REVIEW

Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand

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Abstract

The nuclear receptor family comprises ligand-dependent and orphan receptors. To the latter group belong the estrogen receptor-related receptors (ERRs) for which conflicting results have been published concerning the nature (constitutive or liganded) of their transcriptional activities. ERRs interfere in various ways, positively and negatively, with estrogen signaling. Moreover recent data analyzing ERR expression in human breast tumors have proposed ERRα and ERRγ as prognostic markers of these cancers. The identification of modulators (positive or negative) of ERR activities would therefore be highly useful in our understanding of estrogen-related pathologies. The purpose of this review is to summarize our knowledge of the nature of ERR activities and progresses in identifying synthetic ERR modulators.

Journal of Molecular Endocrinology (2003) **31,** 349–357

On nuclear receptors (NRs) in general and orphan receptors in particular

According to a commonly used definition, NRs are described as ligand-dependent transcription factors (Laudet & Gronemeyer 2002). With very few exceptions, all NR proteins share a similar organization in modular domains, two of which are remarkably conserved between members of the family and across evolution. These are (i) the DNA-binding domain (DBD), localized in the middle of the primary structure and which mediates specific interaction with the target DNA sequences and (ii) the ligand-binding domain (LBD), in the C-terminal part, which is responsible for ligand recognition, dimerization, interaction with coactivators and ligand-dependent transcriptional activation. Three-dimensional (3D) structure determination has demonstrated that ligand entry into the binding pocket of the LBD evokes a conformational change allowing the recruitment of coactivator proteins that directly or indirectly modify chromatin and induce target gene expression. Several coactivators have been described that interact with NRs, the most common being the three widely expressed p160 family members, SRC-1, GRIP1/TIF/SRC-2 and pCIP/AIB1/SRC-3 (reviewed in McKenna *et al.* 1999). A ligand-independent transactivation domain can be found in the non-conserved N-terminal part (A/B domain) of certain, but not all, NRs, such as the estrogen receptor (ER) α (Laudet & Gronemeyer 2002).

Receptors for classic ligands such as steroid hormones, thyroid hormones, retinoic acids or vitamin D were identified on the basis of their capacity to transduce a previously known hormonal signal. Besides them, the NR superfamily also comprises a number of so-called orphan receptors that were isolated on the basis of their sequence similarity and identical domain organization to other NRs (Giguère 1999). Since no ligand has been identified that regulates them, the orphan receptors do not fulfill the above-mentioned definition. One of the remaining questions concerning orphan receptors is how their transcriptional activities are regulated. In other words, do they respond to a natural ligand which remains

Printed in Great Britain
Online version via http://www.endocrinology.org

to be identified, or do they act in a ligand-independent manner?

This question has been at least partially answered for a number of orphan receptors. For instance, certain oxysterols (catabolites of cholesterol) have been shown to act as ligands for LXRs, and to modulate (positively in rodent, negatively and indirectly in humans) the expression of an enzyme (Cyp7a) that catalyzes their degradation, thereby acting in a feed-forward mechanism (reviewed in Repa & Mangelsdorf 2000, Goodwin *et al.* 2003). The LXRs can therefore be considered as ligand-dependent receptors. On the contrary 3D structure determination of Rev-erbα has demonstrated that the putative ligand-binding pocket was actually packed with amino acid side-chains rendering the entry of a ligand very unlikely (Renaud *et al.* 2000). Rev-erbα, which is in fact a transcriptional repressor, is therefore considered as a non-liganded, constitutively acting receptor. However, it should be remembered that a constitutive activity does not exclude the existence of a ligand, as exemplified by CAR. Indeed, the activity of this receptor, originally described as constitutive, can be down-regulated by androstane metabolites (Forman *et al.* 1998), but also further activated by phenobarbital (Tzameli *et al.* 2000).

