Molecular Mechanisms of Estrogen Receptor Alpha inactivation by Antiestrogens

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À vous tous qui avez subit les conséquences de mes choix.

ABSTRACT:

Due to the prominent role of estrogen in breast tumor growth, antiestrogens have been developed to inactivate its signaling pathway, which is dependent on estrogen receptor alpha (ERα). Selective estrogen receptor modulators (SERMs), such as Tamoxifen (Tam) and Raloxifene (Ral), are mainly antiestrogenic in mammary tissues but estrogenic in others. However, full antiestrogens such as ICI182,780 inactivate ERα in all tissues. Crystallographic structures indicate that the side chains of SERMs displace helix 12 (H12) of ERα ligand-binding domain (LBD) into the coactivator-binding groove, while those of full antiestrogens prevent its stable association. To facilitate the development of novel ERα inhibitors, we investigated the molecular mechanisms underlying partial/full antiestrogenicity. Both Tam and Ral side chains contain a tertiary amine. We demonstrated that this tertiary amine is not required for Tam's antagonist activity, but that the presence of a free negative charge is required for partial agonist activity. Investigating the molecular determinants of receptor down-regulation induced by antiestrogens, we demonstrated that long hydrophobic residues of H12 play an important role both in accumulation of ERα in an insoluble fraction and in transcriptional repression by antiestrogens. Both effects appear due to steric hindrance between antiestrogen side chains and residues L536, L539 (Ral and ICI182,780) and L540 (ICI182,780) found in the N-terminus of H12. While inhibition of proteasome-dependent degradation did not increase ERα-dependent transcription in the presence of ICI182,780, it also failed to restore levels of soluble receptor similar to untreated controls. Finally, we mapped the lysine (Lys) residues targeted in the ligand-dependent and independent ubiquitination of ERα to the C-terminal half of the receptor and initiated a systematic mutagenesis of Lys residues in this domain in order to identify ubiquitination sites in the presence of agonists or antagonists. Overall, this research clarifies the importance of the side chain of partial as well as full antiestrogens for their antagonist

capacity. It also identifies specific residues within ERα LBD that contribute to the antagonist actions of antiestrogens or to their partial agonist activity. Finally, identification of the site(s) of receptor ubiquitination should clarify the importance of the ubiquitin/proteasome pathway in modulating ERα transcriptional activation properties.

RÉSUMÉ:

Dû au rôle prépondérant des estrogènes dans le développement de tumeurs mammaires dépendant du recepteur des estrogènes alpha (ERα), plusieurs antiestrogènes ont été développés afin d'inactiver ses voies de signalisation. Les modulateurs sélectifs des ERs (MSREs), tels le tamoxifène (Tam) et le raloxifène (Ral), agissent de façon antagoniste principalement dans le tissu mammaire et de façon agoniste dans d'autres tissus. Par contre, les antiestrogènes totaux, tels le ICI182,780, inactive le récepteur dans tous les tissus. Les structures crystallographiques du récepteur lié aux divers antagonistes, démontrent que la chaine latérale des MSREs déplace l'hélice 12 (H12) du domaine de liaison du ligand (DLL) du récepteur dans le sillon de liaison aux coactivateurs, alors que les antiestrogènes totaux préviennent l'association stable de H12 avec le DLL. Afin de faciliter le développement de nouveaux inhibiteurs du ERα, nous avons étudié les mécanismes moléculaires régissant l'activité agoniste-partielle et l'activité antagoniste totale des antiestrogènes. Une amine tertiaire se retrouve dans la chaîne latérale du Tam et du Ral. Nous avons démontré que cette amine tertiaire n'est pas requise pour l'activité antagoniste de Tam, mais la charge négative en position 351 est requise pour l'activité agoniste-partielle. Grâce à l'étude des déterminants moléculaires régissant la régulation négative du ERα par les antiestrogènes, nous avons établi l'importance des longs résidus hydrophobes de H12 pour l'accumulation du ERα dans une fraction insoluble et pour son inactivation transcriptionelle par les antiestrogènes. Ces effets étant dépendants des résidus L536, L539 (Ral et ICI182,780) et L540 (ICI182,780) retrouvés dans la portion N-terminale de H12. Alors que l'inhibition du protéasome ne permet pas une augmentation de la transcription dépendante du ERα en présence de ICI182,780, elle ne me permet pas de restaurer les niveaux de ERα soluble tels que perçus en absence de traitement. Finalement, nous avons demontré l'importance de la région C-terminale pour l'ubiquitination du ERα en absence et en

présence de ligands. Également, nous avons initié une mutagénèse systématique des Lys dans cette région afin d'identifier les sites d'ubiquitinations en présence d'agonistes et d'antagonistes. Globalement, ces recherches clarifient l'importance de la chaîne latérale dans la médiation de l'activité antagoniste des antiestrogènes partiels et totaux. Nos travaux de recherche permettent également l'identification de résidues spécifiques au sein du DLL du ERα qui contribuent à l'action antagoniste des antiestrogènes ou à leur activité agoniste partielle. Finallement, l'identification de sites d'ubiquitination du récepteur devrait contribuer à élucider l'importance de la voix ubiquitine/protéasome dans la modulation des propriétés d'activation transcriptionnelle du ERα.

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ABBREVIATIONS:

A.A.: amino acid

AE : antiestrogen

AF1 : activation function 1

AF2 : activation function 2

AR : androgen receptor

CBG : coactivator-binding groove

DBD : DNA binding domain

e1 : ubiquitin activating enzyme

e2 : ubiquitin conjugating enzyme

E2 : 17beta-estradiol

e3 : Ubiquitin protein ligase

EDTA : ethylene diamine tetra acetic acid

EGF : epidermal growth factor

ER : estrogen receptor

ERα : estrogen receptor alpha

ERβ : estrogen receptor beta

ERE : estrogen response element

ERKO : estrogen receptor alpha knockout

FBS : Fetal bovine serum

FSK : Forskolin

GFP^2-Ubi : Green fluorescent protein-ubiquitin, lysine 48 and 63 mutated to alanine

GFP^2-Ubi_{AA} : Green fluorescent protein-ubiquitin, glycine 75 and 76 mutated to alanine.

GR : glucocorticoid receptor

H12 : Helix 12

HERS : Heart and Estrogen/Progestin Replacement Study

HSB : High-salt buffer extracts

ICI : ICI182,780

LBD : ligand-binding domain

Luc : luciferase

MAPK : Mitogen Activated Protein Kinase

MR : mineralocorticoid receptor

MCF7: Michigan Cancer Foundation -7

NEM : N-ethylmaleimide

NR boxes : nuclear receptor boxes

NR : nuclear receptor

OHT : 4-hydroxytamoxifen

PBS : Phosphate buffered saline

PI3K : Phosphatidyl-Inositol-3 Kinase

PKA : Protein Kinase A

Pol II : RNA polymerase II

PPARα : peroxisomal proliferator-activated receptor alpha

PPARγ: peroxisomal proliferator-activated gamma

PR : progesterone receptor

Ral : Raloxifene

RAR : retinoic acid receptor

RXR : retinoid X receptor

SERM : selective estrogen receptor modulator

SRA : steroid receptor RNA activator

STAR : study of tamoxifen and raloxifene

STEAR : selective tissue estrogenic activity regulator

Tam : tamoxifen

TR : thyroid receptor

Uba3: ubiquitin activating enzyme 3

VDR : vitamin D receptor

WHI : Women's Health Initiative

wt : wild-type

REMERCIEMENTS:

C'est enfin terminé!

Cette thèse découle de nombreuses heures, réparties sur plus de quatre ans et demi, dévouées à l'avancement des connaissances selon une approche scientifique des plus exostive. De multiples embûches ont freiné la progression de mes travaux. La première fut l'insuccès des projets de recherches qui dura pendant un peu plus d'un an. Par la suite une périodes riches en résultats donna l'espoir d'une publication. Malheureusement la deuxième embûche réduit cet espoir à presque rien. En effet mes résultats furent plubliés par un autre groupe de recherche. L'optimisme, le positivisme, et l'acharnement au travail inculqué par mes proches me permis de rebondir. Les travaux que j'accomplie par la suite, à un rythme effréné, permire l'écriture d'un premier manuscrit. L'inévitable se devant d'arriver due à une surcharge de travail pour mon corps, je me retrouva à l'urgence. Malgré l'incapacité des médecins à trouver la sources de mes problèmes physiques, mes capacités de travail furent réduites pendant les dernières années de ma thèse. Vous pouvez donc imaginer à quel point je suis soulagé de voir la fin de cette formation académique. De plus, je suis très satisfait de mes réalisations. En effet, mes années de thèse mon permis l'écriture de trois manuscrits de recherche novatrice apportant une masse de connaissances supplémentaires qui, je l'espère contribueront significativement à l'avancement de la recherche. J'ai également écris un manuscrits de résumé de recherche. La communauté scientifiques saura juger de l'importance de mes travaux pour en assurer la pérénnité.

Je vous remercie,

Je me dois de souligner la contribution extraordinaire des gens qui m'ont entourés dès le début et qui m'ont supportés tout au long des mes épreuves. En premier lieu, J'aimerais souligner l'apport de ma directrice

de thèse, le Dr. Sylvie Mader. Grâce à mon séjour dans son laboratoire et à ses nombreux conseils, j'ai perfectionné ma rigueur au travail tout en pratiquant de nouvelles approches scientifiques. Le Dr. Mader m'a également démontré la faisabilité des collaborations saines dans le milieu scientifique. Du point de vue personnel, j'ai apprécié les nombreuses conversations scientifiques et non-scientifiques que nous avons eux, un débat d'idées bien argumenté étant toujours bienvenue. Merci donc pour tout les bons conseils et pour les encouragements. Mes recherches ont également été soutenues par les membres du laboratoire. M.Sc Anick Auger, M.Sc Martine Bail, Dr. Véronique Bourdeau, Samuel Chagnon, M.Sc Julie Deschênes, M.D. Shahrooz Emtiazjoo, M.Sc David Ferland-McColough, Elise Hébert, Gricha Hervouet-Zeiber, M.Sc Gisèle Marguier, M.Sc Denis Nguyen, Geneviève-Anne Pinard et M.Sc Walter Rocha, j'ai grandement apprécié votre compagnie. Particulièrement, j'aimerais souligner le travail d'Anick, Geneviève-Anne, Elise et Samuel qui ont directement contribués à ma recherche. Un salut également a Walter Rocha, mon confrère de labo depuis le début de ma thèse, avec qui j'ai eu les discussions les plus enrichissantes au courant des dernières années. Plusieurs personnes de l'extérieur du laboratoire doivent également être mentionnées. Je pense ici à Sandrine Crabé qui a toujours su me surprendre agréablement par sa franchise et son franc parler. Je pense aussi à Louise Cournoyer, toujours très accueillante et partante pour toute discussion. Le MCETC m'a également supporter financièrement à travers une bourse de 17 500 dollars étalés sur deux ans.

Le support de plusieurs proche doit tout autant être mentionné. Premièrement, je te remercie Dr. Geneviève. Notre mariage reste à se jour le plus beau moment de ma vie. A chaque instant tu le rends plus enrichissant. Tu m'as beaucoup apporté afin que je puisse compléter cette thèse. Non seulement as-tu enduré mes horaires du temps mais tu as également su contribuer à mes travaux et à la rédaction de me thèse. Je te remercie aussi pour tout l'amour que tu m'offres, malgré notre pauvreté

financière, tu as su augmenter notre richesse affective. Comme je dirais si je me prenais pour Darwin, tu es définitivement mon Emma. Je tiens également à souligner l'appuie inconditionnel de mes parents Carole et Maître Pierre ainsi que de mon frère Ing. Patrick et son épouse Marie-Josée. Merci de m'avoir supporté et d'avoir crue en mes capacités. J'espère pouvoir en offrir autant à Aimée et Fée. Je remercie aussi ma belle-famille, Denise, Ing. Raymond, Dr. Anne, M.D.Veronique, MBA Mike et "Trivial Pursuit©, Tribond©, etc " Mike. Vous êtes parmis les rares à avoir du soutenir trois thèsart. Vous en aurez peut-être d'autre à soutenir en Kiara, Zoé et ....

Ce qui m'a coûté le plus au courant de ces années fut l'éloignement de mes amis. Malgré tout, je sais que je peux compter sur vous Pascal, Marylin, Sébastien, Isabelle, Simon C., Michelle, Mathieu, Lindsay, Francis, Karen, Geneviève T. Jocelyn, François, Marc, Julie, Catherine, Eric, Simon B. et Edith. Je tiens à souligner d'avantage les trois belligérants par excellence, Simon C. Sébastien et Pascal. Je vous le répète ici, vous êtes mes phares et mes balises, sans vous je ne suis qu'une épave en devenir.

GUIDELINES FOR THESIS PREPARATION:

As stated in the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts* that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (1) a table of contents; (2) a brief abstract in both English and French; (3) an introduction which clearly states the rational and objectives of the research; (4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (5) a final conclusion and summary; (6) a thorough bibliography; (7) Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

* Note: connecting texts that provide the logical bridges between each chapter in this thesis were presented between each chapter requiring such a connection.

CONTRIBUTION OF AUTHORS:

Dr. Sylvie Mader was the candidate's research and thesis supervisor for the past 4 ½ years. She provided scientific direction to the candidate and assisted in the conception and interpretation of the experiments and in the writing of three original research manuscripts. Hence, Dr. Sylvie Mader is co-author on all research manuscripts included in this dissertation.

ORIGINAL RESEARCH ARTICLES

CHAPTER 2. MODE OF ACTION OF ANTIESTROGENS (A REVIEW)

Mathieu Lupien and Sylvie Mader. (in preparation)

M Lupien, wrote the entire manuscript and prepared all figures including the schematic representation of antiestrogen modes of action (Figure 2.1), of antiestrogen structure (Figure 2.2) and of the structural modifications imposed on the estrogen receptor alpha by agonists and antagonists (Figure 2.3) based on crystal structures available from the protein data bank (web: http://www.rcsb.org/pdb/; accession name 1HJ1 for ICI164,384-bound ERβ, 3ERD for the agonist bound ERα stucture in presence of the GRIP1 peptide and 3ERT for the OHT-bound ERα structure.

CHAPTER 3. DIFFERENCES BETWEEN TAMOXIFEN AND RALOXIFENE IN THEIR FUNCTIONAL INTERACTIONS WITH ASPARTATE 351 OF ESTROGEN RECEPTOR ALPHA.

Guila Dayan, Mathieu Lupien, Anick Auger, Sylvia I Anghel, Walter Rocha, Sébastien Croisetière, John A Katzenellenbogen and Sylvie Mader. (2005) ready for submission to Mol. Endocrinol.

G. Dayan conceived and executed the experiments leading to figures 3.2A, 3.4, contributed to the data analysis of all figures and to the writing of the manuscript.

M. Lupien was second author and performed the experiments and the data analysis of figures 3.5, 3.6, 3.8 and 3.9. He also assisted in the analysis of all figures and contributed to the writing of the manuscript.

A. Auger assisted both M. Lupien and G. Dayan in their work and in the data analysis for all figures and table 3.1.

S I. Anghel performed preliminary work with the D351X mutants.

W. Rocha performed the alkaline phosphatase experiments and the data analysis of figure 3.2B and C.

S. Croisetière carried out preliminary experiments on cellular growth leading to figure 3.2A.

J. A. Katzenellenbogen provided the various tamoxifen derivatives used in this study.

CHAPTER 4. ROLE OF AF-2 HELIX 12 POSITIONING IN TRANSCRIPTIONAL REPRESSION OF ESTROGEN RECEPTOR ALPHA BY FULL ANTIESTROGENS

Mathieu Lupien, Anick Auger, Guila Dayan, Geneviève-Anne Pinard, Sylvia I Anghel, Caroline Loch, Jean-Marie Wurtz, Dinos Moras and Sylvie Mader. (2005) ready for submission to Mol. Endocrinol.

M. Lupien conceived, planned and coordinated all of the studies described in this manuscript under Dr. Mader's supervision. He performed the mutagenesis of all hydrophobic mutants in the helix 12 of the ERα LBD and all transactivation and degradation assays as well as the data analysis. He supervised the work performed by the research associate A. Auger. He also assisted in the modeling and the analysis that followed. M. Lupien prepared all the figures and contributed to the writing of the manuscript.

A. Auger assisted M. Lupien in the transfection of cells for the transactivation and degradation assays leading to figures 4.3A-B, 4.6, 4.7A-B and 4.8.

G. Dayan performed preliminary work on the D351X mutants in HeLa cells that served in the development of the project.

G-A Pinard was trained by M. Lupien during her internship in Dr. Mader laboratory. She assisted M. Lupien in the transactivation and degradation assays of the ΔAB ERα mutants leading to figure 4.8.

S. I Anghel performed preliminary work on the D351 mutants.

C. Loch performed the modeling in the laboratory of D. Moras under the supervision of J-M. Wurtz leading to figure 4.4 and 4.5.

J-M. Wurtz supervised C. Loch in the modeling studies and assisted in the analysis of the modeling data. He also contributed to the revision of the manuscript.

D. Moras collaborated in the modeling experiments through the supervision of Dr. J-M Wurtz.

CHAPTER 5. Both agonist and antagonist-dependent ERα ubiquitination and degradation are dependent on C-terminal lysine residues.

Mathieu Lupien, Xavier Mascle, Muriel Aubry and Sylvie Mader. (2005)

M. Lupien conceived, planned and coordinated all of the studies described in this manuscript. He performed all the transactivation, degradation and ubiquitination assays in figure 5.1, 5.2, 5.3 and 5.4. As well, he analysed the data from all figures. He coordinated and supervised the work of Xavier Mascle. He prepared all figures and wrote the manuscript.

X. Mascle contributed to the experiments resulting in figure 5.2B-D and 5.4C.

M Aubry, supervised the work of Xavier Mascle as his research director.

CLAIMS FOR ORIGINALITY:

This dissertation furthers the knowledge concerning the mechanisms governing ERα inactivation by antiestrogens. Overall, It demonstrates the importance of specific interactions between the antiestrogen side chain and residues in ERα LBD to attain receptor inactivation highlighting direct targets for future antiestrogen development. Similarly, the work presented in this thesis demonstrates that full antiestrogens sequester the receptor in an insoluble fraction. It also demonstrates that inhibition of the proteasome-dependent degradation pathway is insufficient to reduce the antagonist activity of antiestrogens and that ERα ubiquitination is dependent on its C-terminus.

SPECIFIC CLAIMS FOR ORIGINALITY:

CHAPTER 3: This chapter is the first to establish the role of the tertiary amine in the side chain of tamoxifen for its antagonist and partial agonist actions. It also characterises a novel compound, compound **9**, with Ral-like actions.

Previous work as demonstrated that the tertiary amine in the side chain of the selective estrogen receptor modulator (SERM) Raloxifene (Ral) is required for its antagonist properties. Through its interaction with the negatively charged residue D351 found in helix H3 of the ERα ligand bindin domain (LBD), this tertiary amine contributes to the stabilisation of Ral side chain in a position that prevents ERα from adopting an active conformation. The antagonist potential of Tamoxifen (Tam), another SERM member, is also influenced by the amino acid D351. As Tam side chain also contains a tertiary amine, we decided to investigate its importance for the inactivation of the ERα. Interestingly, Tam demonstrates a partial agonist activity in certain tissues. We therefore also investigated the impact of Tams' tertiary amine on its agonist activity. We

established that the tertiary amine found in Tam side chain is not essential for its antagonist actions. Tam side chain is therefore not stabilized as for Ral through direct interaction with D351. Our results suggest that in presence of Tam the role of D351 is not to stabilise this antiestrogen side chain but rather to stabilise ERα LBD in a conformation typical of an activation function 2 (AF2) inactivation but that allows for the partial agonist activity dependent on the activation function 1 (AF1) of the receptor. Furthermore, the results presented in this chapter characterize the novel compound **9** as having Ral-like properties in terms of ERα inactivation. Due to the low bioavailability of Ral, future research may establish compound **9** as a functional replacement.

CHAPTER 4: This chapter identifies the importance for full antiestrogens to remove the ERα from the soluble fraction in order to completely inactivate it. It also demonstrates for the first time that proteasome-mediated degradation of ERα in presence of antiestrogens is not required for receptor inactivation. Finally, the work presented in this chapter allowed the identification of specific residues within the receptor LBD that can be targeted by the antiestrogen side chain in order to attain complete receptor inactivation.

Previous reports have demonstrated the capacity of full antiestrogens to lead to ERα elimination, namely through degradation of the protein. In the course of cancer progression numerous cancererous cells have an altered capacity to degrade proteins through the proteasome. We therefore investigated the importance of ERα degradation in presence of full antiestrogens for their antagonist actions. Using mutants of ERα that were no longer degraded by antiestrogens we demonstrated that these compounds maintained their antagonist activity. However, certain mutants, no longer degraded, actually abrogated the antagonist action of the antiestrogens ICI182,780 and Ral and actually demonstrated agonist activity. We demonstrated that this activity was

dependent on the AF1 function. Modeling studies highlighted the structural mechanisms of action of antiestrogens. It demonstrated that the side chain of the antiestrogens could inactivate the receptor by establishing a steric conflict with residues L536, L539 or L540 found in the Helix 12 of ERα ligand binding domain (LBD). The identification of these key residues offers specific targets in the development of novel antiestrogens.

CHAPTER 5: This chapter demonstrated the need for an active 26S proteasome for efficient transactivation of the ERα in presence of estrogen and 4-hydroxytamoxifen (OHT) and establishes that proteasome inhibition does not increase ERα transcriptional activity in presence of full antiestrogens. It also characterises the ubiquitination of ERα in presence of estrogen and antiestrogens and establishes the requirement for the C-terminal end of the receptor for its degradation and ubiquitination.

Multiple reports have demonstrated the importance of ERα degradation by the proteasome for efficient transactivation. Recent reports have indicated that this mechanism is involved in the first 6-8 hours of transactivation following agonist exposure. Although some have reported indirectly ERα ubiquitination, a single report has demonstrated direct ERα ubiquitination. Thus, there is still a debate as to whether the receptor is indeed ubiquitinated. Therefore, we established ERα ubiquitination in absence of presence of ligand through the direct attachment of a GFP^2-Ubiquitin fusion protein to ERα. Interestingly, ICI182,780 and raloxifene which behave as full antiestrogens in our assays, induced the strongest ubiquitination of the receptor. As ubiquitination occurs on lysine residues of the target protein we undertook the identification of this residue. We first demonstrated that ERα C-terminus is required for ubiquitination under all conditions. We next performed an arginine scanning mutagenesis of the lysine residues in this region. Preliminary experiments did not allow for the identification of a single lysine residue responsible for regulating ERα

transcriptional activity in presence of agonists or antagonists. More mutants are presently being tested.

PROLOGUE

« On va toujours trop loin pour les gens qui vont nulle part »

Pierre Falardeau

Any introduction to a thesis on nuclear receptors can only hope but never actually do justice to this vast field of research. I have attempted to present to the best of my knowledge the literature that best describes the field of estrogen signaling that pertains to the topic of this thesis while taking into account the abundant information ascribed to all members of the nuclear receptor family. Therefore, I apologize to all whose work was omitted from this thesis either intentionally, due to space limitations, or unintentionally, due to oversight. Furthermore, even though this research tried to remain unbiased in its design as well as in its analysis, I realize that all members of the scientific community may not accept my final theories derived from this work. As for all theories, they are valid only until disproven and the scientific exercise is to constantly try to disprove them. My only hope is that the readers of this thesis will enjoy the scientific validity of the work performed as much as I enjoyed applying its principles.

Chapter 1: The physiological functions of estrogens and their mode of action

1.1) Estrogen production in pre- and post-menopausal women

Estrogens are lipophilic hormones of the steroid class characterized by a phenolic A ring, mainly produced by the ovarian granulosa cells in premenopausal women which acts on numerous distal target tissues (1). In postmenopausal women, circulating levels of estrogens are much lower and are produced predominantly from a number of extragonadal sites such as brain, bone and adipose tissues as well as skin fibroblasts. At these sites, estrogens act mainly in a paracrine or intracrine fashion (2-5), and their local concentration can reach much higher levels than those found in the blood stream.

The biosynthesis of estrogens in extra-ovarian tissues as well as in ovarian granulosa cells is dependent on the aromatase, a member of the cytochrome P450 superfamily. This protein, encoded by the CYP19 gene (6), binds the C19 androgenic steroid substrates (androstenedione and testosterone) and catalyzes a series of reactions leading to estrogen production (Fig. 1.1). This process, known as aromatisation, is the rate-limiting step in estrogen formation as aromatase expression is under tight regulation while circulating androgens are abundant (7). The most potent naturally occurring estrogen in humans is estradiol (Kd=0.21nM), followed by estrone (Kd=0.35 nM) and estriol (Kd=1.5 nM) (8). Aromatisation results in the production of estrone and estradiol, while estriol is produced following the convertion of estrone by the 16α-hydroxylase (Fig. 1.1). During the premenopausal period estradiol is the predominant estrogen. Throughout pregnancy, estriol is produced in large quantities by the placenta, while after menopause the main estrogen is estrone. Target tissues of estrogens include those constituting the reproductive, skeletal,

cardiovascular and nervous systems (9-12). Estrogens functions differ from one tissue to another.

