as a mutant selective antagonist of ERα(E353A) and ERβ(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 ERα(wt) although with lower potency than Raloxifene. DRL527 did not alter ERα(E353A) and ERβ(E305A) dependent AP-1 response but was rather found to be a antagonist of ERβ(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 ERβ 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 ERα and ERβ on EREs and an antagonist of ERβ 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.

Acknowledgment

This work was supported by the National Institutes of Health (NIDDK R01DK54257).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.151.

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