In spite of the examples quoted above, there are some orphan receptors for which the existence of a ligand is still an open question. Although they were the first orphan receptors to be identified, this is the case for the estrogen-receptor related receptors (ERRs), to which this review is dedicated. Furthermore, even if a receptor is recognized as constitutively active, it could still be (at least theoretically) possible to modulate its activity using a synthetic agonist or antagonist. In this respect, recent data have identified synthetic antagonists of the ERRs. Given the wide variety of developmental, physiological and metabolic processes controlled by NRs, finding a way to modulate the activity of these transcription factors is an issue of considerable importance.

On ERRs

ERRα and β were isolated in 1988 by mean of a low-stringency screen with the DBD of ERα (Giguère *et al.* 1988). More recently, a third member of the subfamily (ERRγ) has been identified (Hong *et al.* 1999). ERRs are present throughout evolution, with a unique homologue existing in the invertebrates *Drosophila*, amphioxus and tunicates (Dehal *et al.* 2002, B Horard & J-M Vanacker, unpublished observations). In terms of sequence, ERRs are fairly similar to the ERs (Fig. 1), particularly in the DBD regions (over 60% sequence identity between human ERRα and ERα). The lower conservation level of the LBDs (less than 35%) is apparently consistent with the incapacity of ERRs to bind estradiol (E2) (Giguère *et al.* 1988, Yang *et al.* 1996). However, *in silico* superimposition of the ligand-binding pocket of ERRα on that of ERα has revealed a greater level of local sequence identity, suggesting that structurally close ligands could be bound by both receptors (Chen *et al.* 2001).

In the past years, numerous interconnections between the ERRs and estrogen signaling have been documented, and these are thoroughly discussed in a recently published review (Giguère 2002) (see Fig. 2 for summary). For instance, ERRs and ERs share common transcriptional target genes, such as lactoferrin, osteopontin and pS2, on which they can either synergize or compete with one another (Yang *et al.* 1996, Vanacker *et al.* 1999a, Lu *et al.* 2001, Kraus *et al.* 2002). Furthermore, Yang *et al.* (1996) found that human ERRα interacts with human ERα at least *in vitro* through protein–protein contacts. Moreover, the aromatase gene, encoding the enzyme catalyzing the conversion of androgens to estrogens, is a transcriptional target of ERRα (Yang *et al.* 1998). Altogether, these finding have raised the possibility that the ERRs (at least ERRα) might directly and indirectly modulate the estrogenic response.