1.2) Estrogens and the reproductive system

The reproductive system constitutes the best-described target for estrogens. Although the female sex hormones are crucial for gender determination, they do not appear to be required for differentiation and initial development of the female reproductive tracts (13, 14). In fact, in female mice unresponsive to estrogens (αERKO mice) the uterus, although hypoplastic, develops normally (13). Rather, estrogens appear essential during sexual maturation of the gonadal ducts. Mice not producing estrogen (ArKO mice) demonstrate a more severe phenotype where the external genitalia and uteri are underdeveloped (15). However, these mice not only lack estrogen production but also have increased levels of androgens. It is thus believed that the hormonal imbalance, and not only the estrogen deprivation, leads to the more severe phenotype. The late implication of estrogens in reproductive organs also pertains to the ovaries' development. In αERKO and ArKO mice normal ovaries are observed at birth and during neonatal development (14-16). It is only later in life that defects in ovaries, characterized by a complete inability to ovulate and become pregnant, are detected (14-16). Similarly, fetal mammary gland development is independent of estrogen actions. Normally, in mammals the mammary gland is essentially underdeveloped at birth and does not undergo full growth until the completion of puberty, and in fact, remains relatively undifferentiated until pregnancy and lactation. It is during these later stages that estrogens are essential (15, 17, 18). They stimulate the growth and differentiation of the ductal epithelium, induce mitotic activity of ductal cylindric cells and stimulate the growth of connective tissues (19). These effects ultimately contribute to

alveolar formation, ductal morphogenesis, and lobulo-alveolar development (20).

1.3) Estrogens and bone homeostasis

Estrogens also impact on the development, maturation and maintenance of the skeleton. Their initial role, during the pubertal growth spurt of both boys and girls, is to contribute to the formation of the peak bone density attained in late adolecence (21). A secondary role for estrogen is to help maintain this bone density, a process that is effective until menopause in women. Bones consist of a highly metabolically active tissue in which the processes of osteoblastic bone formation and osteoclastic resorption are continuous throughout life. Coupling of osteoblast and osteoclast action ensures that normal bone structure is maintained. A decrease in bone mass and deteriation of the microarchitecture of the skeleton, known as osteoporosis, results from a deregulation of bone homeostasis as a direct consequence of estrogen deficiency. Osteoporosis has important health implications as at least 90% of all hip and spine fractures occur in elderly Caucasian women in the United States of America (22). Hormone replacement therapy is commonly recommended to postmenopausal women in order to reduce the risks of developing osteoporosis. It is currently thought that the protective effect of estrogens on bone integrity may be mediated via regulation of bone cell apoptosis. Indeed estrogens induce apoptosis of isolated osteoclasts (23) while they mediate potent anti-apoptotic effects on osteoblasts and osteocytes (24-27).

1.4) Estrogen and the cardiovascular system

In the cardiovascular system, estrogens were first suggested to have beneficial effects, acting with progesterone to rapidly relax small and

large arteries. They were thus believed to prevent or at least reduce the risks of heart attacks, as a significant increase in cardiovascular disease risk is associated with the loss of estrogen and progesterone at menopause (28). Due to the prevalence of cardiovascular disease as the leading cause of death among men and women in Western societies (29), intensive clinical trials have recently attempted to validate the advantageous role of estrogens in the cardiovascular system. Several reports indicate that postmenopausal women who have a higher risk of cardiovascular diseases benefit from a hormone therapy regimen that includes estrogens through an improved outcome for cardiovascular events (30) (31). However, recent results from the Women's Health Initiative clinical prevention trials (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) have indicated that no beneficial effect on cardiovascular morbidity or mortality could be achieved through the treatment of postmenopausal women with a combination of estrogen and progestins. Interestingly, estrogens were shown to regulate the expression of a number of genes affecting the cardiovascular system, namely the AT_1 receptor (32) and Angiotensin-Converting Enzyme (33). Additional studies will thus be required to clarify the role of estrogens in this system.

1.5) Estrogens and the nervous system

The central nervous system is also affected by estrogens (34, 35). This concept was initially supported by studies in laboratory animals where estradiol exerted neurotrophic effects in tissue explants derived from the hypothalamus and preoptic area of the developing mouse (36). Later studies demonstrated the importance of estrogens in cognitive function in the adult brain, facilitating synaptogenesis in regions of the brain such as the hippocampus (37, 38). They were also shown to have neuroprotectant properties in both culture and animal models (39), and to

decrease the risk of developping Alzheimer disease (40), potentially by contributing to the maintenance of the neurotransmitter systems that undergo degeneration in Alzheimer's disease (41). Similar neuroprotective actions for estrogens have been ascribed to Parkinson's disease (42). More recently, estrogens have been recognized for their role in ocular development, where they are believed to provide protection against age-related cataracts (43).

1.6) Estrogen modulators

Recently the GPR30 protein, belonging to the GPCR class of receptors, was identified as a transmembrane mediator of estrogen (44, 45). However, most effects of estrogen are mediated by their cognate intracellular receptors, the estrogen receptor alpha (ERα) and the estrogen receptor beta (ERβ) (12, 46) (Fig. 1.2). Both estrogen receptors (ERs) are members of the nuclear receptor (NR) superfamily (47-49). This family also includes the receptors of Vitamin D (VDR), thyroid hormone (TR), glucocorticoid (GR), progesterone (PR), mineralocorticoid (MR), androgens (AR) and retinoic acid (RAR, RXR), while some orphan nuclear receptors may function in the absence of ligand. The prototypic nuclear receptor is subdivided into six regions, denoted A to F, based on blocks of sequence conservation for a given receptor throughout species and between different receptors. NRs harbour a DNA binding domain (DBD) in the C region and a ligand-binding domain (LBD) in region E. This region also harbors a dimerization interface, the coactivator-binding groove (CBG) and the ligand-dependent activation function 2 (AF2), while the N-terminal B region contains the ligand-independent activation function 1 (AF1). Sequence identity between ERα and ERβ is highest in the DBD (97%) and the ligand-binding domain (59.1%) but weakest in the A-B regions (15.5%) (50). Although conservation of the LBD primary sequence

is much lower throughout the NR family, reflecting ligand diversity, the tridimensional structures of these LBDs are actually very similar.

1.7) Genomic actions of ERα

The current knowledge on estrogen signalling has mainly been derived from research performed on ERα and is therefore the focus in the following sections. In its unliganded form, the ERα is predominantly found in the nucleus but also localises to the cell membrane and the cytoplasm (51-55). As a monomer it is associated with heat shock proteins (HSP90 or 70), the co-chaperone p23 and immunophilins (56-58) or as free dimers (59) (Fig. 1.3). Following ligand binding, ERα undergoes conformational changes releasing heat shock proteins and other accessory proteins, allowing for the activation of AF2. Within minutes ERα redistributes in the nucleus to punctate nuclear-matrix sites (60, 61), where it can homo- or heterodimerise with ERβ. Whether membrane associated ERα can translocate to the nucleus is still unknown.

Once in the nucleus, ligand-bound ERα can interact with specific palindromic DNA sequences, known as estrogen response elements (ERE, consensus sequence PuGGTCANNNTGACCPy) found in the promoter regions of target genes (62-65). This binding may induce sequence specific allosteric changes in the conformation of the receptor LBD, modulating coactivator recruitment (63, 66-74).

One of the first coactivator complexes recruited to DNA-bound ERα is the SWI/SNF ATP-dependent chromatin-remodelling complex (75, 76). It is recruited to DNA-bound ERα through its BAF57 subunit. In the presence of estrogens, BAF57 can directly interact with the receptor's LBD and DBD. This contributes to the remodelling of histones that normally partake in the stabilization of chromatin through its condensation and formation of nucleosomes. This remodelling increases accessibility of the DNA to other molecules.

A second protein aggregate, the CBP/p160 complex, can also be recruited in the first minutes following DNA binding by the ERα. This complex is composed of several subunits with intrinsic acetyltransferase activity and contributes to chromatin opening through histone acetylation (77-79). These subunits include the CBP/p300 which can also acetylate coactivator proteins, such as p160 coactivators namely SRC-1 (Steroid Receptor Coactivator-1), TIF2/SRC-2 (Thyroid receptor Interacting Factor 2) and AIB1/SRC3 (Amplified In Breast cancer 1) (80), contributing to the release of p160 coactivators from the ERα. Interaction of these coactivators with the ERα occurs on the coactivator binding groove (CBG) and involves three to four NR boxes for each p160 coactivator, consisting of LXXLL motifs (L: leucine; X: any amino acid) (81-84). P160 coactivators possess weak histone acetyltransferase activity allowing them to partake in chromatin decondensation. The RNA based steroid receptor RNA activator (SRA) can also be found in this complex as it interacts with the p160 proteins (85). Protein-arginine methyltransferase (PRMT1, 2 and CARM1) recruited to this CBP/p160 complex, also contributes to the chromatin remodelling potential (86). As for acetylation, modification of histones by their methylation opens the chromatin and facilitates access of various proteins to the DNA.

A third ensemble, the TFTC complex, can be recruited to DNA-bound ERα. This complex also harbours histone acetyltransferase activity and consists namely of TRRAP and GCN5, and further opens the chromatin (68).

Finally the non-histone modifying coactivator complex, known as the TRAP/DRIP/SMCC complex, which contains Mediator subunits, is also recruited to the receptor (87-91). It is the TRAP220/PBP/DRIP205/RB18A subunit that binds to the receptor through LXXLL-dependent and independent mechanisms (91-95). This interaction acts to facilitate the recruitment of the basal transcriptional machinery, namely the RNA polymerase II (Pol II), and the formation of the pre-initiation complex

involving the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (96, 97). The C-terminus of RNA Pol II or its RBP1 subunit once phosphorylated by the CDK7 subunit of TFIIH, allows the transition of the active RNA Pol II from mediator-type protein to elongator-type protein (98). This entire coactivator recruitment process lasts for approximately 40 to 45 minutes on a given promoter, such as for the PS2 gene promoter, and is then reinitiated for a second round of transcription (69).

1.8) ERα degradation and transactivation

In order to complete a cycle the receptor needs to be removed from the DNA. In this regard, degradation of the receptor appears to be essential for optimal transcription efficiency of the ERα. Mutants that are no longer degraded fail to induce a response following estrogen treatment, at least regarding regulation of genes with ERE sites in their promoter (99). The ubiquitin-proteasome degradation pathway appears to play a crucial role in E2-mediated ERα degradation within the first seven hours of induction (100). Proteasome inhibitors (lactacystin and MG132) prevent ERα degradation and transactivation function (99, 101). Similarly ERα transactivation and degradation are considerably reduced in cells deficient in elements of ubiquitin tagging (99). A role for degradation in transcriptional activity has also been observed for many other transcription factors including NHR such as PR, AR, PPAR, RAR, RXR, VDR and TR (102-110). However, this is not a required mechanism as degradation plays the reverse role in GR signaling. Preventing GR degradation actually increases its transactivation efficiency (111, 112).

A common feature of the ubiquitin-proteasome degradation pathway is the covalent attachment of ubiquitin, a highly conserved 8.6 kDa protein present both in the nucleus and cytoplasm, to lysine residues within the target protein (Fig. 1.4). This process involves three classes of

enzymes, namely the ubiquitin-activating enzyme (e1), ubiquitin-conjugating enzymes (e2) and ubiquitin-protein ligases (e3). The e1 first activates ubiquitin in an ATP-dependent manner. Ubiquitin is then transferred from the e1 to an e2. It may next be transferred directly from the e2 to the target protein or through an e3 intermediate. The biological specificity of the ubiquitin pathway seems to be modulated by the selective combination of e2 and e3 proteins. Furthermore, various types of ubiquitination will result in differential outcomes. Indeed, ubiquitin residues may be found in a monomer, attached to the lysine residue on the tagged protein. Under such conditions, the ubiquitination plays a role in endocytosis and transcriptional activation. In cases of polyubiquitination, ubiquitin residues may attach to one another through the K29, K48 or K63 of the ubiquitin. K63 polyubiquitin chains are involved in DNA repair. Proteins with a K29 and K48 polyubiquitin chains are recognized and degraded by a multi-subunit protease complex, known as the 26S proteasome. *In vitro* and *in vivo* ubiquitination of ERα has been reported (113, 114). However, the exact lysine of ERα that gets attached by ubiquitin or the e2 and e3 involved is yet to be identified.

In addition to NHR, CBP, corepressors and p160-family coactivators are degraded by the ubiquitin-proteasome pathway (99, 115). Whether the turnover process of coactivators occurs concomitantly with transcriptional activation induced by these same receptors is unknown. Mutations in ERα that prevent coactivator binding inhibit ligand-mediated degradation, suggesting that coactivator recruitment is another signal for degradation (99). Interestingly, a number of proteins actively involved in the ubiquitin-proteasome pathway have been identified as coactivators of ERα. These proteins include the e3 ubiquitin-protein ligases E6-AP and RPF1/RSP5, the ubiquitin conjugating enzyme UBC9, and an ATPase subunit of the 26S proteasome, TRIP1/SUG1 (113, 116-119). However none seem to require their "degradation" function to coactivate ERα.

Additional regulation of ERα degradation may arise from other post-translational modifications. As for ubiquitination, these events can occur on lysine residues of ERα. For instance, several transcription factors, including GR, AR, PR, p53, c-Jun and c-Myb can be covalently modified by SUMO-1, a small ubiquitin-like modifier, through the process of sumoylation. (120-125). This modification regulates protein localisation and activity (for review see (126)). Moreover, as ubiquitin residues cannot access the modified lysines, the target protein is protected from degradation by the proteasome. Although ERα sumoylation has yet to be described, modification of lysine residues by small ubiquitin-like proteins has been reported. Indeed, ERα can be neddylated. In this case, neddylation inhibits ERα-mediated transcription (127). Interestingly, it also impacts ERα protein levels by enhancing its turnover by the 26S proteasome, acting like ubiquitination (128). Other post-translational modifications not occurring on lysine residues may also impact ERα ubiquitination such as phosphorylation and acetylation. The ubiquitination state of other nuclear receptors, such as PR, AR and RAR can be potentiated by phosphorylation of serine, threonine or tyrosine residues. Mutation of MAP kinase phosphorylation sites in these receptors abrogates ligand-dependent degradation of nuclear receptors (108-110).

1.9) Contribution of ERα AF1 function to transcriptional regulation

Although ligand-dependent activation of the ERα involves direct activation of AF2 through coactivator recruitment at the C-terminal CBG, optimal transactivation potential of the receptor is achieved through synergy with the cell and promoter-specific activation function AF1 (47, 129, 130). Multiple mechanisms can be employed to activate the AF1 function but all involve the recruitment of coactivators to the N-terminus of the receptor.

In the absence of ligand, both the AF1 and AF2 of the ERα aporeceptor are believed to be kept dormant through the interaction of the N-terminal A region of the receptor with the C-terminal CBG. This restrains the accessibility of both AF1 and the CBG to their respective coactivators (131). Under estrogen treatment, the ligand-induced conformational changes of the receptor interfere with the capacity of the A region to interact with the CBG. This releases the AF1 function, which becomes free to recruit coactivators. These include two members of the p160 family, SRC1 and TIF2 (132). This interaction occurs independently of the p160 protein NR boxes and is mediated by their Q-rich region (132, 133). The RNA molecule SRA is also indirectly recruited to the receptor AF1 domain through its interaction with the p160 family proteins (85). The RNA helicases p68 and p72 are additional coactivators that associate solely with the ERα AF1 domain (130, 134). The interaction of p68 and p72 with ERα is dependent on the region between amino acid (A.A.) 72 and 127, and 56 and 72, respectively while the p160 coactivators interact with the region between A.A. 41 and 64 of the receptor (130, 132, 134). Therefore these two classes of coactivators interact with different regions of the receptor N-terminus. They can thus act in concert for an optimal AF1-dependent response.

Phosphorylation of S104 and S106, S118, S167 or T311 of the ERα can result in AF1 activation in the absence of ligand (52, 135-141). This may be triggered following exposure to EGF, TGF-α, insulin or insulin-like growth factor (IGF) 1 and 2, heregulin, dopamine, cyclin D1, cyclinA/CDK2, cAMP, okadaic acid or phorbol ester (135, 142-160). Such nonsteroidal activation of ERα through the AF1 is common to other nuclear receptors, namely PR, AR, RAR, RXR and VDR (152, 161, 162) (for review see (59)). The MAP kinase pathway is involved in AF1 activation through the phosphorylation of S118 (144, 163, 164). This phosphorylation can be enhanced by the orphan nuclear receptor COUP-TF (165). The importance of phosphorylation events in ERα AF1 activation

in the absence of estrogens was demonstrated by substitution of serine 118 to alanine. This mutation prevented phosphorylation of the receptor and growth factor-induced ERα activation but did not affect hormone-induced activation (166). Phosphorylation of S167, which is dependent on the serine/threonine protein kinase pp90rsk1, the casein kinase II as well as the PI3Kinase/AKT pathway can also lead to AF1 activation following growth factor stimulation (134, 167-169). S167 phosphorylation enhances DNA binding and coactivator recruitment, optimizing the transcriptional activation of the receptor (167, 170, 171). Similarly, T311 can be phosphorylated by the p38-MAPK pathway contributing to increased AF1 accessibility to coactivators (52).

1.10) Transcriptional regulation by ERα through non-classical genomic pathways

In addition to its role as a transcription factor, ERα can behave as a coactivator for other transcription factors (Fig.. 1.3). This may occur under estrogen stimulation as well as under conditions of ligand-independent activation of the ERα (172). Activated ERα interacts with, and potentiates the action of c-jun/c-fos (AP1) transcription factors even if deleted of its DBD (172-175). Although DNA binding by ERα is dispensable in this tethering mechanism, interaction with coactivators, as in the case of ERE-dependent gene regulation, is crucial for transcriptional activation (175). Interestingly, the overall strength of an ER's action at AP1 sites bears little relationship to its strength at an ERE. For instance, ERα is a weaker activator of AP1-dependent transcription than ERβ, but is a more potent activator of classical estrogen response than ERβ (174, 176-178).

ERα can also interact with Sp1 (179-183) enhancing the transcription of genes containing SP1 binding sites, in a hormono-dependent manner (181). More recently, a crosstalk between ERα and Stat5b was identified. In this case, ERα monomers interact with the Stat5b

transcription factor (184). This interaction requires ERα DBD and hinge region. Even though the DBD region is required, its DNA binding activity does not potentiate Stat5b activity as point mutations in ERα DBD that prevent ERE binding do not compromise its coactivator function towards Stat5b (185).

Direct interaction between ERα and these transcription factors appears to facilitate the recruitment of additional coactivator molecules to the transcription factors. However, others have suggested that ERα does not need to bind to these transcription factors in order to potentiate their activity. In fact, beneficial effects of ERα on various transcription factors can be increased in the presence of antagonists. ERα is known to interact with corepressors, such as NcoR and SMRT, in the presence of antagonists (186). An alternative hypothesis is thus that ERα contributes to the squelching of corepressor molecules that would normally dampen the transactivation potential of other transcription factors.

In addition to its role as a coactivator, ERα can act as a corepressor. This is the case towards the NF-κB and C/EBPβ and GATA-1 transcription factors (187-189). In these cases, ERα binds to the transcription factors and restrains their capacity to bind DNA thus preventing target gene regulation (189-192).

1.11) Activation of non-genomic pathways by ERα

In addition to reprogramming transcription of target genes, which mediate the long-term effects of estrogens, ERα can rapidly modulate the activity of several signalling pathways through direct protein-protein interactions. This mechanism of action modifies the cell biology without a need for *de novo* protein synthesis (Fig. 1.5). These "non-genomic" effects have been reported in all estrogen target tissues, i.e. bones (25, 193), cardiovascular (194), brain and mammary tissues (195-197) (for review see (198-200)).

The non-genomic effects following estrogen treatment include changes in membrane lipid composition (201, 202), modulation of ion channel permeability (203), activation of numerous signalling pathways, namely cAMP/PKA, PI3K/AKT, p38-MAPK and c-Src/p21ras/Erk pathways (25, 52, 196, 204-214), induction of endothelial nitric oxide synthase increasing NO formation (215-217), augmented intracellular calcium levels (213, 218-221) and increased protein secretion (222). These non-genomic actions of ERα are common to other nuclear receptors. For instance, PR can induce c-src/p21ras/Erk activation following progesterone treatment. It has actually been proposed that activation of this pathway by PR is indirect, and uses ERα as a scaffolding protein as it interacts with PR and with c-Src. Of interest, phosphorylation of tyrosine 537 of ERα potentiates its interaction with the Src-Homology 2 (SH2) domain of c-Src (223).

Part of these non-genomic effects are derived from membrane bound ERα, which represents a small fraction of the total intracellular receptor content, but that has been detected in endometrial cells, neurons, myometrium, liver, breast cancer cells (MCF-7), and in osteoblasts and osteoclast-like cells (51, 193, 195, 224-231). Homodimerization of membrane ERα appears to be required for activation of downstream signalling cascades (232). The mechanism of ERα localisation to the membrane is still ill defined. However, ERα localizes to membrane regions rich in signalling proteins known as caveolae (233). Furthermore, ERα activity can be positively potentiated by Caveolin-1 (234). ERα was also reported to interact with striatin, a calmodulin-dependent scaffolding protein interacting directly with caveolin-1 (235, 236). This interaction between ERα and striatin appears necessary to form a membrane signalling complex necessary for the activation of eNOS (236). In addition to membrane-bound ERα, a G-protein coupled receptor (GPCR) has been suggested to modulate the non-genomic effects of estrogens. This was first suggested as inhibitors of G-protein signalling could block second messenger signalling by estrogens. (207, 228, 237, 238). The GPR30 was

identified as this transmembrane GPCR family protein early this year (44, 45).

1.12) Estrogen target genes regulated directly by ERα

The orchestrated induction of genomic and non-genomic events by estrogen impacts on the expression of a number of genes involved in cell proliferation and differentiation. A detailed list of known estrogen target genes can be found on the estrogen responsive genes database (ERGDB) (239).

Two classes of estrogen target genes have been characterized. The first class, known as primary response genes, requires the direct recruitment of either ERα or ERβ to their promoter/enhancer region (for review see (240)). This class is subdivided in two categories, those where ERs directly interact with DNA and those where ERs do not bind DNA but are found in the enhancosome through association with other DNA-binding proteins. As direct recruitment of ERs is required for primary response genes, they often harbor EREs in their promoter/enhancer region (for review see (62, 241)). This consensus sequence first described in the *Xenopus laevis* vitellogenin A2 promoter (64) constitutes a potent ER-binding site characterized as an inverted-repeat with classically 3, and occasionally 5 nucleotides spacing (full-ERE, PuGGTCANNNTGACCPy) (242). Alternatively, ERs will bind to half-EREs (5'-PuGGTCA-3') either cooperatively with a distant half-ERE (243) or with a proximal Sp1 site (181). Having multiple ER-binding sites may partially explain the differential expression pattern of certain genes from one cell environment to another. Indeed, the cell environment influences the chromatin condensation state, which directly affects the accessibility of transcription factors to the DNA, therefore an ER-binding site associated with a given gene could be employed under certain conditions while another site would

be implicated under other conditions. This could in turn modulate differently the expression pattern of a target gene.

Although the consensus ERE sequence established almost 20 years ago has a high binding affinity to ERs, some variations in its sequence still allow for efficient binding *in vitro* (63, 65). However, the *in vitro* binding affinity of ERs for the consensus or nonconsensus EREs in presence of estrogens does not necessarily correlate with the expression level of target genes (63). Furthermore, the efficiency at promoting gene expression from a given ER-binding site varies according to cell type. Noteworthy, the ERE sequence can allosterically regulate ERα structure, function and coactivator recruitement (66, 67, 244-246). The differential expression of genes harboring EREs in their promoter/enhancer region from a cell type to another, or from a cell environment to another may therefore also derive from the variability in coactivator and accessory protein levels (186).

Benefiting from the availability of full genome sequencing in multiple species, a genome-wide screen for consensus and near-consensus (sequences differing from the consensus sequence in one or two nucleotides) EREs identified 660 gene orthologues from the human and mouse genomes with conserved elements in the flanking regions (-10 to +5kb) (65). More than 230 of these genes were known to be estrogen-stimulated in humans (239, 247). Unfortunately, the presence of an ERE does not necessarily infer that a gene is directly regulated by ERs. Direct recruitment of ERα to a number of these EREs was therefore demonstrated by chromatin immunoprecipitation (ChIP), establishing them as primary response genes (65). More recently, an ERE binding activator (EBA) corresponding to two DNA-binding domains joined with the hinge region of the ERα fused to the strong activation domain of VP16 or p65 was developed (248). These constructs behave as constitutively active ERα mutants inducing the expression from an ERE promoter construct in transfected cells in the absence of ligand. Therefore, these constructs will

offer a novel alternative to identify estrogen target genes requiring direct ER binding. Of interest, the EBAs can modify breast cancer cell growth in an equivalent manner to estrogen, suggesting that direct gene regulation contributes to cell growth.

Recently, a spectrum of primary response genes was identified through expression profiling on gene microarray following treatment with estradiol in presence of cycloheximide, which prevents *de novo* protein synthesis (247, 249). Noteworthy, not all genes identified contain an ERE in their promoter/enhancer region. In fact, approximately 35% of the known human primary response genes do not contain an ERE-like sequence (240). Instead they rely on a second DNA binding transcription factor to attract ERs to the enhancosome. Sp1 is the classic example of such a transcription factor. Although it can contribute to ERα binding to ERE-half sites, it also allows ERα to act as a coactivator. In this case the DNA-binding domain of ERα is dispensable (179-183, 250, 251). Similar association occurs between ERα and the activator protein 1 (AP-1) (172-175, 252) and the Stat5b transcription factors (184, 185).

For the last few years tremendous investments have been directed at identifying estrogen target genes through DNA microarray, Serial Analysis of Gene Expression (SAGE) and other hightroughout-put techniques (65, 247, 253-256). Interestingly, this research has focused on the identification of estrogen target genes in both normal and disease state tissues. For instance, both techniques have been used to identify estrogen target genes with potential implication in breast cancer development or progression (253, 257). DNA Microarray was also used in order to identify genes potentially modulating the partial agonist activity of antiestrogens (247, 258, 259). Although these approaches do not differentiate genes regulated by the ERα versus the ERβ, the characterization of the estrogen transcriptome and its modulation in the course of disease progression will benefit the development of novel diagnostic, prognostic and therapeutic tools in the near future.

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1.14) Figure legends

Figure 1.1. Pathways leading to estrogen biosynthesis and metabolism.

Conversions from androstenedione and testosterone to oestrone and oestradiol respectively are catalysed by the enzyme aromatase (p450). Estrone production can also derive from the action of estrone sulfate (ES) on estronesulphate. This can be reverted by the estrone sulphate transferase (EST). Estrone is reversibly converted to estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD). Estriol is derived from the direct action of 16α-hydroxydase (16α-HD) of estradiol.

Figure 1.1

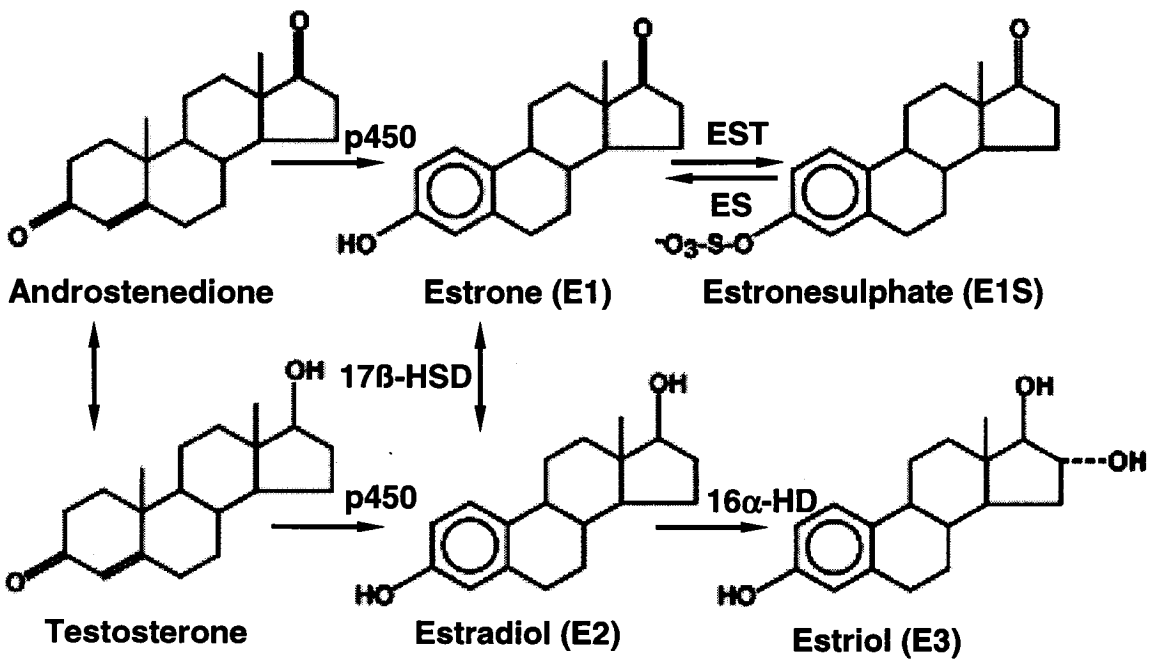

p450
EST
ES
HO
$^{-}O_3$-S-O
Androstenedione
Estrone (E1)
Estronesulphate (E1S)
OH
17ß-HSD
p450
16α-HD
Testosterone
Estradiol (E2)
Estriol (E3)

Figure 1.2: Schematic representation of ERα and ERβ domain distribution.

Nuclear receptors are subdivided into 6 regions lettered A to F according to sequence homology across the family members. The ligand-independent activation function 1 (AF1) of ERα lies in the A/B regions while the ligand dependent activation function 2 (AF2) lies in the C-terminal E/F regions. The C region harbors the DNA binding domain (DBD) while a hinge is located in the D region. The initial and ending amino acid of the various regions in ERα are shown. Homology sequence between ERα and ERβ is presented in %.

Figure 1.2

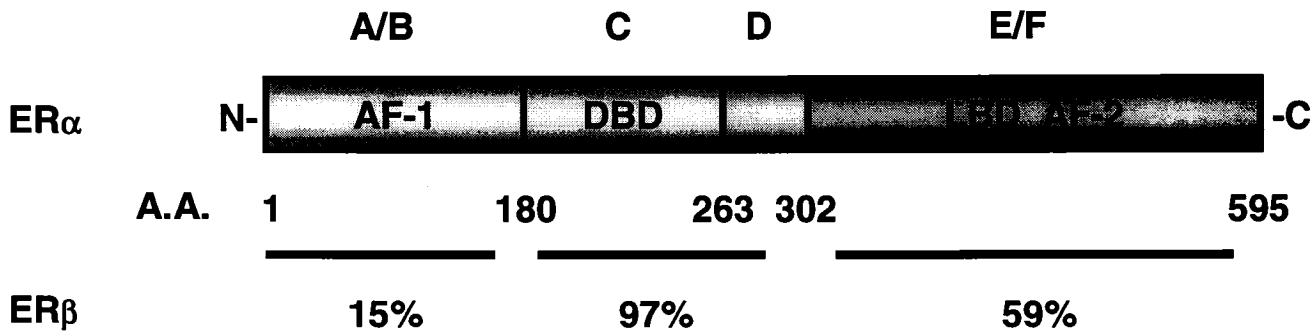


Figure 1.3: ERα genomic signaling cascade.

In absence of ligand ERα can be found in association with heat shock proteins (HSP) and co-chaperone (p23) or as free dimers. The classic genomic response of ERα following agonist binding involves dimerization and interaction with estrogen response elements (EREs) found in the promoter/enhancer region of target genes. This is followed by an ordered recruitment of coactivator molecules (CoA) including proteins with acetyl transferase (CBP) and methyl transferase (CARM) activity conrtibuting to chromatin remodeling. This ultimately favors the recruitment of the pre-initiation complex (PIC) allowing for gene expression. Alternatively, ERα activation may lead to a non-classical genomic response. This involves the interaction of ERα with specific transcription factors, such as Sp1 and AP1, following its binding to an agonist ligand. The transcription factors can interact with their respective DNA recognition sequences. ERα acts as a CoA, contributing to the recruitment of additional CoA to this transcription complex. In this scenario ERα does not necessarily interact directly with the DNA.

Figure 1.3

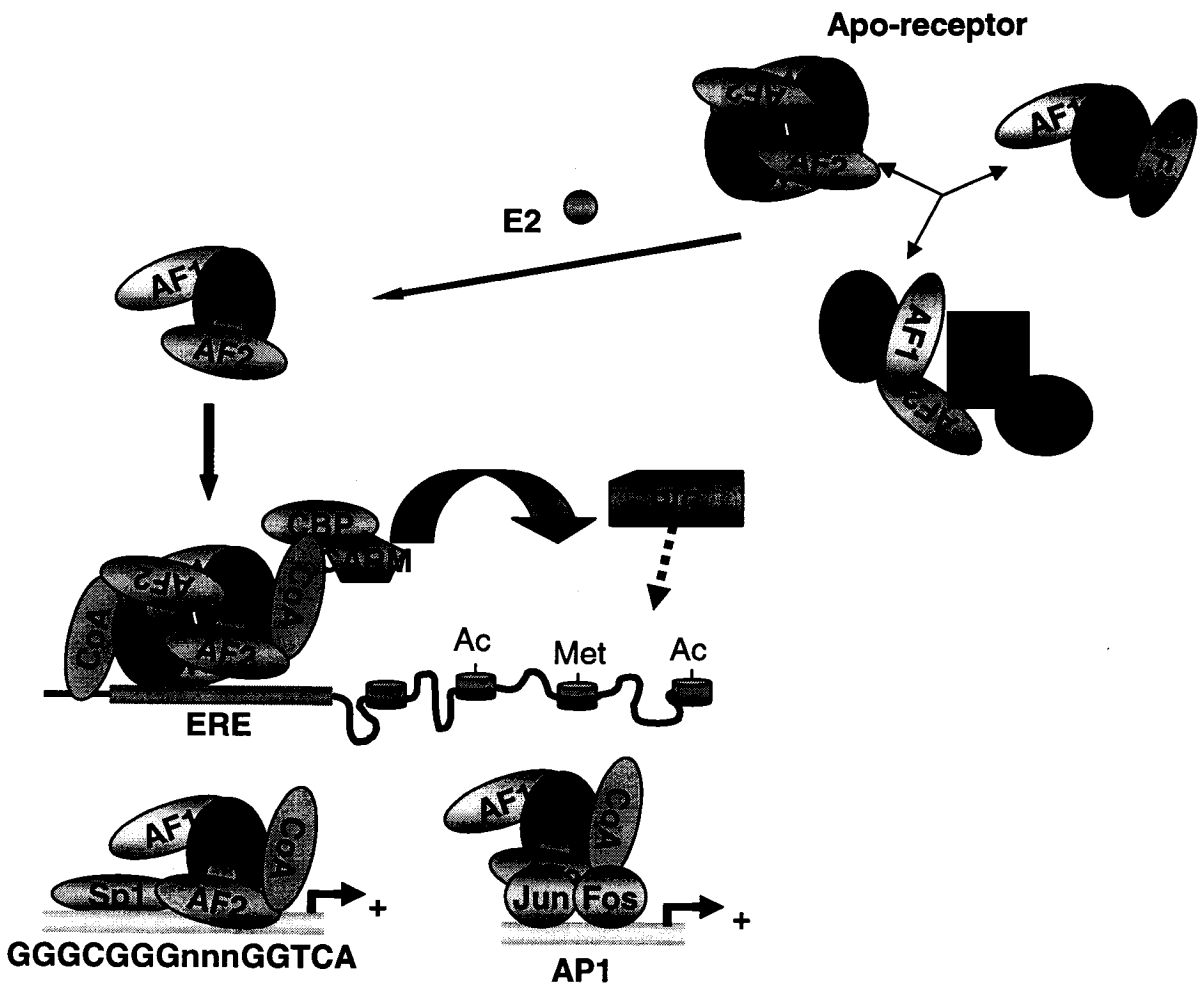

Apo-receptor
E2
AF1
AF2
CBP
p/CIP
SRC-1
Ac
Met
Ac
ERE
Sp1
Jun
Fos
+
GGGCGGGnnnGGTCA
AP1

Figure 1.4: ERα degradation by the ubiquitin proteasome pathway.

The process of ubiquitination first involves the activation of the ubiquitin residue in an ATP dependent fashion. This is performed by the ubiquitin-activating enzyme (e1). The ubiquitin bond to the e1 is then transferred onto the ubiquitin conjugating enzyme (e2). It can than directly transfer the ubiquitin residue onto the target protein, such as ERα, or transfer it onto the ubiquitin protein ligase (e3). This is the last enzyme involved before the actual attachment of ubiquitin on a lysine found in the target protein. This last step may be performed a number of times, where the ubiquitin is actually attached to an ubiquitin residue already bound to the target protein. The polyubiquitin chain can than be recognized by a subunit of the 26S proteasome allowing for the degradation of the target protein.

Figure 1.4

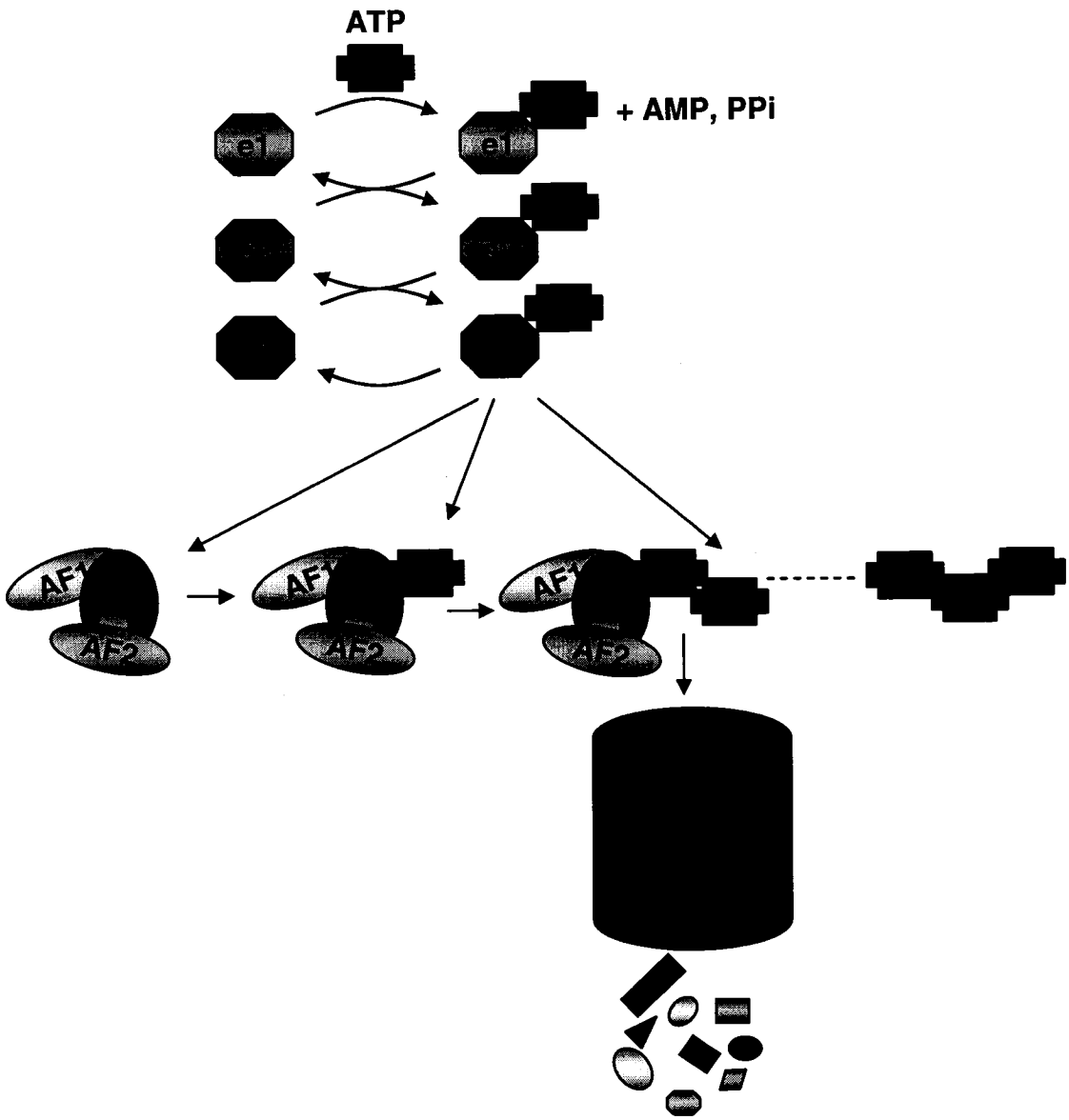
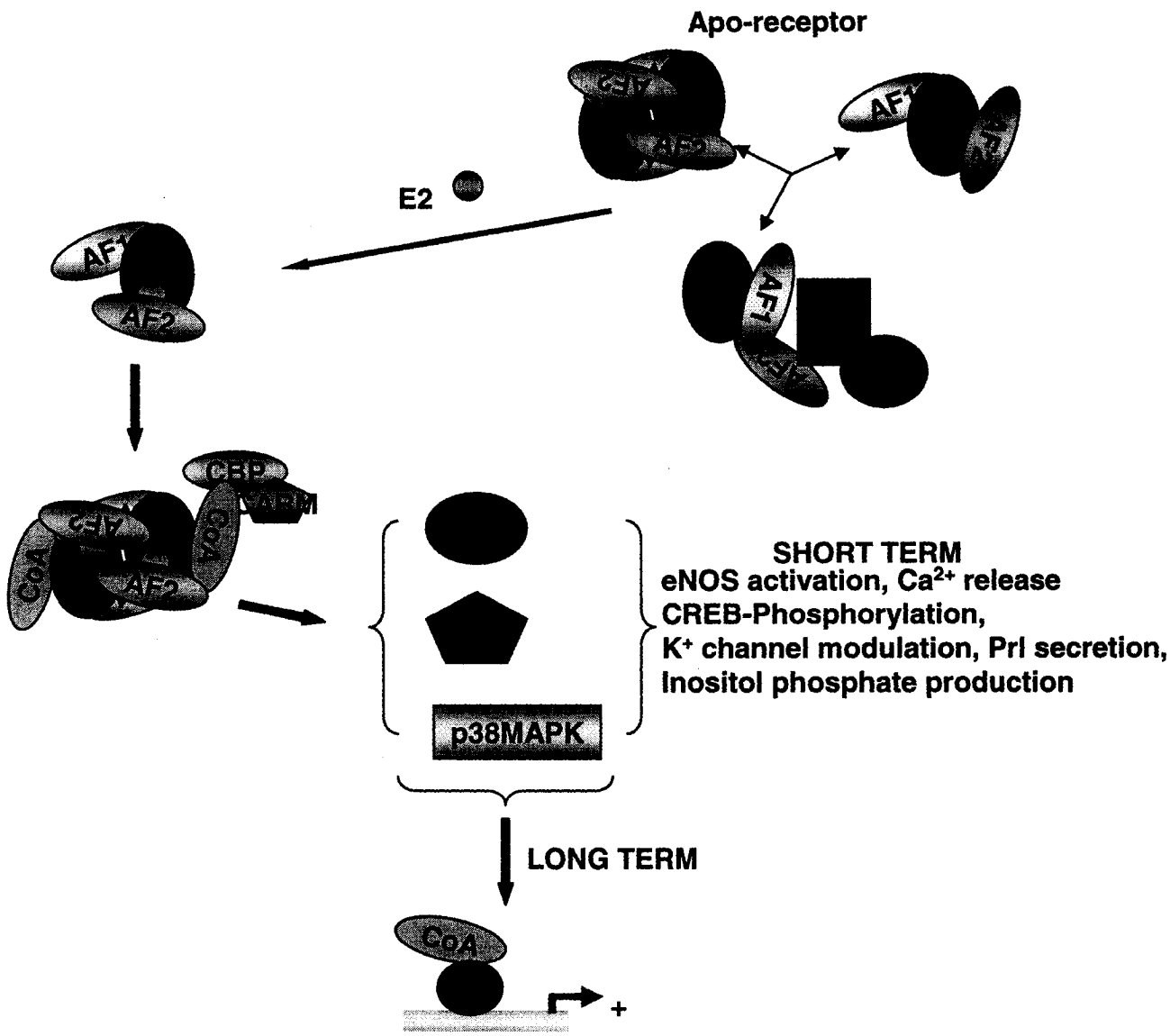


Figure 1.5: ERα non-genomic pathways.

Following agonist binding ERα can interact with cytoplasmic proteins in order to modulate their activity. For instance, ERα can interact with PI3K, c-src or p38MAPK under estradiol treatment and lead to short term effects that do not require *de novo* protein synthesis. These effects include endothelial nitric oxyde synthase (eNOS) activation, calcium release, phosphorylation of CREB, secretion of prolactin (Prl), modulation of potassium channels and production of inositol phosphate. The non-genomic pathways of ERα also involve changes in the PI3K, c-src and p38MAPK signaling pathways afecting specific transcription factors (TF) that lead to the modulation of the expression of their target genes through binding to their respective response elements (RE).

Figure 1.5


Apo-receptor
E2
AF1
AF2
CBP
SRC
CoA
p38MAPK
SHORT TERM
eNOS activation, Ca2+ release
CREB-Phosphorylation,
K+ channel modulation, Prl secretion,
Inositol phosphate production
LONG TERM
+

Chapter 2: MODE OF ACTION OF ANTIESTROGENS:

2.1) Development and clinical use of Tamoxifen

One of the most common and fatal outcomes of defects in estrogen signaling is breast cancer. It is the most frequently diagnosed cancer in women (30% of all cancers diagnosed) and the second highest cause of cancer-related deaths among women in North America (15%). Breast cancer is characterized by the uncontrolled proliferation of mammary gland epithelial cells. Estrogens, such as 17β-estradiol (E2), promote the proliferation of breast tumor cells as well as of mammary gland epithelial cells during normal development through the estrogen receptor alpha (ERα) (1, 2). This receptor and the other member of the ER family (ERβ) are ligand-inducible transcription factors, which modulate gene transcription in epithelial cells following estrogen treatment (1). Overexpression of ERα is detected in approximately two-thirds of all breast tumors (2), suggesting a higher response of these cells to estrogen signaling. Moreover, while ERβ knockout (βERKO) female mice have normal breast development and lactate normally (3), ERα knockout (αERKO) female mice exhibit undeveloped mammary glands characterized by vestigial ducts, indicating that ERα is essential for epithelial cell proliferation (4, 5).

Antagonists of estrogen signaling, known as antiestrogens, were initially developed in the mid 1950s as fertility agents. Unfortunately, long-term usage of the first antiestrogens developed, such as the non-steroidal antiestrogen ethamoxytriphetol (MER-25) and the triphenylethylene derivative chlomiphene, was impossible due to their significant toxicity (6). However, MER-25 and chlomiphene were found to impact on breast tumor progression (7). In the early 1970s, tamoxifen (Tam; ICI46,474; Nolvadex), initially recognized for its antifertility properties in laboratory animals (reviewed in (8)) was tested for the first time in breast cancer patients (9).

Since then, it has been the most widely used antiestrogen in breast cancer treatment, and has become the gold standard in the adjuvant hormonal treatment of all stages of breast tumors (10, 11). Tam is not only effective in the treatment of advanced breast cancer but also as adjuvant therapy for early stage disease (12-15), reducing the risk of primary tumor development in contralateral breast (13, 16-21) and in the prevention of breast cancer in women at risk (22).

2.2) Tamoxifen, a selective estrogen receptor modulator (SERM)

The effectiveness of the antiestrogen Tam in suppressing growth of hormone-dependent breast tumors has been amply demonstrated (23, 24). Tam competes with agonists such as 17β-estradiol (E2) for binding to the ligand-binding pocket of estrogen receptors in a reversible manner (Fig. 2.1). While agonist-bound estrogen receptors behave as ligand-dependent transcription factors and recruit various coactivators capable of contacting the basal transcription machinery or of modifying/remodeling chromatin, Tam induces a conformational change of the receptor ligand-binding domain (LBD) that prevents coactivator recruitment (25). In addition, Tam induces binding of corepressors to ERα. Corepressors interact with some heterodimeric nuclear hormone receptors (NHRs), such as TR/RXR and RAR/RXR, in the absence of ligand, and result in the inactivation of the receptor (26, 27) by recruitment of histone deacetylase proteins, which act by deacetylating the histone in the vicinity of the nuclear receptor response element contributing to chromatin condensation (28). In the case of ERs, the aporeceptor is not recognized by corepressors, possibly due to its interaction with heat-shock protein complexes. Binding of the antiestrogen Tam releases the receptor from these complexes, but the conformation of the LBD in the presence of Tam generates a new interacting surface, which allows for the recruitment of

corepressors such as NcoR or SMRT through amphipatic helical motifs called CoRNR boxes (29-32). Thus, while Tam-bound receptors can bind DNA with the same specificity as the wt ERs, the reduced recruitment of coactivators and increased association with corepressors both prevent transcriptional activation of target genes (31).