Little is known about the *in vivo* functions of the ERRs. It has been proposed that ERRα plays a role in bone formation. Indeed this receptor is expressed in ossification zones of the mouse embryo (Bonnelye *et al.* 1997a) and up-regulates the expression of the osteopontin gene (Vanacker *et al.* 1998) coding for an extracellular matrix protein involved in bone remodeling. More recently, Bonnelye *et al.* (2001) have found that ERRα plays a role in *in vitro* osteoprogenitor cell proliferation and differentiation. Both ERRα and ERRγ are also highly expressed in muscle, heart and adipose tissues (Bonnelye *et al.* 1997b, Hong *et al.* 1999, Heard *et al.* 2000). In adipose tissues, ERRα has been suggested to participate in the control of
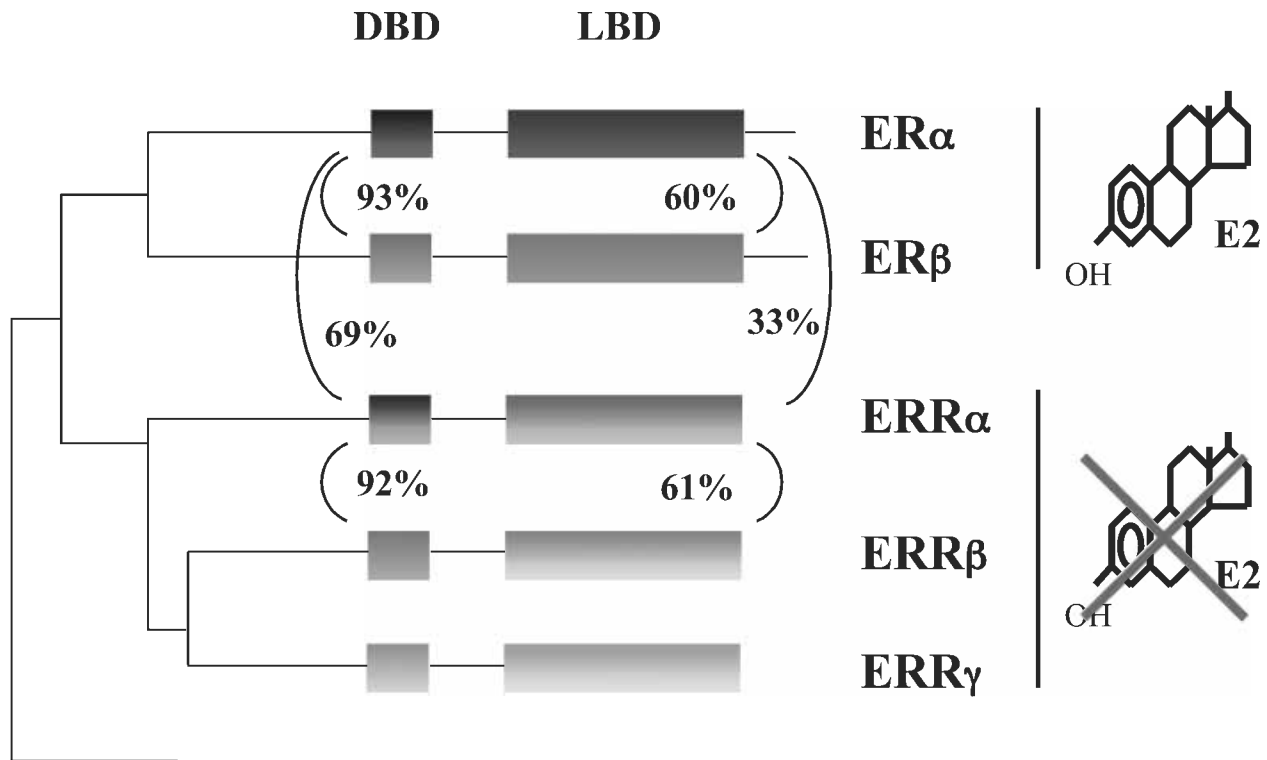
Figure 1 The ER/ERR subfamily A schematic representation of the two mouse ERs and the three mouse ERRs is displayed. Percentage of sequence identity within the DBD and the LBD is indicated.

energy expenditure through the regulation of the expression of the MCAD (medium-chain acyl dehydrogenase) gene (Sladek *et al.* 1997, Vega & Kelly 1997). In the central nervous system, both ERRα and ERRγ possess a complex pattern of expression (Bonnelye *et al.* 1997b, Hermans-Borgmeyer *et al.* 2000, Lorke *et al.* 2000) but no function has yet been attributed to the receptors. ERRβ is mainly expressed during early mouse development (Pettersson *et al.* 1996) where it is necessary for proper trophoblastic cell proliferation and differentiation as demonstrated by the placentation defects observed in mice lacking ERRβ (Luo *et al.* 1997).

A natural ligand for ERRs?