In spite of its efficacy in breast cancer treatment, Tam maintains estrogenic action in a gene- and tissue-specific manner. For instance, Tam treatment decreases the risk of developing osteoporosis in postmenopausal women (33). It also contributes to the maintenance of the lipid profile/cardiovascular system (34-37). Unfortunately, the partial agonist effects of Tam are not all desirable. This antiestrogen demonstrates partial agonist activity in uterine tissues, resulting in an increased risk of endometrial cancer development (38-43), which limits its use as a preventive agent in breast cancer treatment. This tissue-specific partial agonist function has led to the classification of this compound as a Selective Estrogen Receptor Modulator (SERM). In addition, acquired tolerance to Tam is a common failure in breast cancer treatment (44, 45). The partial agonist activity of Tam may be increased during the course of breast cancer treatment, leading to actual stimulation of breast tumor growth as demonstrated by the positive effect of Tam withdrawal on some tumors after long-term treatment (46). Although it has been proposed that specific mutations in the ERα receptor, such as the D351Y mutation, may increase the agonist activity of Tam (47), adaptations in alternate signaling pathways, such as increased expression of the EGF receptor (EGFR) and c-erbB2 (48-51) as well as upregulation of coactivators or downregulation of corepressors (30, 52), would appear to be the most frequent mechanisms resulting in the conversion of Tam into a more potent agonist

The molecular mechanisms of the agonist activity of Tam have been extensively studied and appear to involve effects on several functional properties of ERα. Like agonists, Tam induces ERα binding to

target promoters; however, unlike agonists which induce receptor turnover, Tam increases the steady state levels of ERα (53-62). The AF2 transcriptional activation function appears to be repressed by Tam, but the AF1 function may be activated and induce transcription of target genes in a cell- and promoter-specific manner (63-65). Note however, that a weak implication for AF2 in cooperation with AF1 has not been ruled out (66). Partial agonist activity occurs on classical EREs as well as on alternate response elements such as AP1 (39, 63, 67, 68) and involves coactivator recruitment (69). It has been recently suggested that the agonist versus antagonist function of Tam is derived from the ratio between coactivator and corepressor protein levels in a given cell (70). Indeed, reducing SRC1, 2 or 3 levels in Ishikawa cells through RNAi abrogates the agonist activity of Tam while overexpressing these coactivators in MCF7 cells reveals the agonist activity of Tam. Similarly in HepG2 cells overexpression of SMRT abrogated the agonist activity of Tam while SRC1 overexpression had the opposite effect (71).

Not surprisingly, the negative secondary effects of Tam have fuelled the development of alternative antiestrogens, resulting in the recent introduction of novel compounds for breast cancer treatment and hormonal therapy.

2.3) Raloxifene and the next generation of SERMs

Second generation antiestrogens are now undergoing clinical trials. For instance the SERM raloxifene (Ral) is presently under investigation for breast cancer prevention in the STAR (Study of Tam And Ral) clinical trial. As observed with Tam, Ral behaves as an agonist in certain tissues but as an antagonist in others. Its agonistic properties are apparent mainly in bone tissue and Ral was thus initially developed for treatment of osteoporosis and osteopenia (38, 72, 73). A role in the prevention of Alzheimers disease has also been suggested due to the capacity of Ral to

protect against β-amyloid-induced neurotoxicity (74). Weak uterotrophic or mammotrophic actions have been reported for this antiestrogen (38, 75, 76) but there is no clinical indication for increased risk of developing breast or uterine cancer to this day. At the molecular level Ral behaves like Tam (Fig. 2.1), by competing with agonists for the ligand-binding pocket of ERα, preventing coactivator recruitment through induction of a conformational change of the receptor LBD, and inducing corepressor association with ERα (77). Ral can also increase the steady state protein level of ERα (53, 54), but to levels inferior to those obtained with Tam. This correlates with a general weaker agonist activity of Ral compared to Tam in cell line assays (54, 70, 76-79). As long-term clinical trials have been instigated only recently, the capacity for tumor cells to acquire tolerance to Ral and the extent of secondary effects following prolonged treatment remain poorly characterized.

At the structural level, difference between Ral and Tam can be observed in the steroid backbone that include the presence of phenolic hydroxyls and a corbonyl "hinge" found between the basic amine-containing side chain and the olefin in Ral absent in Tam. Furthermore, the tertiary amine substituent found in these antiestrogens side chain differs (Fig 2.2). As these structural differences impose a different conformation to the receptor, they are believed to impact the tissue selective action of Ral and Tam (73).

2.4) ICI182,780, and full antiestrogens

Some antiestrogens appear to completely antagonise ERα regardless of the cell/tissue or promoter context (80, 81). This class of antiestrogens includes ICI164,384, the related ICI182,780 (Faslodex) and RU58668 (81-84). These compounds are important from a clinical point of view as they can be used as second line therapy after tumour cells

become resistant to SERM treatment (85). This absence of cross-resistance suggests different mechanisms of action for full antiestrogens and SERMs. Like SERMs, full antiestrogens compete with agonists for the ligand-binding pocket of ERα. Their longer side-chain sits in the coactivator-binding groove, as observed in crystallographic studies with ICI164,384 and ERβ. Thus, full antiestrogens may prevent coactivator recruitment by a different mechanism than that used by SERMs, in which H12 occupies the coactivator-binding groove (86). Crystallographic artefacts in the positioning of H12 cannot be excluded however.

The antagonist potential of full antiestrogens may result from their capacity to impair several aspects of estrogen receptor function (Fig. 2.1). ICI182,780 limits ERα intranuclear mobility, promotes a punctuated perinuclear distribution of the receptor, favours its nuclear to cytoplasmic shuttling and induces its degradation by the 26S proteasome (55, 58, 59, 87-91). Accordingly, ICI182,780-bound ERα was not found on EREs *in vivo* (92). The relative importance of the different mechanisms of AF2 inactivation used by full antiestrogens and of their effects on receptor stability/nuclear localization remain to be established.

Because of their lack of partial agonist activity compared to SERMs, full antiestrogens may have more serious side effects than SERMs, rendering their use in first-line therapy and prevention more problematic. Furthermore, development of tolerance to ICI182,780 has been reported in MCF7 cells, the classical cellular model of hormone-dependent breast cancer (45). The development of novel SERMs that completely inactivate the receptor in tissues like breast and uterus while maintaining an agonist function in others is therefore still a major field of research.

2.5) Understanding antiestrogen action at the structural level

The estrogenic or antiestrogenic actions of various ERα ligands can be ascribed to specific conformational modifications occurring in the LBD of the receptor following ligand binding (reviewed in (93) and Fig. 2.3). As for other nuclear receptors (94-97), evidence for structural differences between agonist versus antagonist-bound ERα complexes were initially derived from antibody epitope mapping, partial proteolysis and mobility shift experiments (95, 98-101). These are derived from the different structure of antiestrogens. Indeed, the length of the side chain of SERMs, such as Tam and Ral, is typically shorter than for full antiestrogens including ICI164,384 and ICI182,780 (Fig 2.2). The detailed structural characterization of ligand-bound nuclear receptors was eventually obtained by crystallographic analysis of their LBD. The LBD of the unliganded RXRα was the first to be crystallized (102). Later on, the crystal structure of agonist-bound ERα and ERβ was obtained and indicated that the LBD was composed, as for the other steroid nuclear receptors, of 12 alpha-helices (103, 104). The H5, H6, H9 and H10 establish a tightly packed central core. These are surrounded by H2, H3, H4, H7, H8, and H11. The active conformation of ERα (i.e.: when bound by agonists) is characterized by the positioning of H12 above the ligand-binding pocket formed by H3, H5/6, and H11. This conformation contributes to the establishment of a coactivator-binding groove (CBG) composed of H3, H4, H5 and H12. Residues K362 and E542 found at the ends of the groove establish a charge-clamp (Fig. 2.3A). This facilitates coactivator recruitment as the C-terminus and the N-terminus of the NR box helix are capped (103). The set of residues from helices H3, 4, 5 and 12 which contribute to recruitment of coactivators correspond to the previously defined AF2 transcriptional activation domain. Antiestrogens all repress transcriptional activation by the AF2 activation domain by preventing positioning of H12 in the active conformation (103-105).

Differences in either the LBD structure that is most stable for a given antiestrogen and/or in the dynamics of equilibrium between different LBD conformations are likely to explain the different impact of these ligands on ERα functional properties.

2.5.1) SERM-bound conformation of ERα

When SERMs like the active metabolite of Tam, 4-hydroxytamoxifen (OHT), or Ral, occupy the ligand-binding pocket of ERα, H12 is prevented from adopting the conformation observed in the agonist-bound structure by steric hindrance resulting from the presence of the antiestrogen side chain that protrudes from the ligand binding cavity (Fig. 2.3B). Instead, H12 is reoriented and occupies the coactivator-binding groove in a position that mimics that of the coactivator peptide. Note that H12 contains an LXXML motif that resembles the consensus coactivator motif. As in the case of coactivator peptides, H12 positioning is facilitated by a charge-clamp. While K362 caps the C-terminal turn of H12 much as it does with the coactivator peptide, the N-terminal part of H12 is not capped by E542 (itself part of H12) but by E380 (103) (Fig. 2.3B). This alternative positioning of H12 competitively inhibits coactivator recruitment through the coactivator-binding groove, resulting in AF2 inactivation (103, 105). The crucial role of the antiestrogen side chain in their antagonist action is demonstrated by the fact that its removal allows the receptor to adopt an agonist-like conformation where H12 sits on top of the ligand-binding pocket favoring the recruitment of coactivator peptides (106). More recently, fluorescence anisotropy assays have been used to monitor differences in the dynamic conformation of ERα when bound to SERMs. This technique, also employed with other nuclear receptors (107), was adapted to follow the degree of receptor helicity near the C-terminus of H11 (108). This was achieved using Cysteine (Cys)-bound fluorophores in a receptor LBD mutant where Cys 381 and 417 were replaced with serine.

Increased levels of anisotropy at the end of H11 (Cys 530) were observed for the ERα bound to OHT, suggesting a shortening of H11 compatible with an extension of the H11-H12 loop necessary for H12 to occupy the coactivator-binding groove. However, this was not observed for the Ral-bound ERα, demonstrating differences in the mode of action of SERMs, which may explain their distinctive biological activities in different estrogen target tissues (38). Note however, that both crytallography and fluorescence anisotropy techniques only use the ligand-binding domain of the receptor which represents only ~40% of the entire receptor. On the other hand, identification of peptides that interact differentially with different conformations of the full-length receptor has been achieved using the phage-display technique (109-112) (reviewed in (113)). Interestingly, peptides associating with the ERα in the presence of OHT but not Ral were identified (110-112), suggesting that the SERMs can induce distinct conformations to the receptor exposing a unique peptide-binding surface. Note however, that a possible contribution of the antiestrogen side chain itself to peptide binding cannot be ruled out.

Although crystallography studies suggest that the conformations of the ERα LBD bound to Tam or Ral are very similar, the differential partial-agonist activity of Ral, Tam and other SERMs may be derived from slight differences in receptor LBD conformations, or from a different conformational equilibrium. Indeed, it has been suggested that ligand binding does not result in a switch from one inactive to an active conformation but rather from a multitude of conformational states to fewer conformations (114, 115)

2.5.2) Conformation of ERα in the presence of full antiestrogens

Although there is no published crystallographic analysis of the ERα in presence of full antiestrogen, crystals of ERβ LBD bound to the full-

antiestrogen ICI164,384, a compound closely related to ICI182,780 (81, 83), reveals distinct features compared to SERM-bound ERα (86). Indeed, coactivator recruitment by AF2 appears to be prevented by occupancy of the coactivator-binding groove by the longer side chain of full antiestrogens rather than by H12, which is not visible in the crystal structure (Fig. 2.3C). This suggests that ICI164,384 prevents H12 from associating with the LBD in either the agonist or SERM-induced conformation. Fluorescence anisotropy assays with either ICI164,384 or ICI182,780 also indicate a higher helical content at the end of H11, which is not compatible with occupancy of the coactivator-binding groove by H12 (108).

2.5.3) Role of H12 positioning in receptor inactivation

A role for H12 in ERα degradation and/or nuclear export has been suggested by the restoration of receptor nuclear levels following deletion of H12 or double mutation in H12(L539A/L540A) (116, 117). Whether this is linked to the an unstable association of H12 with the LBD as observed in the ERβ-ICI164,384 structure remains to be investigated. Interestingly, the N-terminus of H12 is also unresolved in Ral-bound ERβ (105).

Displacement of H12 from the ligand-binding pocket can also be achieved through the recruitment of corepressors. Crystallographic studies on the GW6471 antagonist bound to PPARα indicate that inactivation of the receptor is attained by the binding of the SMRT corepressor in the pocket formed by H3, H4 and H5 (118). As the three turns of the alpha helix of the corepressor LXXXIXXXL motif (CoRNR box) is accommodated, H12 is prevented from stabilizing above the ligand-binding pocket or the coactivator-binding groove. In fact, H12 loosely packs along H3 on the outside rim of the receptor (Fig. 2.3D). A similar conformation was recently demonstrated for ERα when bound to the antagonist GW5638. Indeed, the H12 in GW5638-bound ERα structure

was exposed to the environment outside of the LBD. This forced the presence of hydrophobic residues from H12 outside of the LBD which was correlated with a destabilization and inactivation of the receptor (119).

2.6) Development of alternative therapies

As resistance to both SERMs and full antiestrogens can be observed in preclinical models, other methods to inhibit estrogen signaling in breast cancer patients have been investigated in parallel with the development of novel antiestrogens. Aromatase inhibitors are metabolic modulators that prevent synthesis of estrogens from androgens. Estrogens (mainly estradiol) are produced by ovarian granulosa cells from testosterone in premenopausal women, but conversion from adrenal androgens in peripheral tissues can also take place in post-menopausal women (producing mainly estrone). Aromatase activity is necessary in both cases and is also frequently overexpressed in tumor cells or in adjacent tissue. Aromatase inhibitors, which are competitive inhibitors of the steroid- (type I) or cofactor- (type II) binding sites of the enzyme, will prevent signaling by estrogen receptors by abolishing production of its natural ligands. A potential limitation is the capacity of the receptor to be activated in a ligand-independent manner (see Chapter 1). Therefore, combined inhibition of estrogen signaling by inhibition of endogenous production of natural agonists and use of synthetic ligands that repress its transcriptional activation may seem promising. However, combined treatment with aromatase inhibitors and Tam has not revealed advantages over treatment with aromatase alone. This is likely to be due to the fact that aromatase inhibitors could not prevent tumor resistance due to conversion of Tam into a partial-agonist. Of interest however, preclinical models (xenografts of aromatase-overexpressing MCF7 cells) have indicated that association of aromatase inhibitors and full antiestrogens leads to a more complete inhibition of breast tumor growth (120).

While total blockade of estrogen signaling may be desirable in ERα positive breast cancer patients, the pleiotropic effects of estrogens in non-reproductive tissues including bone, brain and the cardiovascular system need to be better characterized in order to define the ideal profile of compounds used in the preventive setting, as full estrogenic blockade may lead to severe long-term side effects. SERMs with optimized activity profiles in different systems and/or combined treatments aiming at alleviating side effects may be the key to the prevention of breast cancer and possibly also to safer hormonal replacement therapies.

Compounds that specifically activate estrogen receptors in tissues other than breast and endometrium could thus provide a useful alternative in combination with aromatase inhibitors. For instance, tibolone has been shown to prevent bone-loss, to have beneficial effect on the brain and to relieve climacteric symptoms as effectively as estrogens, without stimulating endometrial and breast cell proliferation (121-123). This compound, classified as a Selective Tissue Estrogenic Activity Regulators (STEAR), differs from SERMs since the steroid metabolism of the target tissue establishes its tissue selective estrogenic activity. Indeed, this compound is converted into a steroid agonist by enzymes found in target tissues. The conversion of tibolone to its active metabolites does not appear to require the aromatase (123). However, further investigation is required. As aromatase knockout mice have been generated in the past, it will be of interest to establish the activity profile of tibolone in these mice. It may be hoped that with the plethora of novel approaches developed recently for the modulation of estrogen signaling, imminent improvements in the treatment of associated pathologies, including breast cancer, will be revealed.

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2.8) Figure legends:

Figure 2.1: Mechanisms of inactivation of the ERα by antiestrogens.

Apo-receptor may be found in a dynamic population of variable conformations of agonist and antagonist nature. Agonists, such as 17β-estradiol (E2) interact with ERα through its ligand-binding domain favoring an active conformation (1). This allows for dimerization and interaction with estrogen response elements (EREs) in the promoter of target genes (2). Coactivators (CoA, CBP, CARM, etc) are then recruited and favor chromatin decondensation through acetylation and methylation of histone (3). The SERMs (tamoxifen and raloxifene) and the full antiestrogen (Full AE) (ICI164,384, ICI182,780 and RU58668) employ multiple mechanisms in order to inactivate the ERα. First, they compete for the ligand-binding pocket with the agonist (4) and by so doing prevent coactivator recruitement (5). In addition, the SERMs can specifically favor the recruitment of corepressor complex (CoR, HDAC, etc) in order to facilitate chromatin condensation (6). As well, they prevent agonist-bound ERα from interacting with the DNA as SERM-bound ERα occupy the ERE (7). On the other hand, full antiestrogen force the nuclear export and degradation of the receptor (8).

Figure 2.1

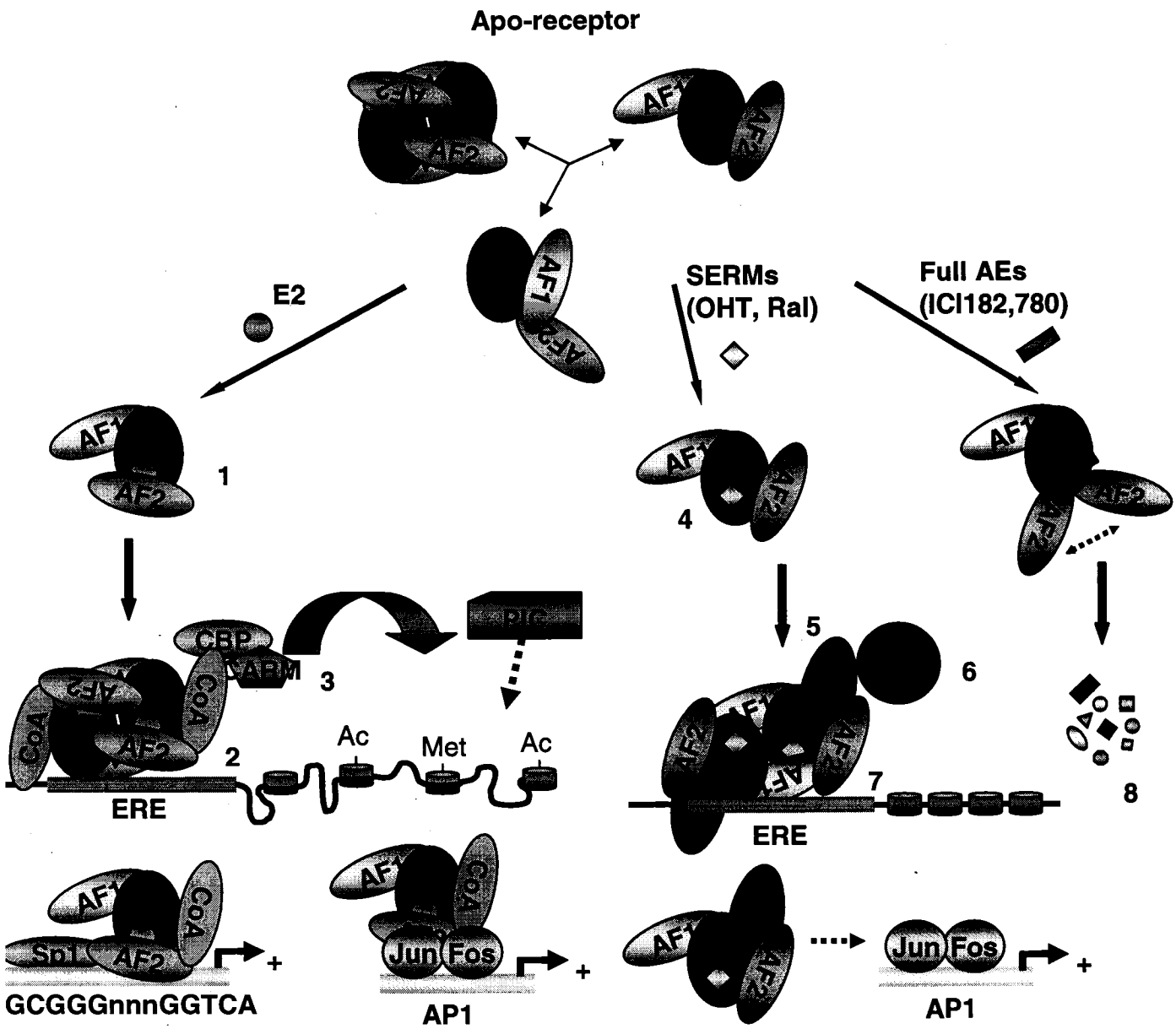
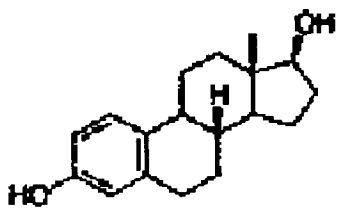

Apo-receptor
E2
SERMs
(OHT, Ral)
Full AEs
(ICI182,780)
AF1
AF2
1
4
CBP
SRC-1
3
CoA
2
Ac
Met
Ac
ERE
5
6
7
8
Sp1
GCGGGnnnGGTCA
Jun
Fos
AP1
+

Figure 2.2: Estrogen receptor ligands.

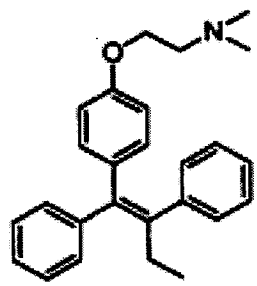
Shematic representation of estrogen recetors agonist (estradiol) selective estrogen receptor modulators (SERMs, tamoxifen and raloxifene) and full antiestrogens (ICI163,384 and ICI182,780)

Figure 2.2

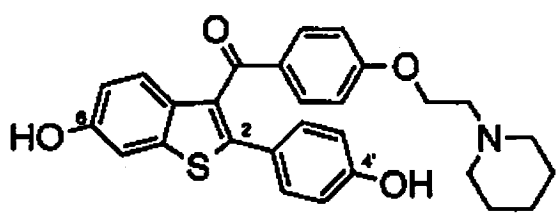
Estradiol

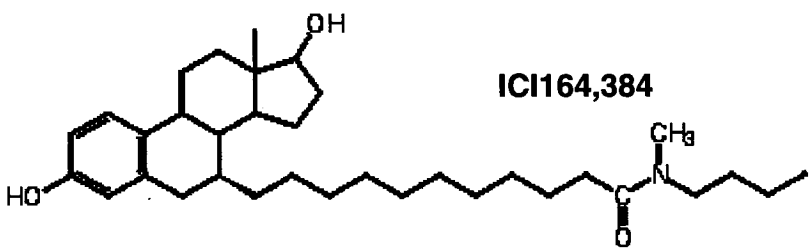


Tamoxifen

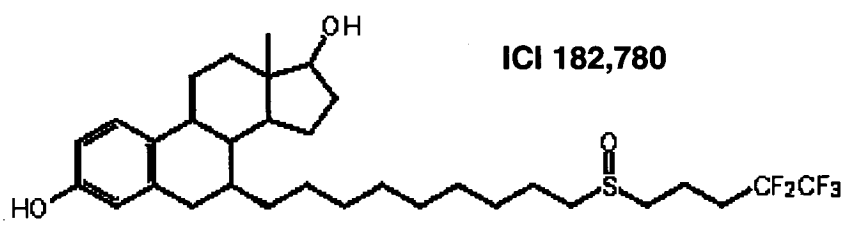


Raloxifene





ICI164,384



ICI 182,780

Figure 2.3: Crystal structure of ligand bound ERα

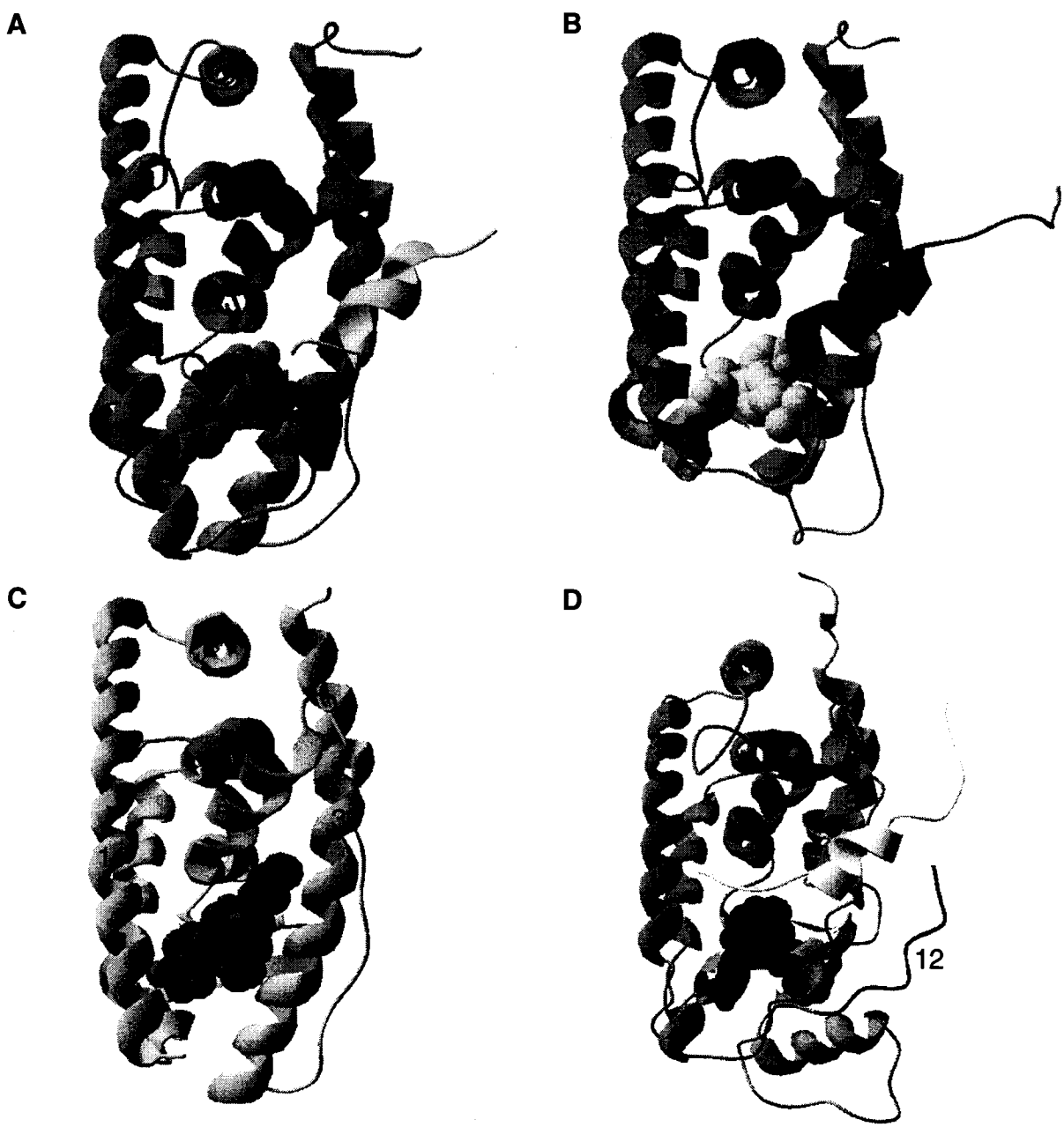
A: In presence of agonists, such as diethylstilbestrol (DES, green), the helix H12 (purple) of ERα ligand binding domain (LBD, blue) is characteristically positioned above the ligand-binding pocket. Coactivators (orange) may be recruited to the coactivator-binding groove established by H3, H4 and H5.

B: When SERMs, such as 4-hydroxytamoxifen (OHT, yellow), bind to ERα H12 is forced above the coactivator-binding groove.

C: When bound by the full antiestrogen ICI164,384 (ICI, dark orange), the H12 of the ERβ LBD is unresolved suggesting mobility of this helix. The antiestrogen side chain occupies the coactivator-binding groove preventing coactivator recruitment.

D: Antagonist (GW6471, red) bound PPARα indicates the stabilization of H12 in a novel conformation, along the outside rim of H3 while the corepressor peptide (yellow) occupies the groove established by H3, H4 and H5.

Figure 2.3



Main research goals:

In order to facilitate the development of novel antiestrogens, which will increase choices in the armamentarium of available treatments for breast cancer or other estrogen receptor derived diseases, we investigated the molecular and structural determinants required for inactivation of the receptor. *In chapter 3*, we investigated how the structure of the side chain of SERMs could influence their antagonist potential on ERα. The SERM Ral has a tertiary amine in its side chain essential for its antagonist activity. The side chain of Tam also harbors a tertiary amine but its function had not been thoroughly investigated. This is the object of the studies described in chapter 3, which make use of Tam derivatives with a modified side chain. *In chapter 4*, our goal was to clarify the mechanisms for ERα inactivation by full antiestrogens. Prior research had demonstrated that full antiestrogens could lead to receptor degradation. From a structural point of view, the side chain of ICI182,780 prevents helix H12 of the LBD from associating with the LBD and inhibits coactivator recruitment. We observed that in HepG2 cells full antiestrogens do not lead to degradation of the receptor but to accumulation in an insoluble fraction. We examined the role of long hydrophobic amino acids at specific positions in H12 in this phenomenon and investigated the structural constraints between antiestrogen side chains and these amino acids. *In chapter 5*, our final goal was to further characterize the role of receptor degradation in the mechanims of action of full antiestrogens and to map the key residues of ERα that direct its ubiquitination and degradation by the 26S proteasome in the presence of different ligands. Indeed, ERα is ubiquitinated and degraded through the proteasome-degradation pathway. However, no elements of this pathway have clearly been identified. Therefore, we mapped the domain involved in ubiquitination and investigated the implication of various lysine residues as potential ubiquitin attachment sites. *Chapter 6* finally discusses the overall contributions

resulting from these studies and their impact in the field of antiestrogen development.

Chapter 3: DIFFERENCES BETWEEN TAMOXIFEN AND RALOXIFENE IN THEIR FUNCTIONAL INTERACTIONS WITH ASPARTATE 351 OF ESTROGEN RECEPTOR ALPHA.

RUNNING TITLE: Antagonist activity of tamoxifen derivatives.

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Keywords: SERMs, Tamoxifen, Raloxifene, antagonist activity, agonist activity, estrogen receptors.

The abbreviations used are: AF1, activation function 1; AF2, activation function 2; CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; ERα, estrogen receptor alpha, ERβ, estrogen receptor beta; H12, helix 12; LBD, ligand binding domain; LUC, luciferase activity; OHT, 4-hydroxytamoxifen; Ral, raloxifene; SERM, selective estrogen receptor modulator; Tam, tamoxifen; wt, wild type.

3.1) Abstract

The bulky side chains of antiestrogens hinder folding of the ligand-binding domain (LBD) of estrogen receptors (ERs) in a transcriptionally active conformation. The presence of a tertiary amine in the side chain of raloxifene (Ral), which interacts with a negatively charged residue in helix H3 of the ER LBD (D351 in hERα), is important for antiestrogenicity in animal and cellular models. Here we have examined the influence of tertiary amine substituents and of mutations at position 351 in hERα on the activity profiles of tamoxifen (Tam) derivatives. Results obtained in several cellular model systems suggest that the degree of antiestrogenicity of Tam derivatives is neither significantly decreased by preventing interaction with D351, nor maximized by increasing the basicity of the side chain. However, suppression of the negative charge at position 351 abolishes the agonist activity of Tam in HepG2 cells. Moreover, the low levels of agonist activity of Ral and of a specific Tam derivative, compound 9, were increased to levels similar to those observed with Tam by mutation of D351 to glutamate. This agonist activity of compound 9 and Ral is dependent, as for Tam, on the activation function 1 (AF1). Our results suggest that contrary to Ral, Tam and most of its derivatives do not interact with D351 in an optimal manner, although this can be improved by modifying tertiary amine substituents.

3.2) Introduction

Antiestrogens are synthetic estrogen analogs used clinically in breast cancer treatment to inhibit the proliferative action of estrogens (1-3). Antiestrogens compete with estrogens for binding to estrogen receptors ERα and ERβ (4-6), which act as ligand-dependent transcription factors. ERs, like other members of the nuclear receptor superfamily, contain two transcriptional activation functions flanking a central DNA binding domain (7-9). The C-terminal activation function (AF2), which is part of the ligand binding domain (LBD), recruits coactivators in the presence of estrogen, but not of antiestrogens (6, 10-16).

Typically, antiestrogens contain a bulky side chain attached to a steroid or steroid-like skeleton. The importance of the side chain for the antagonist activity of antiestrogens has been demonstrated in pioneering studies using uterotrophic assays in immature rats. A tamoxifen (Tam) derivative lacking its alkylaminoethoxy side-chain was fully estrogenic in this assay (17). Accordingly, crystallographic studies of the estrogen receptor LBD complexed to various ligands (18-22) have demonstrated that while antiestrogens interact with estrogen receptors in a manner similar to that of estradiol, the bulky side chain cannot be accommodated within the ligand binding cavity. As a result, the structures of the ERα LBD complexed to estrogen or to the antiestrogens Tam or raloxifene (Ral) differ markedly with respect to the position of the C-terminal helix 12 (H12), which in the agonist structure acts as a lid to the ligand binding cavity and participates to the coactivator recruitment surface (18, 19). In the antagonist-bound structures, the side chain of antiestrogens interferes with the positioning of H12 over the ligand binding pocket and H12 is repositioned over the coactivator binding groove, thus preventing recruitment of coactivators by the AF2 function.

The length and composition of the side chain can differ markedly between antiestrogens. Selective estrogen receptor modulators (SERMs)

like Tam and Ral, which behave as partial agonists in a tissue-specific manner, usually have a shorter side chain compared to full antiestrogens such as ICI164,384 and ICI182,780. Tam and Ral are estrogen agonists in bone, and Tam but not Ral is markedly estrogenic in the uterus of ovariectomized rats or mice (23-26). Tam and Ral both contain alkylaminoethoxy side chains, with different tertiary amine substituents. Consistent with the importance of the amine in the side chain of SERMs, replacement of the nitrogen in Ral with a carbon or a non basic nitrogen atom abolished the capacity of Ral derivatives to antagonize estrogen-induced increases in uterine wet weight (26) and resulted in increased estrogenic activity on ER-dependent transcription in stably-transfected MDA-MB-231 cells (27). In Tam derivatives, conversion to a non-basic aromatic amine also abolished antagonist activity in a rat uterotrophic assay (28), consistent with a possible role of the tertiary amine in the antagonist activity of Tam.

The position of the nitrogen atom of the Ral side chain in the crystal structure was found to be compatible with establishment of a hydrogen bond with the carboxyl group of amino acid D351 (18). In the case of Tam, the distance between the two groups is greater but compatible with electrostatic interactions (19). The functional importance of D351 in the antiestrogenic activity of SERMs has been independently supported by the demonstration that a D351Y mutation, characterized in a Tam-stimulated breast tumor (29), results in increased agonist activity of Ral on ERα-dependent transcription in transfected MDA-MB-231 cells (30). Mutation D351E had a similar effect (27). On the other hand, mutation of D351 into hydrophobic amino acids (A, V, G) that cannot establish a hydrogen bond with the tertiary amine preserved antiestrogenicity of Tam in cellular systems where this SERM is fully antiestrogenic (31), and mutation D351G was observed to reduce the agonist activity of both Tam and Ral in MDA-MB-231 cells (32). A model whereby neutralization of the charge of D351 is important for antiestrogenicity of SERMs has been proposed (27).

To further address whether Tam and Ral interact with D351 in a similar manner or whether differential interaction with D351 may be responsible for their different degree of agonist activity, we have examined the importance of interaction with D351 in a series of Tam derivatives in human cellular models. We have characterized the antiestrogenic properties of Tam derivatives containing different tertiary amine substituents on growth of ERα-positive MCF7 cells, on alkaline phosphatase activity in uterine Ishikawa cells, and on the transcriptional activity of hERα in HeLa and HepG2 cells. Our results indicate that the degree of basicity of the side chain in Tam derivatives did not correlate significantly with antiestrogenicity in several experimental systems. However, partial agonist activity was sensitive to the side chain structure, as a pyrrolidine derivative of Tam was observed to have lower partial agonist activity than the parental compound in HepG2 cells. Addition of a single carbon in the antiestrogen side chain was sufficient to restore partial agonist activity. Mutation D351E increased the agonist activity of the pyrrolidine derivative and that of Ral in HepG2 cells to levels of activity observed with Tam with the wild type (wt) receptor, but did not affect the activity of Tam. Similarly, the activity of Tam was not affected in cells where this antiestrogen has mostly antagonist activity (HeLa cells). Our results suggest that Tam and Ral interact differentially with D351 due to differences in side chain structure/conformation. The antagonist/agonist activity of Tam appears mostly independent of interaction with D351 in the cellular systems tested, but the lower agonist activity of Ral or, importantly, of some Tam derivatives are dependent on interaction with D351 and on the activation function 1 (AF1). Models for the role of D351 in modulating the agonist properties of SERMs are discussed.

3.3) Results

Interaction with D351 affects minimally the affinity of Tam derivatives for estrogen receptor alpha.

SERMs, but not full antiestrogens, contain a tertiary amine in their side chains that interacts with amino acid D351 in hERα. Structure-function analyses (26, 27, 29, 30, 32) have pointed to the importance of this group in modulating the antagonist/agonist properties of raloxifene (Ral). In tamoxifen (Tam) derivatives, antagonist activity was observed in a uterotrophic assay in immature rats (28) when the tertiary amine was replaced by a hydroxyl group, but not when it was part of a pyrrole group (compound **14,** see Fig. 3.1). As the hydroxyl but not the pyrrole group can engage in hydrogen bond interactions, these results are compatible with a requirement to establish a hydrogen bond with D351 in order to achieve antiestrogenicity. However, only low levels of agonist activity could be observed at the maximal concentrations of compound **14** used, and thus the absence of estrogen antagonism could also be explained by the low affinity of this compound for estrogen receptors in rat uterine tissue extracts. To further examine to which extent the structure of the tertiary amine modulates the potency of Tam derivatives in the inhibition of human ERα transcriptional activation properties, we measured the relative IC50s in the inhibition of estrogen-dependent expression in HeLa cells for a series of Tam derivatives with decreasing basicity of the tertiary amine (Fig. 1). The most significant effect was a 4.5-fold increase in the IC50 value for the non-basic, aromatic compound **14** (Table 3.1). This result was confirmed in hormone-binding assays and is consistent with, but less marked than the ~20-fold reduction in affinity for rat estrogen receptors (28). A 2.3-fold increase in the IC50 for compound **13**, which has the second-lowest pKa in this series of derivatives (8.7), was also observed

(Table 3.1). These results suggest that the basicity of the tertiary amine affects the affinity of interaction with human ERα minimally.

These results are consistent with our previous report that mutations of D351 to G, A, V, E or Y did not grossly affect the IC50 of the active metabolite of Tam, 4-hydroxytamoxifen (OHT), versus E2 in transactivation assays in Hela cells (31). To further substantiate these results, we compared IC50s for Tam, compounds **9** and **14** in both transactivation and hormone binding assays. Mutations D351A, D351E, D351V and D351Y were found to have little effect on affinity for E2 (Fig. 3.2A), and IC50s for Tam and compound **9** were only minimally affected by mutations D351A and D351V both in competitive transactivation assays (Fig. 3.2B-D, white bars) and in hormone binding assays (dark bars). The only substantial effect of D351A or D351V mutations was a ~20-fold increase in binding of compound **14**, which had reduced affinity for the wt receptor compared to Tam and other derivatives (table 3.1). This suggests that D351 destabilizes the aromatic side chain of derivative **14**, which may form other contacts in the absence of an acidic residue at position 351. A small, but reproducible increase in IC50 values (2- to 4-fold) observed for compound **9** with mutants D351A or D351V could also indicate a role for D351 in stabilizing binding of this compound.

A tamoxifen derivative with a tertiary amine that cannot engage in interaction with D351 retains antiestrogenicity in MCF7, Ishikawa and HeLa cells.

To further investigate whether the nature of the tertiary amine group in the antiestrogen side chain affects the antagonist/agonist activity of Tam derivatives, we characterized the activity of compound **14** and other Tam derivatives (Fig. 3.1) in different human cell lines. In human breast tumor ERα-positive MCF7 cells, Tam and one of its derivatives with a basic side chain, compound **9** (pyrrolidine derivative), fully suppressed the

stimulatory effect of estrogen (E2, 0.1 nM) on cellular growth (Fig. 3.3A). In this system, compound **14** also fully competed, albeit at higher concentrations, returning growth rates to those observed in the absence of ligand. While Tam and compound **9** had growth repressive effects at the maximal concentrations tested, the effect of compound **14** at 2000 nM was comparable to those observed with Tam at 1000 nM and with compound **9** at 100 nM. Thus, whether the lack of repressive effect on growth of compound **14** reflects its lower affinity for ERα or the absence of specific growth-repressive properties remains unclear. However, these results indicate that contrary to E2, compound **14** did not display significant agonist properties on MCF7 cell growth.

Compound **14** was also observed to repress estrogenic stimulation of alkaline phosphatase activity in uterine Ishikawa cells (Fig. 3.3B), although competition was only partial due to the higher concentrations of E2 used in this assay (10 nM). Importantly, compound **14** alone did not have increased basal activity compared to Tam and compound **9** in Ishikawa cells at all concentrations tested (Fig. 3.3C). Finally, we also tested the capacity of Tam, compound **9** and compound **14** to modulate expression of a minimal ERE3-TATA-Luc reporter vector in transiently transfected HeLa cells expressing hERα (Fig. 3.4A-C). There was no dose-dependent increase in transcriptional activity with any of the ligands including compound **14**, while full competition of estrogen-induced activity was observed with all compounds, although with different potencies. Similarly, no agonist activity was observed with compound **14** in cells expressing hERβ (data not shown).

These results suggest that the capacity of the side chain of Tam to engage in interaction with D351 is not required for its antiestrogenic properties in these experimental systems.

The agonist activity of Tam derivatives in HepG2 cells does not correlate with the degree of basicity of their side chains

In order to better characterize the levels of agonist activity of the different compounds on ERα-dependent transcription, we used transiently transfected HepG2 cells as a model (37, 38). Agonist activity of Tam was detected in this system, while transcriptional activity was fully repressed in the presence of Ral (Fig. 3.5, grey bars). Most Tam derivatives also displayed agonist activity, while compound **9** completely repressed transcription. No correlation could be observed between the levels of agonist activity and the basicity of the tertiary amine in the side chain of Tam derivatives (which decreases from compounds **7** to **14**). Variations in IC50s between the different compounds did not influence these results since saturating concentrations of antiestrogens were used in this assay (see Fig. 3.6), Note also that compounds with similar IC50s had variable levels of agonist activity (**#9**, **10**, **12**).

Compound 9 is devoid of partial agonist activity in HepG2 cells, but contrary to full antiestrogens does not down-regulate ERα levels.

In contrast to most Tam derivatives, compound **9** like Ral almost fully repressed basal activity (Fig. 3.6A), This repression occurred in a dose-dependent manner and was almost as effective as that achieved by ICI182,780 at saturation (Fig. 3.6B). Compound **9** had also greater efficacy than Tam at repressing E2-mediated reporter gene expression, although with lower potency compared to Ral and Tam (Fig. 3.6A, grey bars). It is noteworthy that compound **8**, which contains one more carbon in the side chain and compound **10**, with one more carbon in the cyclic amine (Fig. 3.1), both led to intermediary levels of activity between compound **9** and Tam. These results suggest that the specific structure of

the side chain of compound **9** is responsible for its capacity to repress fully ERα-dependent transcription.

Full antiestrogens such as ICI182,780, which completely antagonizes estrogen transactivation in HepG2 cells (Fig. 3.5 and 3.6B), induce degradation of ERα (33-36). We thus examined whether the increased antagonist activity of compound **9** in HepG2 cells resulted from depletion of ERα. Steady state levels of transiently transfected receptor were similar in the presence of Tam and compound **9** (Fig. 3.7), suggesting that the enhanced antagonist activity of compound **9** results from induction of a transcriptionally inactive conformation of the receptor rather than from induction of receptor degradation.

A charge at position 351 is necessary for the agonist activity of all Tam derivatives.

Since abolition of the charge at position 351 has been shown to suppress the agonist activity of Tam on estrogen target gene transcription in MDA-MB-231 cells (27,32), we examined the effect of mutations D351A and D351V on the partial agonist activity of Tam and its derivatives in transfected HepG2 cells. As we reported previously (31), loss of activity was observed with these mutants in the absence, but not in the presence of hormone (Fig. 3.5). We have proposed that the lack of basal activity of these mutants is likely due to a role of the charge of D351 in maintaining an active conformation in the absence of hormone. Stabilizing interactions include hydrogen bonds involving D351 and the peptide backbone of the N-terminus of H12 in its agonist conformation and/or to the stabilization of the H12 N-terminus through an electrostatic capping effect involving the charge of D351. Mutations D351A or D351V also completely abolished transcriptional activity in the presence of Tam and all Tam derivatives with agonist activity (Fig. 3.5). Thus the presence of a negative charge at

position 351 appears important for the agonist activity of all Tam derivatives in HepG2 cells.

Mutations D351E and D351Y restore the agonist activity of compound 9 and Ral while minimally affecting Tam activity.

Contrary to mutations D351A/V, mutation D351E conserves the negative charge at position 351. Consistent with a stabilizing role of a negative charge at this position in the apo-receptor, this mutation did not abolish basal activity. However, the additional carbon in the side chain is likely to modify the relative positioning of this charge relative to the tertiary amine in the side chain of the antiestrogen. It has previously been shown that this mutation increases the agonist activity of Ral in MDA-MB-231 cells (27,32). The D351E mutant was also stimulated by Ral in our HepG2 assay (Fig. 3.5, white bars). Further, transactivation levels in the presence of compound **9** were also increased by this mutation, reaching levels observed with Tam (Fig. 3.5). Note that derivative **9** and Ral can compete out E2 (0.5 nM) under the same experimental conditions (Fig. 3.6B), indicating full occupancy of the wt and mutant receptors at maximal concentrations. Remarkably, mutant D351E had similar levels of transcriptional activity in the presence of saturating concentrations of all Tam derivatives tested in HepG2 cells (Fig. 3.5, white bars). However, this mutation did not lead to major increases in the agonist activity of Tam or OHT in HepG2 or HeLa cells (Fig. 3.5 and data not shown). These results suggest that a free charge at position 351 mediates the agonist activity of SERMs, and that this charge is masked by the tertiary amine in the side chains of Ral or compound **9**, but not of OHT or Tam, unless it is released by replacement of aspartate 351 by glutamate

Mutation D351Y, like the alanine or valine mutations, represses basal activity in HepG2 cells. Contrary to the D351A/V mutants however, this mutation was reported to increase markedly the agonist activity of Ral

on ERα-dependent transcription in stably transfected MDA-MB-231 cells, suggesting that the capacity to engage in hydrogen bonds rather than a free charge at position 351 is important for agonist activity (27, 29, 30, 32, 39). In HepG2 cells, we observed only a minimal increase in the agonist activity for Ral (Fig. 3.5). A slightly more pronounced increase in the transcriptional activity was observed with compound **9**. Overall, the levels of agonist activity observed with mutant D351Y at micromolar concentrations of the Tam derivatives were widely variable, ranging from low levels of activity with derivatives **7**, **8**, **12** and **14**, intermediate levels with compounds **9**, **10**, **11** and **13** and high levels with Tam (Fig. 3.5). The variable degree of activity observed with this mutant in the presence of the different Tam derivatives suggests steric interference between the terminal group in the antiestrogen side chain and the tyrosine residue.

In conclusion, our data indicate that charged (aspartate or glutamate), or to a lesser extent uncharged (tyrosine) hydrogen bond acceptors at position 351 contribute to the partial agonist activity of Tam derivatives on ERα-dependent transcription in HepG2 cells. Further, a precise relative positioning of the negative charge of D351 and of the positive charge of the tertiary amine seems required for maximal suppression of the agonist activity observed with compound **9** and Ral, whereas the agonist activity of Tam appears insensitive to changes in the position of the charge at position 351.

AF1 is essential for the agonist activity of antiestrogens.

The agonist activity of Tam has been associated with its capacity to transactivate through the activation function 1 (AF1) (40, 41). We have investigated the implication of this activation function in mediating the agonist activity of SERMs with D351 mutants. We transiently transfected HepG2 cells with the full-length receptor, with D351 mutants (D351A, E or Y) or with derivatives thereof truncated in the AB region containing AF1

(ΔAB). Deletion of the AB region eliminates the AF1 activity and inactivates the wt receptor on minimal reporter vectors (data not shown). Transcriptional activity can be rescued by cotransfection of the core-domain of coactivator TIF2 (TIF2.1), member of the p160 family of coactivators (42, 43) in the presence of E2 and in the absence of ligand, but not in the presence of OHT (Fig. 3.8). Deletion of the AF1 function also abrogated the agonist effect of OHT on the permissive D351E and D351Y mutants (Fig. 3.8). Ral-dependent transactivation of D351E and D351Y was lost after AF1 removal (Fig 3.8). These results indicate a common mechanism of activation for Ral with D351 mutants and for Tam with the wt receptor, which is dependent on the activation of the AF1 function. Similar results were obtained with compound **9** (data not shown).

The full repression of transcriptional activation in the presence of Ral and compound **9** suggests that transcriptional activation function AF1 is repressed in the presence of these antiestrogens, but not of OHT. We next investigated whether addition of an independent activation function could release transcriptional activation in the presence of these compounds. Fusion of the wt or D351E mutant receptor with the VP16 transcriptional activation function generated a constitutively active receptor, which was also fully active in the presence of OHT or Ral, but not in the presence of ICI182,780. The D351Y and D351A were still ligand-dependent for transcriptional activation, demonstrating a need for activation function AF2 for the basal activity of the fusion protein. These mutants are however partially active in the presence of OHT and Ral. These results indicate that an exogenous activation domain can relieve transcriptional activity in the presence of Ral, suggesting that repression of AF1 activity is occurring through a mechanism specific to this activation function. Further, these results demonstrate that agonist activity can be observed with OHT and to a lesser extent Ral in the absence of a charge at position 351 in the ligand binding domain, indicating that the repressive

effect of the D351A mutation is at least in part specific to the receptor transcriptional activation functions.