ERRs bind as homodimers to at least two types of target DNA sequence, the estrogen response element (ERE: AGGTCAnnnTGACCT) and an extended half site originally characterized as SF1 (steroidogenic factor 1, another orphan NR) response element (SFRE: TCAAGGTCA) (Pettersson *et al.* 1996, Bonnelye *et al.* 1997a, Johnston *et al.* 1997, Hong *et al.* 1999, Vanacker *et al.* 1999b, Hentschke *et al.* 2002a). Transcriptional activation through each of these sites occurs in an apparent ligand-independent manner as no external compound needs to be added to the culture medium. However, at least in the case of ERRα, these transcriptional activities are exerted in a cell-specific manner (Bonnelye *et al.* 1997b). This could indicate the requirement for a ligand present in the permissive cells and not in the restrictive ones. In support of this hypothesis, rat osteosarcoma ROS 17·2/8 cells cultured for 2 weeks with charcoal-treated serum (i.e. depleted of lipophilic compounds including hormones) do not support ERR-driven transcriptional activation in contrast to cells cultured in normal medium (Vanacker *et al.* 1999b). However, since the normal medium could promote ligand-unrelated events (e.g. activating phosphorylation cascades), identification of the active compound will be necessary in order to conclude that a ligand exists.
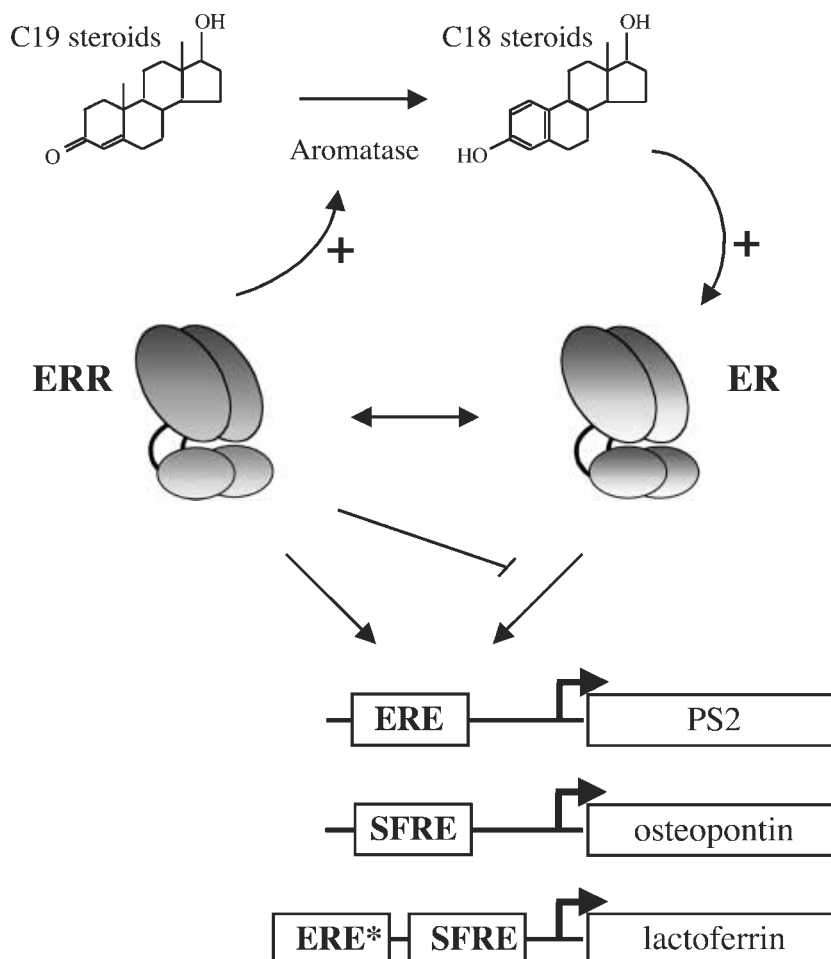
Figure 2 Interconnections between ER and ERRs. Interplay between ER and ERR includes physical contacts and transcriptional regulation of common target genes. Examples of such are the pS2 gene, osteopontin and lactoferrin, on which a perfect SFRE and an imperfect ERE (ERE*) have been identified. ERR can act as a transcriptional regulator *per se* or compete with ER for common binding sites. ERR also regulates the expression of the aromatase gene, whose product converts C19 (androgens) to C18 (estrogens) steroids such as estradiol-17β, the most active natural ER ligand.

Alternatively the cell-dependent transcriptional activities of the ERRs could also reflect the presence or absence of a specific transcriptional coactivator. In this respect, ERRs have recently been shown to interact with members of the PGC-1 coactivator family (Hentschke *et al.* 2002*b*, Huss *et al.* 2002, Ichida *et al.* 2002, Schreiber *et al.* 2003), the expression of which is restricted to certain tissues and cells (Puigserver *et al.* 1998, reviewed in Knutti & Kralli 2001). Orphan ERRs can also be coactivated by the widely expressed members of the