3.4) Discussion

Numerous antiestrogens have been synthesized and characterized in various pre-clinical models to date, and several of these have important clinical applications for breast cancer treatment/prevention or for hormonal therapy in post-menopausal women (1, 38, 46, 47). Antiestrogenicity requires high affinity binding to estrogen receptors, and low intrinsic estrogenic activity. Although the presence of a side chain in estrogen receptor ligands is not an absolute requirement to achieve antiestrogenicity (48), most known ERα antagonists were initially designed as estrogen analogs with a bulky lateral chain. In addition, early evidence pointed to the conclusion that antiestrogenicity of SERMs is dependent not only on the presence of the side chain, but also on the position of the tertiary amine in this side chain (17, 49). Recent progress in our understanding of the effect of estrogen receptor conformation on recruitment of coactivators/corepressors (6, 18, 19, 38) has led to a new surge of interest in understanding the molecular determinants of SERM antagonist/agonist activity. Although the tertiary structures of estrogen receptors LBDs complexes to OHT or Ral are very similar, it is possible that small differences in these structures are at the basis of the differential activity of Tam and Ral in experimental models. Interaction between the tertiary amine in the side chain of SERMs and amino acid D351 of hERα (18, 19) appears to play an important role in the balance of antagonist/agonist activity in partial antiestrogens such as Ral (27, 30, 32). The purpose of this study was to investigate to which extent interaction with D351 affects the activity of Tam or of its derivatives carrying various tertiary amine groups. The previous observation that a pyrrole derivative of Tam, which contains a non-basic tertiary amine, did not antagonize estrogen activity in a uterotrophic assay (28), was compatible with a role of the tertiary amine in the antiestrogenicity of Tam derivatives. However, a previous study of D351 mutations in HeLa cells did not reveal loss of

OHT antagonist activity when mutations were introduced at position 351. To reconcile these apparently conflicting observations, we tested the antagonist/agonist activity of Tam derivatives in various estrogen-responsive cellular model systems.

Characterization of the potencies and affinities of various Tam derivatives and of the effect of mutations of D351 on these parameters indicate that interaction with D351 appears to contribute to the affinity of receptor/antiestrogen interaction only in a minor way compared to the 4-hydroxyl group (~50-fold difference in affinity between Tam and OHT in our assays). An exception was the pyrrole derivative, which had a markedly increased affinity (about 20-fold) for mutants where D351 was replaced by small hydrophobic amino acids, although its affinity was only 4-fold lower than that of Tam. Molecular modeling suggests that specific stabilizing interactions between the aromatic side chain of compound **14** and amino acids of the ligand binding domain can take place when D351 is replaced by alanine or valine, explaining the resulting higher affinity for these mutants compared to Tam (C. Loch and J.M. Wurtz, personal communication).

Results from this study demonstrate that when tested in cells in culture, the pyrrole derivative antagonized estrogenic action and did not display increased agonist activity compared to Tam in three different assays, i.e. (i) E2-dependent proliferation of breast MCF7 cells, (ii) E2-induced alkaline phosphatase activity in uterine Ishikawa cells and (iii) E2 transactivation of an ERE3-TATA-CAT reporter plasmid in transiently transfected HeLa cells. In addition, the pyrrole derivative repressed recruitment of LXXLL motifs by ERα to the same extent as Tam in a bioluminescence resonance energy transfer experiment (data not shown). Therefore, our data suggest that neither the charge of the tertiary amine nor its capacity to engage in hydrogen bond is crucial to the levels of antagonist activity obtained with Tam in these cellular models. Note that although the pyrrole derivative had weaker affinity than Tam for human

ERα, this effect was less marked than previously observed in rat uterine cytosolic assays (4-fold rather than 21-fold, 28). Weaker affinity for rat estrogen receptors compared to human receptors and/or metabolism of this compound *in vivo* may explain the lack of antagonist activity observed in uterotrophic assays, especially in view of the fact that this compound did not display marked agonist activity even at the highest dose. We note however that possible effects of the tertiary amine on other estrogenic functions such as non-genomic effects, or on pharmacokinetic properties may differ in the *in vitro* versus *in vivo* experimental settings.

Our study with Tam derivatives indicates that modulation of the basicity of the tertiary amine failed to increase the agonist activity of Tam. This contrasts with results obtained with Ral derivatives, since conversion of the piperidine group of Ral into a cyclohexane generated a derivative with estrogenic activity in stably transfected MDA-MB-231 cells (27). D351 mutations also had a differential effect on the activity of Tam/OHT and Ral. Whereas mutation D351Y and D351E were not sufficient to increase the agonist activity of Tam/OHT on minimal reporter vectors, even in cell systems where Tam was mostly an antagonist, these mutations converted Ral into a partial agonist comparable to Tam/OHT in HepG2 cells. These results are compatible with results reported in MDA-MB-231 cells stably expressing ERα derivatives carrying mutations at D351. In these cells, OHT behaved as a full agonist on expression of TGF-α, while Ral was repressive. Mutations D351E and, to a lesser extent, D351Y, increased the agonist activity of Ral on TGF-α expression. In addition, mutations D351A/V/G abolished the agonist activity of Tam/OHT in HepG2 cells, and mutation D351G abolished the stimulation of TGF-α expression by Tam. Therefore, results obtained in the two systems are similar but for a few observations. In HepG2 cells, mutation D351Y did not increase the agonist activity of Ral significantly compared to the effects of this mutation on TGF-α expression in MDA-MB-231 cells. This may be due to cell- or promoter-specific differences in coactivator/corepressor expression

profiles, or result from the differences in the sequence of the target gene response element, as allosteric effects of binding sites sequences on coactivator recruitment profiles have been described (51, 52).

Characterization of Ral derivatives has implicated the hinge region between the side chain and the steroid-like skeleton as the main determinant for the differential uterotrophic activity of Tam and Ral, but conversion of the piperidine ring of Ral into a dimethylamine increased agonist activity (26). Conversely, our results also indicate that the agonist activity of Tam can be lowered in some experimental settings by modification of the structure of its side chain. Compared to Tam, the pyrrolidine derivative of Tam had reduced agonist activity in the HepG2 system, and was a slightly more potent compound in all assays. Contrary to what is observed with antiestrogens such as ICI182,780, RU58,668 and GW7604 (33-36), this reduction in agonist activity occurred independently of induction of receptor degradation, suggesting that it reflects adoption of a receptor conformation that is less transcriptionally active. Interestingly, this decreased level of agonist activity was highly dependent on the precise position of the cyclic tertiary amine relative to the charge at position D351. Indeed, either addition of a carbon in the side chain or in the ring, or replacement of D351 by glutamic acid or, to a lesser extent, by tyrosine, led to increased agonist activity. This suggests that the side chain of derivative **9** is more optimally positioned than that of Tam for antagonist activity, in a manner that depends on the precise relative positioning of the tertiary amine and the charge of D351. Thus the profile of activity of derivative **9** with D351 mutants resembles that of Ral, rather than Tam. In addition, other derivatives with side chain containing tertiary amine with more bulky substituents than those of Tam had either similar or lower levels of agonist activity compared to Tam, but behaved in all cases like Tam, Ral and derivative **9** when assayed with mutant D351E. Thus, our results demonstrate that precise interaction with D351 is not critical in

the context of Tam, but appears to be the basis for the lower agonist activity of Tam derivatives with modified tertiary amines as well as of Ral.

The observation that Tam and its derivatives are antagonists even in the absence of interaction with D351 is likely to be explained by the steric clash generated by the antiestrogen side chain with helix 12 of the ER LBD in the agonist position, preventing recruitment of coactivators with LXXLL motifs through the AF2 function. The role of interaction of the tertiary amine with D351 in further suppressing the agonist activity in some Tam derivatives and in Ral is not completely clear at this point, but appears to involve suppression of the activity of both the AF2 and the AF1 function. Of interest, Ral and Tam differ in their capacity to recruit N-CoR corepressors (55), and it has been observed that Tam releases an inhibitory interaction between the ERα LBD and the A region, contributing to activation of AF1 in permissive systems, while the effect of Ral is not characterized (54). It is therefore possible that amino acid D351 may directly modulate some of these interactions through establishment of electrostatic or hydrogen bond interactions. In addition, we cannot rule out that the charge of D351 may contribute to weak AF2 activity, detectable only in the presence of the AF1 function through cooperative recruitment of coactivators. Experiments to address systematically the effect of D351 mutations on corepressor recruitment and on interaction with the AB region are underway in our laboratory, and should allow to better understand the role of D351 in modulating the partial agonist activity of SERMs.

3.5) Materials and Methods

Reagents and hormones

17β-estradiol (E2) and tamoxifen (Tam) were purchased from Sigma, Inc. [2,4,6,7- ^{3}H] Estradiol was purchased from Amersham Biosciences. ICI182,780 (ICI) and raloxifene (Ral) were kindly provided by Dr T. Willson, Glaxo-Wellcome Research Institute (North Carolina). Tam derivatives were provided by John A. Katzenellenbogen (University of Illinois, USA). Cell culture media were purchased from Wisent Inc. and fetal bovine serum was purchased from Sigma.

Cell culture and transient transfection assays:

HeLa and HepG2 cells were maintained in DMEM supplemented with 5% or 10% FBS, respectively, and switched 3 days before initiating experiments to medium without phenol red containing charcoal-stripped serum. Transient transfections for chloramphenicol acetyl transferase (CAT) assays in HeLa cells were carried out in 10 cm plates by the calcium phosphate coprecipitation method (31). DNA mixes contained typically 0.5 μg expression vector for wt ERα (pSG5-HEG0), for ERα mutants at position 351 (29), together with 2 μg reporter vector (ERE3-TATA-CAT/EBV, 32), and 2 μg internal control plasmid pCMV-βGal, and were supplemented to 15 μg total with pBluescribe-M13+ (Stratagene). Hormones were added 18-20 h after transfection, after removing the calcium-phosphate precipitates. For luciferase assays, HeLa cells (5 10^6 cells) or HepG2 cells (2 10^6 cells) were transfected by electroporation (0.24 kV, 950 μF) in a BioRad Gene Pulser II apparatus. DNA mixes contained typically 1 μg expression vector for wt ERα (pSG5-HEG0) or for ERα mutants at position 351 (31), together with 2 μg reporter vector (pERE3-TATA-Luc), and 2 μg internal control plasmid pCMV-βGal, and were supplemented to 40 μg total with Salmon Sperm DNA (Invitrogen). Cells were plated in 6-well plates (seeding density 8 10^5 or 3 10^5

cells/well, respectively) in phenol red-free DMEM containing 10% charcoal-stripped serum. Hormones were added after electroporation. Cells were harvested 48 h after transfection and lysed in 200 µl of lysis buffer (Tris-HCL 100 mM pH 7.9, 0.5% NP40, 50 mM DTT). Luciferase activities were measured in presence of luciferin substrate with Fusion Universal Microplate Analyser (Packard) and β-galactosidase activities were measured at 420 nM with a Spectramax 190 (Molecular Devices). Luciferase activities were normalized for β-galactosidase activities. For competition assays with ERα, estradiol (0.01 or 0.03 nM) and serially diluted antiestrogens were premixed in 1 ml medium prior to adding to cells. Cells were harvested by scraping with a rubber policeman and extracts were prepared for CAT assays by three cycles of freeze-thawing in Tris-HCl pH 8.0 (0.25 mM). CAT activity was measured after standardization for β-Galactosidase activity. IC50 values were calculated using the GraphPad Prism 3 software (GraphPad Software, Inc.). For western blot assays, HeLa cells were transfected with 20 µg of pSG5-HEG0 expression vector using the electroporation technique (10^7 cells, 0.24 kV, 950 µF in a BioRad Gene Pulser II apparatus). Hormones were added 24 h after transfection and incubated for 16 h. Whole cell extracts were analyzed by 8 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transfered onto PVDF membranes, and incubated with the anti-hERα mouse monoclonal antibody B10 (obtained from Prof. P. Chambon). Complexes were revealed by ECL (NEN Life Science Products) as recommended by the manufacturer.

MCF7 cell proliferation assays:

MCF7 cells were maintained in DMEM containing 5% serum. For growth curves, MCF7 cells were plated in 6-well plates (seeding density 3 10^4 cells/well) in phenol red-free DMEM containing 5% charcoal-stripped serum. Cells were supplemented with fresh medium containing hormones every 48 h throughout the course of the experiment (8 days). Cells from

triplicate wells were solubilized in 0.1 N NaOH and protein concentration were measured using a DC protein assay (BioRad).

Alkaline phosphatase assays:

Ishikawa cells were maintained in αMEM supplemented with 5% FBS. Three days before assaying for alkaline phosphatase activity, cells were switched to phenol red-free DMEM containing 5% charcoal-stripped serum, and then plated in 96 well-pates (2.5 10^4 cells/well). Treatments were performed in triplicate for 48 h, after which cells were washed in PBS twice, frozen at -80°C for 15 min, and incubated with 50 µl reaction buffer (p-nitrophenyl phosphate, 5 mM; MgCl2, 0.24 mM; diethanolamine pH 9.8, 1M). Plates were incubated at room temperature until production of a yellow color, and levels of p-nitrophenyl were quantified by measuring absorption at 410 nm.

Hormone binding assays:

HeLa cells were transfected by electroporation as described above for western blot assays. DNA mixes contained 20 µg expression vector for wt hERα (pSG5-HEG0) or for ERα mutants at position 351 (31), supplemented to 80 µg total with salmon sperm DNA. Cells were plated in 6-well plates (seeding density 1.6 10^6 cells/well) in phenol red-free DMEM containing 10% charcoal-stripped serum. Hormonal treatments were performed 24 h after transfection. For saturation binding assays, cells were incubated with increasing concentrations of ^{3}H-labeled estradiol (92.0 Ci/mmol, Amersham Biosciences, Inc.) for 2 h at 37°C. To determine non-specific binding, levels of bound ^{3}H-labelled estradiol were measured in the presence of 500-fold excess of non-labeled estradiol. Specific binding was obtained by subtracting non-specific binding from total levels of bound ^{3}H-labelled estradiol. For competition binding assays, cells were incubated with 0.5 nM ^{3}H-labelled estradiol with increasing concentrations of antiestrogens. Cells were incubated for 2 h at 37°C and radioactivity

was quantified after extraction by scintillation counting. Kd values for estradiol and IC50 values for antiestrogens were calculated using the GraphPad Prism 3 software (GraphPad Software, Inc.).

3.6) Acknowledgements

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3.8) Figure and table legends

TABLE 3.1 The basicity of antiestrogen side-chain does not significantly affect their affinity for hERα.

pKa values correspond to reported values for the corresponding secondary amine (28). Relative potencies of Tam derivatives were calculated from competition curves performed as described in Fig. 3.4A-C in HeLa cells transfected with expression vectors for wild-type (wt) ERα. Values (IC50s expressed as a percentage of the IC50 value measured for Tam) are the average of three independent experiments and are reproducible within ±30%. Relative affinities of Tam, compound **9** and compound **14** were obtained in competitive hormone binding assays in HeLa cells transiently transfected with an expression vector for wt ERα. Tritiated estradiol (0.5 nM) was premixed with increasing concentrations of Tam, derivative **9** or **14** (0.05 nM to 3 μM). Values represent the mean of four different independent experiments and are reproducible within ±30%.

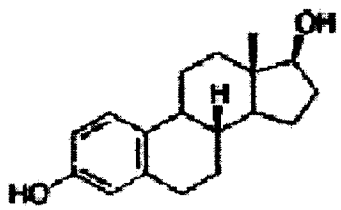
Table 3.1

	# 7	# 8	# 9	# 10	# 11	Tam	# 12	# 13	# 14
pKa	13.6	11.3	11.3	11.2	10.9	10.8	9.8	8.7	3.8
IC50 (Transactivation)	80.2	117.8	39.6	38.8	108.6	100.0	39.4	232.4	448.6
IC50 (Hormone Binding)			33.6			100.0			415.0

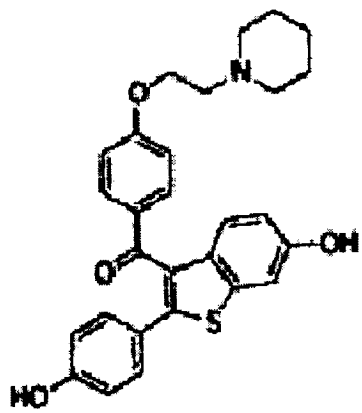
Figure 3.1: Tam derivatives

The structures of the Tam derivatives used in this study and of the parent compound are shown. The triphenylethylene skeleton is identical in all compounds.

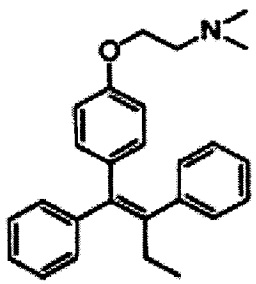
Figure 3.1



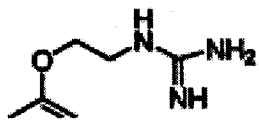
Estradiol



Raloxifene

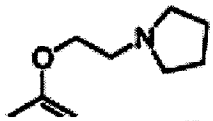


Tamoxifen



#7

#8



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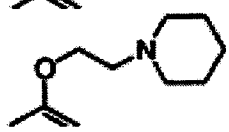

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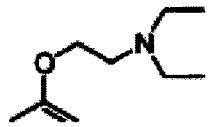

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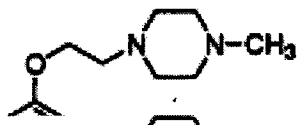

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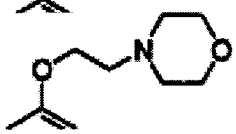

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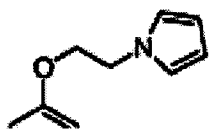


#14

Figure 3.2: Effect of mutations of amino acid D351 of hERα on the binding affinity of Tam derivatives.

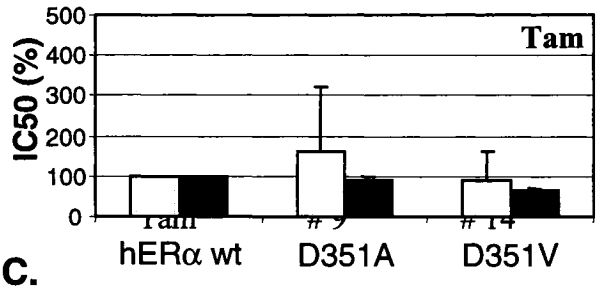
A: Kd values of hERα wt receptor or mutant receptors for estrogen as calculated from saturation binding assays in HeLa cells were transiently cotransfected with expression vectors for wt hERα. (pSG5-HEG0) or mutants D351A, D351V, D351E or D351Y (20 μg). Cells were treated 18-20 h after transfection with increasing concentrations of tritiated estradiol (0.03 to 16 nM), and harvested 2 h later. Panels **B-D** IC50 values for Tam, derivative **9** and **14** respectively, in competitive transactivation assays (light bars) or in competitive hormone binding assays (dark bars). For each compound, IC50 values are expressed as relative values for the mutant versus wild type receptor. Error bars indicate the standard deviation between three different independent experiments.

Figure 3.2

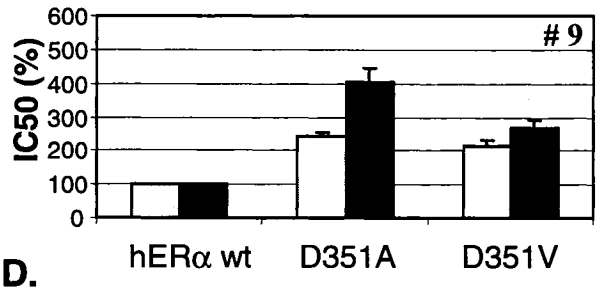
A.

hERα	WT	D351A	D351E	D351V	D351Y
Kd (nM)	0.35	0.75	0.28	1.16	0.53

B.



C.



D.

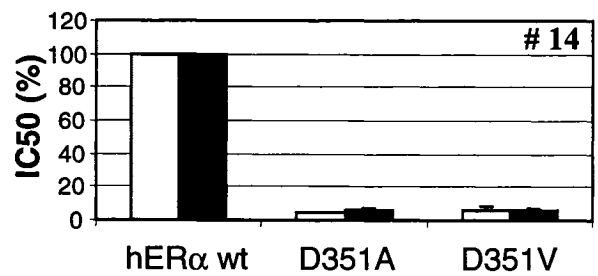


Figure 3.3: Agonist/antagonist activities of Tam derivatives in MCF7 and Ishikawa cells.

A. Repression of estradiol-induced growth of MCF7 cells by Tam derivatives. MCF7 cells were seeded at 3 10^4 cells/well in 6-well plates in phenol red-free DMEM and incubated in the presence or absence of estradiol (0.1 nM), either alone or premixed with increasing concentrations of Tam, derivative **9** or **14** (0.01, 0.1, 1, 10, 100, 1000 or 2000 nM). After 8 days, cells were harvested and protein concentrations were quantified. Error bars indicate the standard deviation between three different experiments with triplicate measurements for each point. **B.** Antagonist activity of Tam derivatives in Ishikawa cells. Cells were seeded at 2.5 10^4 cells/well in 96-well plates, and incubated with estradiol alone (0.1, 1 or 10 nM) or premixed (10 nM) with increasing concentrations of Tam, derivative **9** or **14** (1, 10, 100 or 1000 nM) for 48 h. Cells were then lyzed in the plate and alkaline phosphatase activity was assayed using production of p-nitrophenyl as a colorimetric assay. This experiment was reproduced three times with similar results. A typical experiment is shown, error bars representing the standard deviation between triplicate samples. **C.** Agonist activity of Tam derivatives in Ishikawa cells. Cells were incubated with increasing concentrations of E2 (1, 10, 100 or 1000 nM) or Tam, derivative **9** or **14** (10, 100 or 1000 nM) for 48 h and alkaline phosphatase activity was assayed as described above.

Figure 3.3

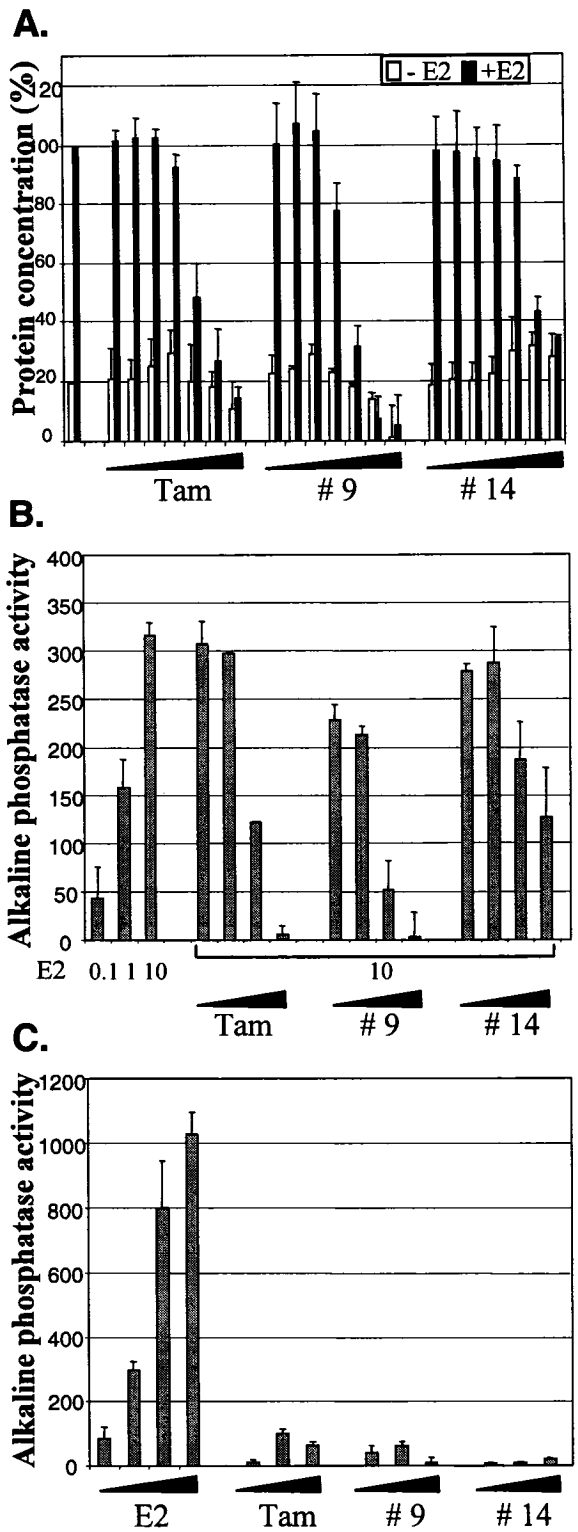

A.
- E2
+E2
Protein concentration (%)
120
100
80
60
40
20
0
Tam
9
14
B.
Alkaline phosphatase activity
400
350
300
250
200
150
100
50
0
E2
0.1 1 10
10
Tam
9
14
C.
Alkaline phosphatase activity
1200
1000
800
600
400
200
0
E2
Tam
9
14

Figure 3.4: Antagonist activity of Tam derivatives on hERα-dependent transcription in HeLa cells.

A-C An expression vector for ERα (pSG5-HEG0, 0.5 μg) was transiently cotransfected into HeLa cells along with the reporter vector ERE3-TATA-CAT/EBV (2 μg) and the internal control vector pCMV-βGal (2 μg). CAT activity was measured in extracts from cells treated for 48 h with increasing concentrations of Tam (**A**), derivative **9** (**B**) or **14** (**C**) administered either alone or premixed with estradiol (0.03 nM).