p160 family, at least in transient transfection assays (Hong *et al.* 1999, Xie *et al.* 1999, Zhang & Teng 2000). Whether this is also true *in vivo* remains to be investigated. *In vitro* experiments have demonstrated that interactions between ERR proteins and coactivators do not require the addition of any external compound. Since, in the case of classic receptors, this type of interaction is ligand-dependent, this suggests that ERRs are constitutive transcription factors. Nevertheless it cannot be formally excluded that a ligand is present and

incorporated when the ERRs are translated *in vitro* or in bacteria. In this respect, the presence of certain fatty acids in the ligand-binding pocket of bacterially synthesized RAR (retinoic acid receptor) has been demonstrated by 3D structure determination (Bourguet *et al.* 2000). As a consequence, RAR is locked in an active conformation by the fatty acid moiety, which acts as a ligand. On the contrary, 3D structure determination of the LBD of ERRγ has shown that this domain spontaneously (i.e. without any fortuitous ligand) adopted an active conformation (Greschik *et al.* 2002). These experiments have revealed the existence of a hydrophobic pocket that is, to date, the smallest one identified in NRs (roughly half the volume of that of ERα). As a consequence, putative agonist ligands that could further stabilize ERRγ in an active conformation should be smaller that half the size of E2. However, the reduced size of the hydrophobic cavity does not seem to be the major determinant of ERRγ's constitutive activity. Mutants displaying an enlarged putative ligand-binding pocket (i.e. in which protruding amino acid side-chains were removed) are still transcriptionally active in a ligand-independent manner. Noteworthy, some of these mutants bind E2 but are not regulated by this hormone. Altogether this suggests that the overall LBD structure contributes to the spontaneous active conformation.

Crystallographic structures of ERRα and ERRβ have not been determined. The close sequence proximity of ERRβ and ERRγ suggests identical transcriptional properties for both receptors (i.e. ligand-independence). ERRα's LBD is more divergent. Computer-assisted modeling has been performed for this receptor using its similarity to ERα (Chen *et al.* 2001). A striking difference is the presence of a phenylalanine side-chain across the binding pocket of ERRα where an alanine is found in ERα. The phenylalanine residue is thought to lock the LBD in an active conformation, since mutating this residue to an alanine results in a dominant negative mutant. This finding has also led to the conclusion that no steroid ligand (E2 in particular) could enter the binding pocket. However, ligands can adapt to the particular constraints of a given ligand-binding pocket. This is, for instance, the case for 9-cis retinoic acid that adopts different conformations when bound to RARγ or retinoid X receptor α (Egea *et al.* 2000). Furthermore, the above-mentioned phenylalanine

residue is not conserved throughout evolution: the single amphioxus ERR and the zebrafish ERR versions bear an alanine at the equivalent position (P-L Bardet, B Horard & J-M Vanacker, unpublished observations). These receptors act as *bona fide* transcription activating factors and in spite of a greater proximity to ERα's LBD, these ERR orthologs are still not activated by E2.

What could be the nature of an eventual endogenous agonist ligand of ERRs, in particular of ERRα, for which the question is more open that for ERRβ/γ? The expression of aromatase, which converts C19- to C18-steroids, is stimulated by ERRα. It could be that this up-regulation is necessary to clear the cells from an unwelcome compound by enhancing its degradation. By analogy with the rodent LXR, whose potentially toxic ligands promote their own degradation by activating the expression of a catabolic enzyme (reviewed in Repa & Mangelsdorf 2000), one can imagine that a substrate (or precursor thereof) of the aromatase could act as an ERRα ligand, a hypothesis which remains to be tested.

Synthetic ligands for ERRs

Reports have been published identifying synthetic modulators of the activities of the ERRs. Strikingly, all these compounds are more or less connected to estrogen signaling and repress ERRs' transcriptional activities. They can be thus considered as antagonists (if ERRs possess natural ligands) or inverse agonists (if ERRs' transcriptional activities are truly constitutive). Two organic pesticides, toxaphene and chlordane, are indeed endocrine disrupters that can display weak estrogen-like activities. Both these molecules have been shown to down-modulate the transcriptional activities of ERRα, probably by impairing its interactions with coactivators such as GRIP1 (Yang & Chen 1999).