Figure 3.4

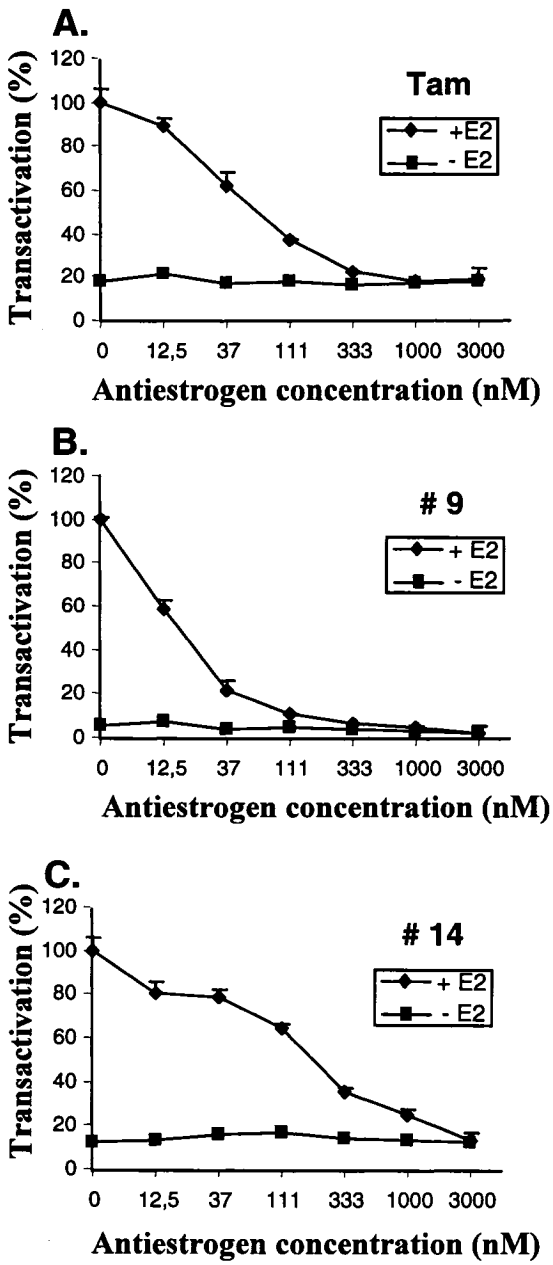

A.
Tam
+E2
- E2
Transactivation (%)
120
100
80
60
40
20
0
0
12,5
37
111
333
1000
3000
Antiestrogen concentration (nM)
B.
9
+ E2
- E2
Transactivation (%)
Antiestrogen concentration (nM)
C.
14
+ E2
- E2
Transactivation (%)
Antiestrogen concentration (nM)

Figure 3.5: Mutation D351E increases the agonist activity of tamoxifen derivative **9** and of raloxifene to levels observed with tamoxifen in HepG2 cells.

Expression vectors for ERα wt (pSG5-HEG0, 1 μg) or D351 mutants (pSG5-HEG0-D351E, Y, A or V, 1 μg) were transiently cotransfected into HepG2 cells along with the reporter vector p-ERE3-TATA-Luc (2 μg) and the internal control vector pCMV-βGal (2 μg). Luciferase activity was measured in extracts from cells treated for 48 h with vehicle (0), E2 (25 nM), ICI 182,780 (100 nM), Ral (100 nM), Tam or its derivatives (1000 nM). This experiment was performed two times with similar results. Error bars indicate the standard deviation between triplicate samples.

Figure 3.5

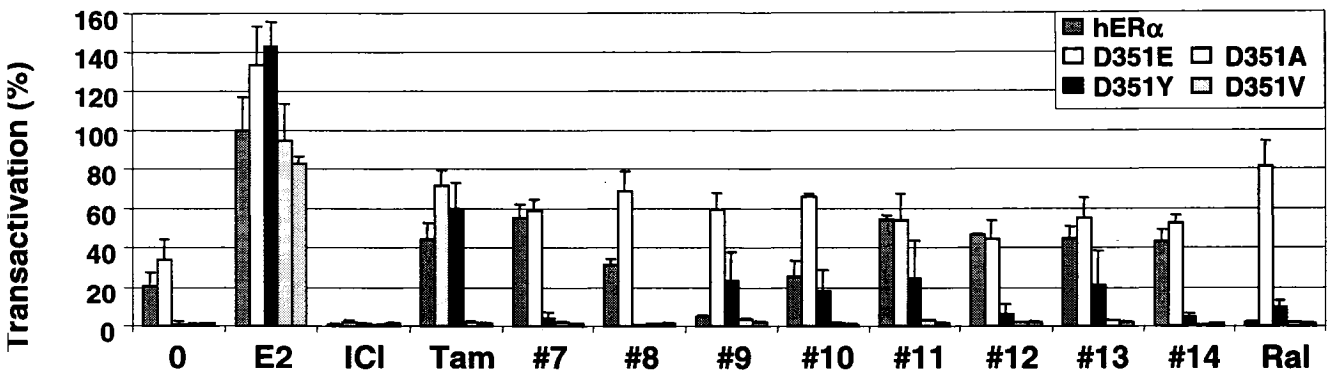

Transactivation (%)
160
140
120
100
80
60
40
20
0
hERα
D351E
D351A
D351Y
D351V
0
E2
ICI
Tam
#7
#8
#9
#10
#11
#12
#13
#14
Ral

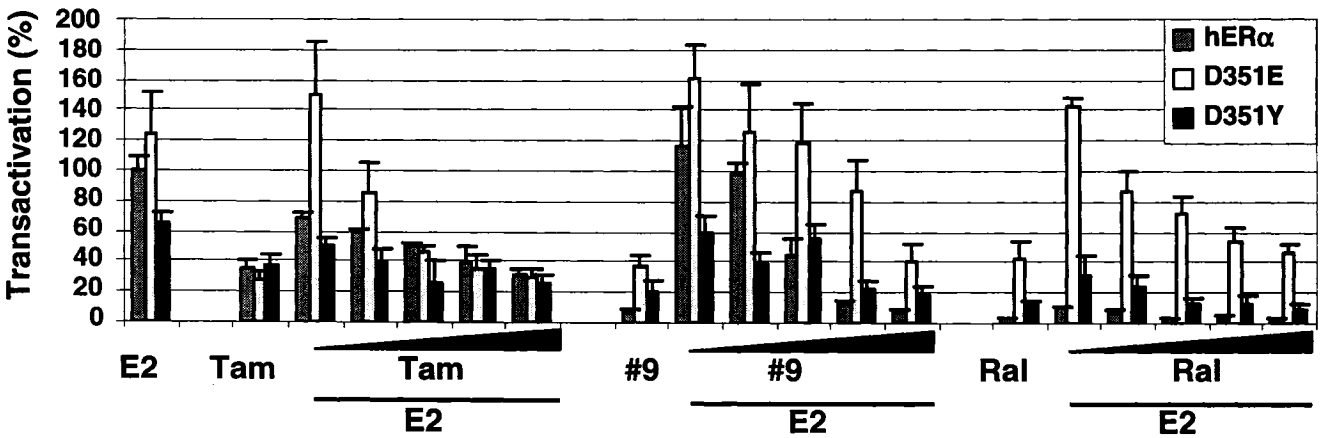
Figure 3.6: Agonist activity of Tam, derivative 9 and 14 on ERα-mediated transcription in HepG2 cells.

A. Competition assays performed as in Fig. 3, but using HepG2 cells transiently cotransfected with expression vectors for ERα wt (pSG5-HEG0, 1 μg) or D351 mutants (pSG5-HEG0-D351E or pSG5-HEG0-D351Y, 1 μg), reporter vector p-ERE3-TATA-Luc (2 μg) and the internal control vector pCMV-βGal (2 μg). Increasing concentrations of Tam, Ral or compound **9** were added either alone (1000 nM) or premixed at different concentrations (0.1, 1, 10, 100 and 1000 nM) with E2 (0.5 nM) before addition to cells in culture. Error bars indicate the standard deviation between two different experiments, each performed in duplicate.

B. An expression vector for ERα (pSG5-HEG0, 1 μg) was transiently cotransfected into HepG2 cells along with the reporter vector pERE3-TATA-Luc (2 μg) and the internal control vector pCMV-βGal (2 μg). Luciferase activity was measured in extracts from cells treated for 48 h with vehicle (0), E2 (25 nM), ICI 182,780 (100 nM) or increasing concentrations of Tam, derivative **9** or **14** (respectively 0.1, 1, 10, 100 or 1000 nM). Error bars indicate the standard deviation between triplicate samples. This experiment was performed three times with similar results.

Figure 3.6

A.


Transactivation (%)
200
180
160
140
120
100
80
60
40
20
0
hERα
D351E
D351Y
E2
Tam
Tam
E2
#9
#9
E2
Ral
Ral
E2

B.

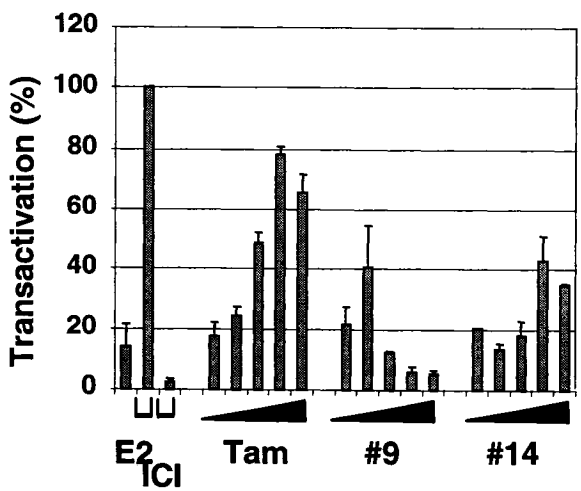

Transactivation (%)
120
100
80
60
40
20
0
E2
ICI
Tam
#9
#14

Figure 3.7: Compound 9 does not induce degradation of hERα.
An expression vector for wt ERα was electroporated in the HeLa cells (pSG5-HEG0) and hormonal treatment (16 h, 2.5 nM E2 or 100 nM antiestrogens) was performed 24 h after electroporation. ERα wt and β-actin protein levels were assessed by Western blot analysis using antibody B10.

Figure 3.7

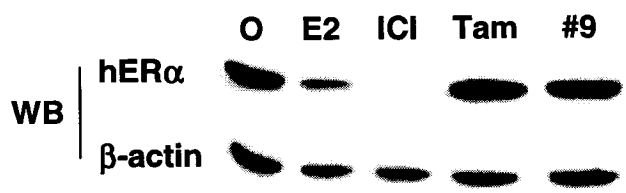
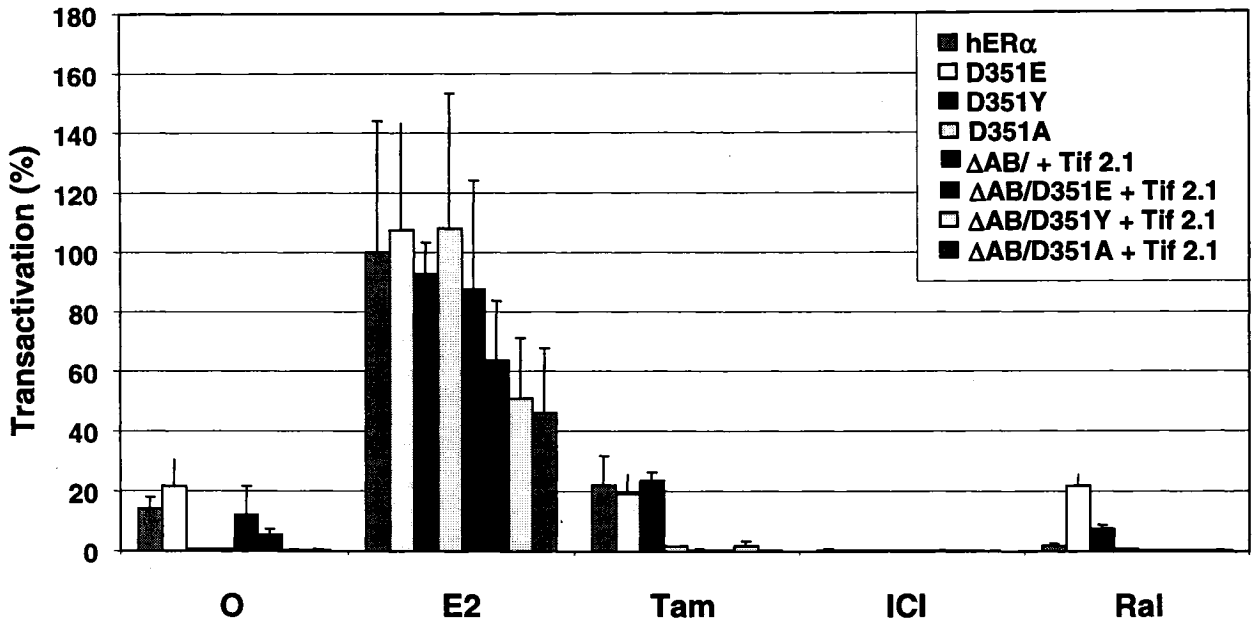


Figure 3.8: The AF-1 activity is required for the agonist activity of SERMs. Transient transfection analysis in HepG2 cells of the transcriptional activity of the wt ERα, the D351E, Y and A mutants or derivatives thereof deleted of the AB region in the absence or presence of overexpressed TIF2.1 (4 μg) was performed as in Fig. 3.6B. Relative luciferase activity is shown.

Figure 3.8


Transactivation (%)
180
160
140
120
100
80
60
40
20
0
O
E2
Tam
ICI
Ral
hERα
D351E
D351Y
D351A
ΔAB/ + Tif 2.1
ΔAB/D351E + Tif 2.1
ΔAB/D351Y + Tif 2.1
ΔAB/D351A + Tif 2.1

Connecting statement: bridging chapter 3 and 4

In chapter 3 we demonstrated that the side chain of SERMs can interact differentially with residue D351 in the LBD of the ERα and that the nature of this interaction plays a role in modulating the transcriptional activity of the receptor in the presence of these antiestrogens.

In the following chapter we investigated the interactions between the antiestrogen side chain and the ERα LBD that are required for full antiestrogen action. As full antiestrogens lead to ERα degradation we also investigated the need for this process for the full antagonist activity of antiestrogens.

Chapter 4 : ROLE OF AF-2 HELIX 12 POSITIONING IN TRANSCRIPTIONAL REPRESSION OF ESTROGEN RECEPTOR ALPHA BY FULL ANTIESTROGENS

Running title: Mechanisms of full antiestrogenicity

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Key words : estrogen receptor alpha, antiestrogens, degradation, Helix 12

4.1) Abstract

The basis for the differential degree of activity of estrogen receptor alpha (ERα) in the presence of different antiestrogens remains incompletely understood. Here we show that in HepG2 cells, a model system for the partial agonist activity of antiestrogens, raloxifene (Ral) shares with the full antiestrogen ICI182,780 the properties of drastically repressing ERα-dependent transcription and of inducing ERα accumulation in an insoluble fraction. Although positioning of helix H12 in the receptor ligand binding domain (LBD) is comparable in the crystal structures with Ral or the partial antiestrogen 4-hydroxytamoxifen (OHT), our mutagenesis results reveal that replacement of several long hydrophobic H12 residues increased the partial agonist properties of Ral and/or ICI182,780, but not OHT, in a manner correlating with increased in soluble ERα content. Transcriptional activity of receptor mutants in the presence of ICI182,780 or Ral required, in addition to increased solubility, integrity of the charge at position 351 in the LBD and the N-terminal transcriptional activation region, similar to requirements for OHT activity with the wt receptor. Molecular modeling suggests that differential steric hindrance between the side chains of OHT, Ral and ICI182,780 and long hydrophobic amino acids in H12 contributes importantly to their effects on receptor solubility and activity.

4.2) Introduction

Estrogens, such as 17-β-estradiol (E2), have pleiotropic actions on a number of target tissues, including in the skeletal, reproductive, cardiovascular and central nervous systems (1-4). These actions are mediated by two estrogen receptors, ERα and ERβ (4, 5), members of the nuclear receptor superfamily of ligand-inducible transcription factors (6-9). Like other unliganded steroid hormone receptors, ERs are thought to interact in the absence of hormone with molecular chaperone complexes including the heat shock protein hsp90, the cochaperone p23 and immunophilins (10, 11). Hormone binding induces conformational changes resulting in binding to DNA (12-15) and in the ordered recruitment of a series of coactivator complexes responsible for histone acetylation, chromatin remodeling, and enhanced recruitment of the basal transcription machinery (16-21). Binding to DNA is achieved through specific interactions between the central DNA binding domain, corresponding to homology region C (22, 23), and palindromic estrogen response elements (EREs, (24-27)). Two transcriptional activation functions are localized on either side of the DNA binding domain. Activation function AF-2 is located in the C-terminal ligand-binding domain (LBD, region E), and recruits coactivators in a ligand-dependent manner. Activation function AF-1, in the N-terminal A/B region, can function in a ligand-independent manner and is very variable both in length and sequence in the nuclear receptor superfamily (5, 6, 28, 29).

The observation that estrogen induces proliferation of mammary epithelial cells and of ERα-positive breast tumor cells has led to the development of antiestrogens for the treatment and prevention of breast cancer (30-32). Antiestrogens are competitive antagonists of estrogen, and block the transcriptional activation properties of ERs. However, some antiestrogens display partial estrogenic activity in a tissue- and gene-dependent manner, hence their description as "selective estrogen receptor

modulators" (SERMs). In animal models, both 4-hydroxytamoxifen (OHT) and raloxifene (Ral) have a favorable, estrogen-like action in bone (33). However, OHT has marked estrogenic activity on the rodent uterus, while Ral has only low activity in this model (33, 34). On the other hand, full antiestrogens such as ICI164,384, ICI182,780 and RU58,668 (35-37) completely block transcriptional activity of ERs in breast and uterine tissues.

Transcriptional activity of ERs in the presence of OHT has also been observed in different cellular models, and correlates with activity of the AF-1 region (38, 39). OHT also stabilizes the ERα protein (40, 41), whereas estradiol stimulates its turnover. On the other hand, full antiestrogens induce a rapid loss of nuclear ERα, resulting in depletion of the receptor ERα from estrogen responsive promoters *in vivo* (15). Clearance of nuclear ERα correlates with proteasome-dependent degradation and/or formation of peri-nuclear aggregates (40-48). In ERα-expressing cells or in transiently transfected cell lines, Ral has often more limited agonist activity than OHT (49-54), but the effect of Ral on receptor levels in different cell models remains poorly characterized to date.

Antiestrogens have been shown by crystallography studies to bind to ERs in a manner similar to that of estrogen, but to prevent folding of the LBD into its agonist conformation due to steric hindrance of the antiestrogen side chain (55-57). In particular, helix 12 (H12), which is crucial for AF-2 activity, is displaced by the antiestrogen side chain from its position in the agonist conformation on top of the ligand-binding cavity. The crystal structures of ERα complexed to antiestrogens OHT or Ral are similar, with H12 associating with the coactivator binding groove formed by helices H3-H5, thus preventing coactivator recruitment by AF-2 (55, 56). On the other hand, in the crystal structure of rat ERβ complexed to ICI164,384, the longer side chain characteristic of full antiestrogens (35-37) interacts directly with the coactivator binding groove (57). The position of H12 is undefined, suggesting conformational flexibility. Thus, there is

evidence for differences in receptor conformation in the presence of different antiestrogens, although these structural differences could result from crystallization constraints. However, the precise functional consequences of these observations remain unclear, as both types of structures should lead to inactivation of AF2. In addition, the basis of the differential activity of receptors bound to Ral and OHT, which induce similar crystal structures of the LBD, is also currently unclear.

In this study, we have sought to analyze the functional mechanisms underlying the more pronounced antagonist action of Ral versus OHT in HepG2 cells. We observe that Ral, like full antiestrogens, induces accumulation of the receptor in an insoluble fraction, and that point mutations in the long hydrophobic amino acids of H12 led to increased transcriptional activity in the presence of Ral or ICI182,780 in a manner correlating with stabilization of the receptor, but necessitating additional determinants such as the integrity of the AF-1 region. The patterns of mutations in H12 residues leading to activation of the receptor in the presence of Ral or ICI182,780 were not identical, and modeling studies support the hypothesis that full antiestrogenicity result from higher degrees of steric hindrance between the antiestrogen side chains and specific long hydrophobic residues of H12, resulting in loss of soluble nuclear receptors.

4.3) Results

Raloxifene and full antiestrogens modulate levels of soluble ERα in HepG2 cells.

HepG2 cells are a well established model system to study the partial estrogenic activity of antiestrogens (14, 58-61). In these cells, co-transfected with an expression vector for human ERα and the ERE3-TATA-Luc reporter vector, transcriptional activity was observed in the absence of hormone, due to basal activity of the receptor, and was induced ~ 5-fold in the presence of estradiol. Saturating concentrations of OHT (which fully displace estradiol in competition experiments, data not shown) also led to transcriptional activation of the reporter vector, whereas either the full antiestrogen ICI182,780 or the SERM Ral fully repressed the receptor transcriptional activation properties (Fig. 4.1A). Thus, in this model system, Ral behaves more like a full than a partial antiestrogen. Transcriptional activation by the unliganded or agonist-bound receptor was dependent on the presence of helix H12, consistent with the role of H12 in recruiting coactivators when positioned on top of the ligand binding cavity (55, 56). Deletion of H12 also decreased activity in the presence of OHT, possibly due to increased recruitment of corepressors as previously suggested (62-64). Activity in the presence of ICI182,780 and Ral remained low, although a small but reproducible increase was observed in the presence of ICI182,780 compared to the wild-type (wt) receptor (Fig. 4.1A). Western analysis of the receptor levels in high-salt buffer extracts (HSB) confirmed the known effects of OHT and ICI182,780, on receptor levels, i.e. stabilization and depletion, respectively (40-43, 47, 48, 65-67). Of interest, Ral also induced disappearance of the receptor from HSB, resembling full antiestrogens in this respect. We then examined receptor levels in Laemmli buffer, which extracts not only high-salt soluble receptor found in the HSB but also insoluble receptor aggregates. Western analysis

revealed accumulation of the receptor in the presence of OHT, but also ICI182,780 or Ral, suggesting that the latter two antiestrogens induce accumulation of the receptor in an insoluble form (Fig. 4.1B). Western analysis of HSB from transfected HeLa cells and from MCF7 cells, which express endogenous ERα, indicated that Ral yielded receptor levels intermediary between those observed in the presence of OHT and of ICI182,780, thus supporting the generality of the conclusion that Ral and OHT differentially affect receptor levels (Fig. 4.1C). Of interest, deletion of H12 stabilized receptor levels in the presence of both Ral and ICI82,780, leading to patterns that were indistinguishable in HSB or Laemmli extracts (Fig. 4.1B). This suggests that H12 plays an important role in modulating receptor solubility in the presence of Ral or ICI182,780.

Specific long hydrophobic amino acids of H12 are important for transcriptional repression and insolubility of the receptor in the presence of Ral or ICI182,780.

Helix H12, which is present at the C-terminus of the LBDs of all nuclear receptors (Fig. 4.2A-B), is an amphipatic helix containing several long hydrophobic amino acids, which play a role in contacting the rest of the LBD to stabilize H12 positioning either in the agonist or antagonist conformation. Indeed, in the presence of agonists, residues 540 and 544 contact the rest of the LBD, and residues 539 and 543 make van der Waals contacts with coactivator LXXLL motifs (56). In addition, residues 540, 543 and 544 form an LXXLL-like motif which allows binding of H12 to the coactivator binding groove in the presence of SERMs in a manner that mimics that of the coactivator LXXLL motifs, and residues L536 and 537 form additional contacts with areas of the LBD outside of the coactivator binding groove (55, 56).

To analyze the amino acids of H12 that are important for accumulation of the receptor in an insoluble fraction in the presence of Ral

or ICI182,780 in HepG2 cells, and the relationship between this property and transcriptional repression, we performed an alanine scanning mutagenesis of all hydrophobic amino acids in H12. Note that although amino acids 536-537 are not part of H12 in the agonist conformation, they are incorporated into H12 in the presence of SERMs (55, 56), and therefore were included in this analysis. The activity of the different mutants was tested in HepG2 cells in the presence of agonists and antagonists. Activity in the presence of OHT was not drastically affected by any of the mutations (Fig. 4.3A). Marked increases in transcriptional activity in the presence of ICI182,780 were observed with several mutations (at positions 536, 539, 540, 543, 544). A subset of these mutations also increased agonist activity in the presence of Ral (536, 539, and to a lesser extent 544). Interestingly, while Ral had more agonist activity than ICI182,780 with mutants at position 536 and 539, mutant L540A had the opposite activity profile. Increased activity in the presence of antiestrogens was observed with mutants that had normal as well as reduced levels of estrogen-induced transcription. Similarly, there was no correlation between levels of basal activity, which were either reduced or increased by these mutations, and levels of activity in the presence of antiestrogens. This suggests that molecular determinants of activity in the presence of antiestrogens differ from those controlling transcriptional activity in the absence of ligand or in the presence of agonists. Finally, note that not all mutations in long hydrophobic residues generated increased activity of the receptor in the presence of Ral or ICI182,780, as mutants Y537A and L541A had an activity profile similar to that of wt receptor.