Diethylstilbestrol (DES)

DES, a synthetic estrogen, was widely used between the 1940s and the 1970s to prevent spontaneous abortion in women. However, patients exposed *in utero* to high doses of DES have a range of gynecological troubles, including a higher incidence of vaginal cancers and malformations of the reproductive tract. These phenotypes closely
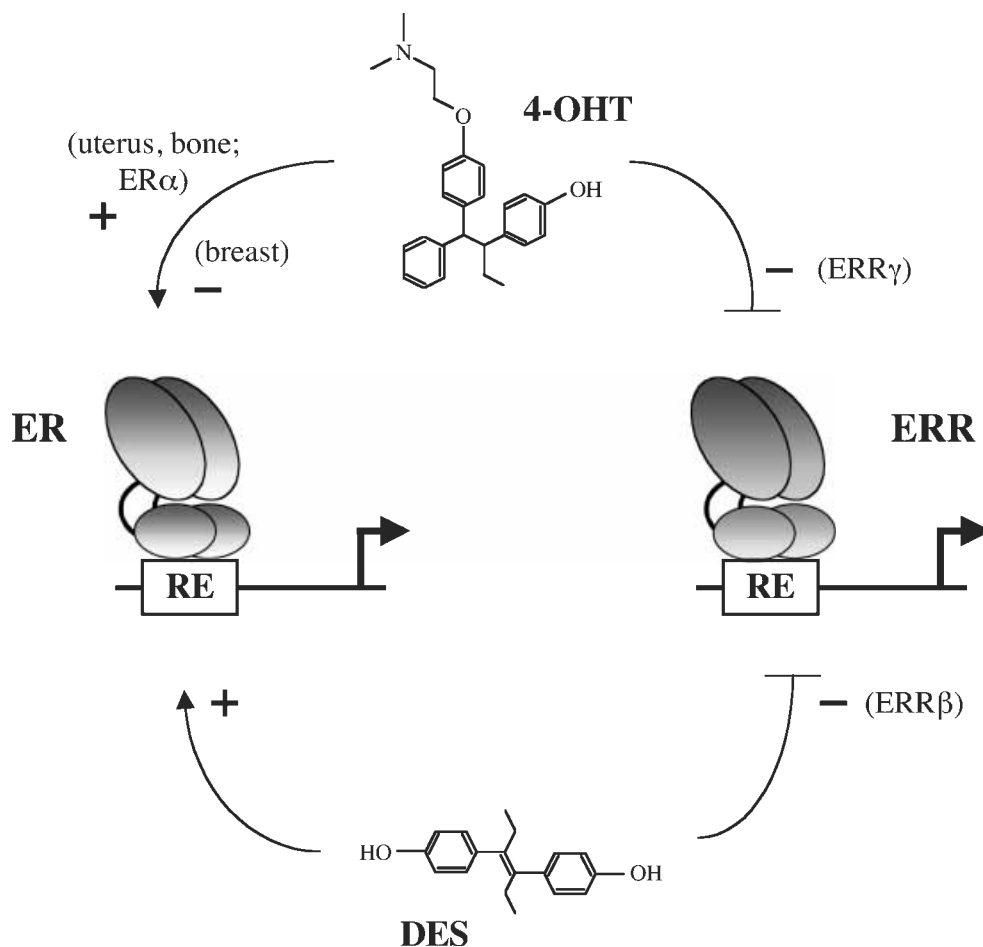
Figure 3 Contrasting effects of SERMs on ERs and ERRs. 4-OHT activates ERα in the uterus and bone, but acts as an estrogen antagonist in breast. 4-OHT also deactivates ERRγ. DES is a potent ER agonist and inhibits ERRβ's transcriptional activities. RE: response element. References are given in the text.

resemble those observed in null mouse models of some Wnt gene family members (Miller & Sassoon 1998, Parr & McMahon 1998). These data raised the proposition that disruption of developmental gene expression accounts for the DES syndrome. On this line, it was effectively shown that DES represses the expression of Wnt7a when administered perinatally (Miller *et al.* 1998). DES is a potent estrogen agonist, it can therefore be intuitively thought to act through ER and mimic the broad spectrum of E2 action. This view has recently been challenged by the finding that DES also represses the molecular activities of ERRs (Fig. 3), such as interaction with a coactivator fragment and reporter gene transactivation (Tremblay *et al.*

2001). Furthermore, administration of DES to pregnant mice partially phenocopies the absence of ERRβ, resulting in an absence of labyrinth and spongiotrophoblast layers and an increase in the giant cell layer. However, it should be noted that the DES concentration necessary to achieve a half-maximal inhibition of ERR activities (1–10 μM range, depending on the assay) is elevated compared with its ER-activating potential (nanomolar) (Kuiper *et al.* 1997). In another study, DES was independently confirmed to bind to all three ERRs but did not modify any of the receptors' transcriptional effects in cell-based assays (Coward *et al.* 2001), which could reflect cell-specific differences in the action of this compound.

Tamoxifen (TAM) and its derivative 4-OH tamoxifen (4-OHT)

Coward *et al.* (2001) also show that TAM and its derivative 4-OHT disrupt the interactions between ERRβ or ERRγ and a SRC-1-originating peptide *in vitro*. TAM is a selective ER modulator (SERM), and as such displays estrogenic or antiestrogenic actions according to the target tissue (see Cosman & Lindsay 1999, Lonard & Smith 2002 for reviews). In particular, TAM acts as an estrogen antagonist in the mammary gland and is clinically used for the treatment of breast cancer. In cell-based assays, only 4-OHT, but not TAM, acts as an antagonist and only vis-à-vis ERRγ (Coward *et al.* 2001). 3D structure analysis of ERRγ predicts that 4-OHT could be fitted into its ligand-binding cavity only if one assumes an antagonist conformation of the LBD (Greschik *et al.* 2002). Again a micromolar range is necessary to obtain half-maximal inhibition in transcriptional assay (Coward *et al.* 2001) whereas nanomolar concentrations deactivate ERβ (Kuiper *et al.* 1997). This micromolar concentration could still be pharmacologically relevant given the doses administered in breast cancer treatment. Along this line, a recent publication has documented a correlation of expression between ERα and ERRγ in breast cancer patients (Ariazi *et al.* 2002). Although performed on a low number of samples, this study proposed ERRγ as a favorable prognostic factor for breast tumor outcome. Altogether this could also suggest that some of the antitumor effects of TAM might be mediated by ERRγ. This hypothesis is to be tested, as it remains to be investigated whether ER and ERRγ share target genes that can be regulated by 4-OHT. It will be also of the utmost importance to define the role of ERRγ in cellular proliferation in the context of breast cancer.

Conclusion

Orphan ERRs have been suggested to interfere with estrogen signaling at various levels. It could therefore be expected that compounds displaying estrogen agonist or antagonist activities should also regulate ERR transcriptional activities. Inverse agonists of ERRβ/γ have been identified. Although the *in vivo* effects of DES and 4-OHT on ERRβ/γ remain to be documented, these discoveries, taken together with the availability of ERRγ's 3D structure, offer promising perspectives in the pharmacology of these receptors. In contrast, no ERRα-interacting molecule has been identified to date that is relevant in cell-based assays (Coward *et al.* 2001). Expression of this receptor in human breast tumor was recently shown to be inversely correlated to estrogen responsiveness (Ariazi *et al.* 2002). In contrast to ERRγ, ERRα has thus been proposed as an unfavorable breast tumor marker which may activate estrogen-responsive targets in an estrogen-independent manner (as has been shown for the pS2 gene). Estrogen-unresponsive breast tumors often appear as relapse after a period of remission following 4-OHT treatment. These tumors represent a crucial problem, since per definition no antiestrogen-based treatment can be efficient. Confirming the expression of ERRα in these tumors, together with determining its effects on cell proliferation, could therefore lead to the proposal of ERRα as a new therapeutic target to design treatments against estrogen-independent tumors. This single example illustrates the importance of finding a regulator of the activity of ERRα. In other words, ERRα needs to meet a ligand, be it natural or synthetic.

Acknowledgements

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