To assess whether mutant receptors with increased activity in the presence of antiestrogens have altered solubility profiles, we performed western analyses on all above-described ERα mutants. Receptor levels in the soluble fraction in the presence of ICI182,780 or Ral correlated well with transcriptional activity of the receptors (Fig. 4.3B). All mutants with

increased activity in the presence of Ral (L536A, L539A, and to a lower extent L544A) were present at markedly increased levels in HSB. In particular, protein levels and transcriptional activity were both as high in the presence of Ral as of OHT for the L536A and L539A mutants (Fig. 4.3A and B). In addition, mutants with increased activity in the presence of ICI182,780 (L536A, L539A, L540A, M543A and L544A) were all detected at higher levels in HSB than the wt receptor in the presence of this antiestrogen (Fig. 4.3B). Notably, mutant L540A had higher solubility in the presence of ICI182,780 versus Ral, while the reverse was true with mutant L536A (Fig. 4.3B), correlating with the transcriptional profiles in the presence of these antiestrogens (Fig. 4.3A). Finally, mutants Y537A and L541A had similar patterns of extraction in HSB as the wt receptor, as well as similar patterns of transcriptional activation, in the presence of OHT, Ral and ICI182,780. These observations suggest that mutations in H12 affect simultaneously receptor solubility and transcriptional activity in the presence of antiestrogens ICI182,780 or Ral.

Mutations in long hydrophobic amino acids of H12 relieve steric hindrance with the side chain of Ral or ICI182,780.

Since some mutations in hydrophobic residues (Y537A, L541A) had no effect on receptor solubility/activity, and others had antiestrogen-specific effects, our results suggest a specific role of individual H12 residues on receptor conformation in the presence of Ral or ICI182,780. To investigate this hypothesis, we superimposed complexes obtained in the presence of OHT (56), Ral (55), or ICI16,384, a compound closely related to ICI182,780 (57) to the ERα-E2 complex (68), and assessed the impact of antiestrogen binding on the agonist structure of the receptor. The side chain of antiestrogens created steric clashes with H12 in the agonist position at the level of amino acid L536 (OHT, Fig. 4.4B), L540 (Ral, ICI164,384 and to a lesser extent OHT, Fig. 4.4B-D) and/or M543

(ICI164,384, Fig. 4.4D). Depending on the extent of the clash, replacement of these residues by alanines may directly relieve steric hindrance. For instance, steric conflict between L540 and the side chain of ICI182,780, but not the more extensive overlap with the side chain of Ral, can be relieved by mutation to alanine (Fig. 4.4C-D), correlating with a gain in transcriptional activity in the presence of ICI182,780 but not Ral for this mutant. Replacement of L536 by alanine was also insufficient to relieve the steric clash with the OHT side chain, and did not generate increased levels of transcriptional activity in the presence of this antiestrogen. Note that in addition to direct relief of steric hindrance, mutations removing the long hydrophobic side chains of neighbouring amino acids, such as L536A, L539A or L544A may allow rearrangement of the side chain of Ral or ICI182,780 in a manner that may better accommodate H12 in the agonist position.

We also examined the effect of the Ral or ICI164,384 side chains on positioning of H12 in the coactivator binding groove by superimposing the corresponding structures (55, 57) with that of OHT-bound ERα (56). The side chain of Ral was not found to generate important steric conflicts with H12, since the closest amino acids, L536 and L539, could be accommodated to form van der Waals contacts (Fig. 4.5C) as observed in the crystal structure of ERα with Ral (55). The ICI164,384 side chain on the other hand led to steric clash with L536, and less critical hindrance with L540 (Fig. 4.5D), amino acids whose replacement by alanine residues increases agonist activity of ICI182,780.

Thus, many of the alanine mutations studied here appear to reduce steric hindrance between antiestrogen side chains and H12 positioned either on top of the ligand binding cavity or in the coactivator binding groove and may thus facilitate association of H12 with the LBD.

Mutations that increase transcriptional activity in the presence of Ral or ICI182,780 do not lead to detectable AF2 activity.

Since mutations in several long hydrophobic amino acids of H12 may facilitate positioning of H12 in an agonist-like conformation in the presence of Ral or ICI182,780, we examined whether gains in AF2 activity would explain increased receptor activity by assessing recruitment of an LXXLL peptide in a mammalian two-hybrid assay (69). As expected, the α/βI peptide was recruited to the wt ERα receptor only in the absence of ligand or the presence of E2, but not in the presence of any antiestrogen (Fig. 4.6A). Recruitment in the presence of E2 was not drastically affected with the L536A, Y537A and L541A mutants, all of which transactivated an ERE3-TATA-Luc reporter vector at least as well as the wt receptor. Loss of E2-dependent recruitment was observed with the mutants affecting amino acids 539, 540, 543 and 544, which are known to be involved in stabilization of H12 in the agonist position and/or in interactions with the coactivator LXXLL motif (55, 56). This effect is consistent with the decrease in transactivation capacity observed with these mutants (Fig. 4.3), although cooperativity of transcriptional activation between the three EREs in the reporter vector probably blunts the reduction in transactivation. No recruitment of the LXXLL peptide was observed in the presence of either Ral or ICI182,780 with any of the mutants that had increased transcriptional activity with these antiestrogens, i.e. L536A, L539A, L540A, M543A and L544A (Fig. 4.6A). These results indicate that the conformation of ERα mutants with increased agonist activity in the presence of Ral or ICI182,780 does not result in detectable AF-2 activity in this assay, and suggest involvement of additional functional determinants in the observed gains in transcriptional activity.

Increased solubility of ERα in the presence of Ral or ICI182,780 is not sufficient for partial agonist activity.

To further investigate the link between accumulation of ERα in an insoluble fraction and the antagonist activity of antiestrogens, we tested the effect on both parameters of mutations in other structural determinants of ERα known to affect the agonist activity of antiestrogens. Aspartate 351 in helix 3 of ERα LBD interacts with the tertiary amine present in the side chain of OHT and Ral (55, 56). Mutations D351E or D351Y increased transcriptional regulation of an ER target gene (TGFα) in the presence of Ral in stably transfected MDA-MB-231 cells, while on the other hand mutation D351G abolished activity in the presence of OHT (70, 71). In HepG2 cells, activity in the presence of ligand was not affected with the ERE3-TATA-Luc reporter vector, but basal activity was repressed by mutations D351G, D351V and D351Y (Fig. 4.7A) as previously observed in HeLa cells. This is consistent with a role of a negative charge at position 351 in stabilizing H12 in the agonist conformation by helix capping interactions at the N-terminus of H12 and by hydrogen bonds with the peptidic bonds of L539-L540 and L540-541 (72). In the presence of Ral, gain of activity with D351E, and to a smaller extent with D351Y, but not with D351G/V was observed (Fig. 4.7A), in agreement with reports in MDA-MB-231 cells (70, 71). Of interest, we observe here that the levels of extractable receptor in HSB in the presence of Ral are increased by all mutations, whether the corresponding receptor is transcriptionally active or not in the presence of Ral (Fig. 4.7A and B). Similar results were obtained in HeLa cells (data not shown). This suggests that conformational changes induced either by mutations that do not conserve the charge (D351G/V), or by a replacement that preserves the charge but increases the length of the 351 side chain (D351E), can both induce alterations in the LBD structure resulting in protection from aggregation, likely by affecting the relative positioning of H12 and the Ral side chain. However, the capacity of residue 351 to engage in H-bond interaction appears important for transcriptional activity of the stabilized receptor in the presence of Ral. Note that the mutations at D351 affected neither the

solubility or the activity of the receptor in the presence of ICI182,780, whose side chain does not interact with D351. However, mutation D351A introduced in the context of an L536 mutant with increased agonist activity in the presence of ICI182,780 (mutant L536P) abolished this gain in transcriptional activity (data not shown), suggesting that activity in the presence of all antiestrogens is dependent not only on solubility of the receptor but also on the charge of D351.

Since transcriptional activity in the presence of the partial antiestrogen OHT has been correlated with activity of the AF-1 region (38, 39), we have assessed whether this is also the case for activity of ERα mutants in the presence of full antiestrogens. In HepG2 cells, removal of the AF-1-containing AB region (ΔAB construct) practically inactivates the wt receptor, with only residual activity detectable in the presence of E2 on the minimal ERE3-TATA promoter. Transactivation in the presence of E2 and in the absence of ligand, but not in the presence of OHT, can be rescued by cotransfection of the core-domain of coactivator TIF2 (TIF2.1), member of the p160 family of coactivators (73, 74). Removal of the AF-1 activation function in mutant L536A, which displays increased levels of transcriptional activity in the presence of both ICI182,780 and Ral as well as in the absence of ligand, also led to loss of detectable activity in the presence of all ligands except for residual transcription in the presence of E2 (Fig. 4.6). While TIF2.1 expression could restore activity in the presence of E2 and the absence of ligand, it was unable to increase the activity of the truncated receptor ΔAB/L536A in the presence of OHT, ICI182,780 or Ral. Overexpression of the full length SRC1 coactivator, another member of the p160 family, also led to a partial rescue of activity in the presence of E2 or the absence of ligand, but failed to rescue activity in the presence of antiestrogens (data not shown). Similar results were obtained with ΔAB/L539A (data not shown). Together, these results suggest that the AF-1 region is required for cofactor recruitment mediating

the agonist activity of Ral and ICI182,780 with mutant receptors as well as for that of OHT with the wt receptor.

Altogether, these results suggest that increased levels of soluble receptor are necessary, but not sufficient to yield increases in partial agonist activity in the presence of antiestrogens ICI182,780 and Ral. Integrity of the AF1 region and of the charge at position D351 are both necessary for gains in transcriptional activity in the presence of these antiestrogens.

4.4) Discussion

Our goals in this study were to analyze the basis of the near complete transcriptional repression of ERα by the antiestrogen Ral in HepG2 cells, where OHT has partial agonist activity, to assess the contribution of the reduction in ERα levels induced by full antiestrogens in their antagonist activity, and to identify the molecular determinants playing a role in either or both properties. Our results show that in spite of the similar structures of the receptor ligand binding domain in the presence of OHT and Ral (55, 56) levels of ERα in whole cell extracts in the presence of Ral were severely reduced in HepG2 cells, similar to what is observed with full antiestrogens, while OHT stabilized the receptor. These observations are not specific to HepG2 cells, as receptor levels in the presence of Ral were intermediary between those observed with OHT and full antiestrogens in HeLa and MCF7 cells, although the extent of the reduction with respect to basal levels was variable depending on the cell line. In HepG2 cells, the observed reduction in receptor levels in whole cell extracts in the presence of Ral or full antiestrogens did not correspond to a proteasome-mediated degradation of ERα as described for full antiestrogen ICI182,780 in MCF7 cells (47, 48) but rather in the accumulation of the receptor in an insoluble fraction. Such an accumulation of the receptor in an insoluble fraction is also observed in MCF7 cells in the presence of the proteasome inhibitor MG132 ((48); and our own observations), suggesting saturation of the proteasome machinery in HepG2 cells. Note that perinuclear aggregates of receptors have been described in the presence of full antiestrogens in a number of studies (40-43, 47, 48, 66), and probably correspond to badly conformed receptors accumulating when the proteasome degradation pathway is saturated. Altogether, these results suggest that the near total lack of agonist activity of Ral in HepG2 cells may result from reduced levels of functional nuclear ERα compared to OHT.

In support to this hypothesis, our results indicate that all mutants with increased agonist activity in the presence of Ral had increased solubility in whole cell extracts (H12 mutants and mutants at position D351). The same correlation was established with several point mutants in H12 in the presence of ICI182,780, suggesting that increased solubibity of the receptor in the presence of these antiestrogens is a prerequisite for transcriptional activity. Note that gains in transcriptional activation were not limited to our synthetic reporter vector, as increased transcription of the endogenous estrogen target gene pS2 in the presence of Ral or ICI182,780 was observed in MDA-MB-231 cells stably transfected with the L536A mutant (data not shown). Nevertheless, mutants that were found in the HSB in the presence of Ral and/or ICI182,780 did not necessarily gain transcriptional activity on the minimal promoter tested. Complete deletion of H12 increased solubility in the presence of either antiestrogen, but led to a mostly inactive receptor under our experimental conditions. Similarly, the double mutation L539-540A was very weakly active under our experimental conditions although soluble receptor levels were increased in the presence of ICI182,780 or Ral (data not shown). Note however that activity of the latter mutant with ICI162,384 or ICI 182,780 has been reported in HepG2 cells cotransfected with the GRIP1 coactivator or in Cos-1 cells with an ERE-tk-CAT reporter vector (59, 75). Mutations D351V/G also resulted in increased levels of soluble receptor in the presence of Ral, without increased activity in the presence of this antiestrogen. Thus is appears that solubility of the receptor in the presence of Ral or full antiestrogens is necessary, but not sufficient for agonist activity on minimal promoters. This suggests that partial agonist activity may require additional functional determinants compared to those responsible for insolubility/aggregation.

Crystal structures obtained with ERs in the presence of agonists or antagonists differ most strikingly in the positioning of H12. This helix acts as a lid to the ligand binding cavity in the presence of estrogen (55, 56),

but is positioned in the coactivator binding groove in the presence of OHT or Ral (55, 56), while it is unresolved in the presence of the full antiestrogen ICI164,384 (57), a close parent of ICI182,780 (35, 36). However, these differences in LBD structures in the presence of different antiestrogens may reflect specific crystallographic constraints for each complex and dynamic exchange between different positions of H12 is likely to take place. For instance, location of H12 in the coactivator binding groove can be observed in the presence of estrogen when point mutations are introduced in three Cys residues, even though the resulting mutant is transcriptionally active *in vivo* (76). Recent NMR spectroscopy experiments also suggest that the LBD of nuclear receptors in absence of ligand can adopt a number of conformations generating a dynamic ensemble of conformational populations. Conversely, ligand binding is associated with a marked conformational stabilization of the ligand binding cavity and coactivator binding groove (77). Therefore, different antagonists may differ in their capacity to stabilize the LBD structure as well as in their capacity to induce a preferred conformation.

Hydrophobic residues of H12 establish ligand-specific contacts with residues in the LBD and play an important role in stabilizing H12 association with the LBD (55, 56). Our alanine-scanning mutagenesis in H12 indicates that mutations in a cluster of amino acids on the hydrophobic side of the amphipathic helix (see Fig. 4.2C) increase both ERα levels in HSB and agonist activity in the presence of the full antiestrogen ICI182,780. However, two hydrophobic residues (Y537, L541) did not affect activity in the presence of antiestrogens, demonstrating the importance of specific amino acids (L536, L539, L540, M543, L544) rather than of global H12 hydrophobicity. In addition, mutations in only a subset of the latter residues increased activity in the presence of Ral, suggesting an impact of structural determinants specific to each ligand.

Our modeling studies from available crystal structures indicate that the side chains of Ral and ICI164,384 create steric clashes with H12 in the agonist or antagonist position at the level of amino acids L536 (ICI164,384 with H12 in the coactivator binding groove), L540 (Ral and ICI164,384 with H12 in the agonist position and ICI164,384 with H12 in the coactivator binding groove) and M543 (ICI164,384 with H12 in the agonist position). There was a positive correlation between the capacity of mutation L540 to relieve local steric hindrance with the side-chain of ICI164,384, but not Ral, and gains in receptor solubility/activity. In addition, while some of the residues whose mutation generated increased stability and activity of the receptor do not appear to be involved in direct steric clashes, removal of their long hydrophobic chains may increase space available for rearrangement of the antiestrogen side chain or of neighboring bulky amino acids themselves sources of steric constraints. In addition, L544 points towards the Ral backbone in the ligand binding cavity when H12 is in the agonist position, and its mutation may allow for a better accommodation of Ral, which is bulkier than those of steroid derivatives or of OHT in this region. Overall, replacement of long hydrophobic amino acids of H12 by alanine residues may reduce structural changes due to the antiestrogen structures in a ligand- and residue-specific manner, resulting in stabilization of H12 and possibly of the overall LBD structure.

Lack of stable association of H12 with the LBD may play an important role in accumulation of ERα in insoluble fractions and/or degradation. Receptors with unstable H12 may be recognized as badly folded and targeted to aggresome/proteasome pathways. This hypothesis is consistent with the observation that either H12 removal or mutations that should stabilize H12 association with the LBD prevent this process. On the other hand, additional determinants were also important for agonist activity in the presence of Ral or ICI182,780, including the integrity of H12, the charge at position D351 and the presence of the AF1 region. All these determinants are also important for partial transcriptional activity with

OHT, suggesting similar mechanisms of action involving cross-talk between specific residues in the ligand binding domain and the AF1 region. Lack of detectable recruitment of LXXLL motifs in a two-hybrid assay, suggestive of inactive AF2 function, was also similarly observed either with OHT-bound wt receptor or with mutants with increased activity in the presence of Ral or ICI182,780. Nevertheless, we cannot exclude that antiestrogens allow only weak AF-2 activity, necessitating cooperativity with AF-1. Alternatively, the LBD surface formed in the presence of partial antiestrogens, or of full antiestrogens in permissive ERα mutants, may recruit specific coactivators via non-LXXLL motifs through an altered type of AF-2 activity. Additional experiments will be required to better define the LBD conformation necessary for agonist activity in the presence of antiestrogens, and to test possible models for the interplay between H12 conformation and AF-1 function.

4.5) Materials and Methods

Plasmids and reagents

17β-estradiol (E2), 4-hydroxytamoxifen (OHT) were purchased from Sigma (Sigma, Oakville, ON, Canada), ICI182,780 (ICI) was purchased from Tocris Cookson Ltd (Ballwin, MO, USA), and raloxifen (Ral) was obtained form Dr. T. Willson (Glaxo-Wellcome, Research Triangle Park, NC, USA). MG132 was purchased from EMD Biosciences, Calbiochem (EMD Biosciences, La Jolla, CA, USA). pSG5-ERα, pSG5-ERαΔ535-547 (ΔH12) and pSG5-HEG19 (ERαΔAB) and pSG5-TIF2.1 were kind gifts from Prof. P Chambon (38, 78). Mutants D351E, D351Y, D351G, and D351V (72) were described previously. Mutations at positions 531, 536, 537, 539, 540, 541, 543, 544 and 539-540 were introduced by site-directed mutagenesis using polymerase chain reaction (PCR) amplification of the ERα cDNA (the sequence of oligonucleotides used for mutagenesis is available upon request). Expression plasmids for ERα mutants were generated by subcloning the digested PCR fragments into the pSG5-ERα expression vector (792 base pair HindIII/BamHI fragment). Clones for each mutant were characterized by restriction digest and sequencing. The ΔAB/L536A mutant was generated by subcloning a 834 base pair *XbaI* fragment from pSG5-L536A into the pSG5-ER□□AB expression vector. Vectors pVP16-ERα, pM-peptide α/βI, and 5x GAL4-tata-Luc were generous gifts from Dr. D.P. McDonnell (69). Mutations L536A, L539A, L540A, L541A, M543A and L544A were introduced in the pVP16-ERα by exchanging a 1611 bp NotI-BamHI fragment.

Cell culture and luciferase assays.

Hela cells were maintained in DMEM (Wisent, St-Bruno, QC, Canada) supplemented with 5% fetal bovine serum (FBS, Sigma, Oakville, ON, Canada). HepG2 cells were maintained in DMEM supplemented with 10% FBS, and MCF7 cells in α-MEM supplemented with 10% FBS. Three

days before experiments HeLa cells were switched to phenol red-free DMEM medium containing either 5% charcoal-stripped serum while HepG2 and MCF7 cells were switched to phenol red-free DMEM medium containing 10% charcoal-stripped serum.

For luciferase assays, HeLa and HepG2 cells were electroporated (0.24 kV, 950 μF in a Biorad Gene Pulser II apparatus) and plated in 6 well plates ($8x10^5$ cells/well). Typically, a DNA mix contained 1 μg expression vector, 2 μg ERE3-TATA-Luc reporter vector, 2 μg internal control pCMV-βGal and 35 μg carrier salmon sperm DNA (Invitrogen, Burlington, ON, Canada); in addition 4 μg of the pSG5-TIF2.1 vector were used in experiments described in Fig. 6. 17-β estradiol (E2, 25 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI182,780 (ICI, 100 nM) or raloxifene (Ral, 100 nM) or vehicle (ethanol) were added immediately after electroporation. Cells were harvested 48 h later in lysis buffer (Tris-HCl 100mM pH 7.9, 0.5% NP40, 1mM DTT). For proteasome inhibition, HeLa and HepG2 cells were pretreated for 1 h with MG132 (10 μM) the day after transfection and subsequently treated with E2 (25 nM), OHT (100 nM), ICI (100 nM) or Ral (100 nM) for 6 h. Luciferase activity was measured in the presence of luciferin with a Fusion Universal Microplate Analyser (Perkin-Elmer, Woodbridge, ON, Canada) and was normalized for β-galactosidase activity, measured at 420 nm with a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). Each transfection was carried out in triplicate and repeated at least three times.

Two-Hybrid assays

HeLa cells were electroporated (0.24 kV, 950 μF in a Biorad Gene Pulser II apparatus) and plated in 6 well plates ($8x10^5$ cells/well). The DNA mix contained 1 μg of the expression vector for the Gal4-pep α/β I fusion protein, 1 μg expression vector for full-length wild-type ERα or mutants of helix 12 fused to VP16, 1 μg 5x GAL4-tata-Luc reporter, 1 μg internal control plasmid CMV-β̃/gal and 36 μg carrier salmon sperm DNA

(Invitrogen, Burlington ON, Canada).17-β estradiol (E2, 25 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI182,780 (ICI, 100 nM) or raloxifene (Ral, 100 nM) or vehicle (ethanol) were added immediately after electroporation. Cells were harvested 48 h later in lysis buffer (Tris-HCl 100 mM pH 7.9, 0.5% NP40, 1 mM DTT). Luciferase activity was measured and normalized for β-galactosidase activity as described above. All transfections were carried out in triplicate and performed a minimum of three times.

Western analysis of receptor levels

For western blotting, HeLa or HepG2 cells were transiently transfected by electroporation ($5x10^6$ cells) with 10 μg of pSG5 expression vectors containing wild-type or mutant ERα cDNAs and 30 μg carrier salmon sperm DNA (Invitrogen, Burlington, ON, Canada) and plated in 10 cm plates. Cells were treated with E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), Ral (100 nM) or vehicle overnight. Cells were harvested in ice-cold PBS, and whole cells extracts were prepared from half the cells by three freeze-thaw cycles in high salt buffer as previously described (79). The other half of cells harvested was resuspended in Laemmli sample buffer (80) and incubated at 100 °C for 5 min.

For western blotting of endogenous ERα in MCF7 cells, cells were plated in 6 well plates ($5x10^5$ cells/well). The following day, cells were pretreated for 1 hour with MG132 (10 μM) or vehicle (DMSO). E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM) or Ral (100 nM) were then added for 4 hours. Cells were harvested in ice-cold PBS, and whole cells extracts were prepared as described for HeLa and HepG2 cells or by resuspension in Laemmli sample buffer and incubation at 100 °C for 5 min.

Whole cell extracts from ERα–expressing cells (HeLa, HepG2 or MCF7) were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (7,5% acrylamide), transfer onto nitrocellulose, and incubation of membranes with an anti-ERα mouse monoclonal

antibody (B10, kind gift from Prof. P. Chambon). Complexes were revealed by ECL (NEN Life Science Products) as recommended by the manufacturer.

Modeling

In order to compare the structural effects of the various mutations on the agonist and antagonist conformations of ERα, the crystal structures of ERα complexed with E2 (PDB code 1GWR, (68), OHT (PDB code 3ERT, (56)), Ral (PDB code 1ERR (55)) and ICI164,384 (PDB code 1HJ1, (57)) were first superimposed, using the Lsq-man module of the O package (version 6 (81)). Mutations were introduced in each crystal structure using the O package.

4.6) Acknowledgements

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[1]The abbreviations used are: E2, 17β-estradiol; ER, estrogen receptor; SERM, selective estrogen receptor modulator; OHT, 4-hydroxytamoxifen; H, helix; LBD, ligand binding domain; Ral, raloxifene; AF, activation function; ERE, estrogen response element; HSB, High-salt buffer extract.

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4.8) Figure legends

Figure 4.1: The SERM raloxifene and the full antiestrogen ICI182,780 both efficiently repress ERα transcription and decrease soluble receptor levels in HepG2 cells.

A. Transient transfection experiments were performed in HepG2 cells by electroporation with an ERE3-TATA-Luc reporter, the internal control plasmid CMV-β-gal and expression vectors for wild-type ERα or mutant ΔH12, as indicated. Cells were treated with vehicle (0), estradiol (E2, 25 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI 182 780 (ICI, 100 nM) or raloxifene (Ral, 100 nM), for 48 h. Luciferase activity and β-galactosidase activity were quantified, and relative luciferase activity was calculated. Results from at least three experiments performed in triplicates are shown, error bars reflecting the standard deviation between independent experiments.

B. After transient transfection with expression vectors for wild-type or mutant ERα, HepG2 cells were cultured in steroid-free medium for 36 hours and subsequently treated with vehicle (0), estradiol (E2), 4-hydroxytamoxifen (OHT), ICI182 780 (ICI) or Raloxifene (Ral) for 16 hours (ligand concentrations as in A). High-salt buffer extracts (HSB, 50 μg) or Laemmli sample buffer were analyzed by SDS-PAGE and Western analysis using anti-ERα antibody B10. A representative result of three independent experiments is shown.

C. HeLa cells transiently transfected by electroporation with an expression vector for ERα (10 μg per 10 cm plate) or MCF7 cells were treated with estradiol (E2, 25 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI182,780 (ICI, 100 nM) or raloxifene (Ral, 100 nM) for 16 or 5 h, respectively. HSB were analyzed by SDS-PAGE and Western analysis as in B.

Figure 4.1

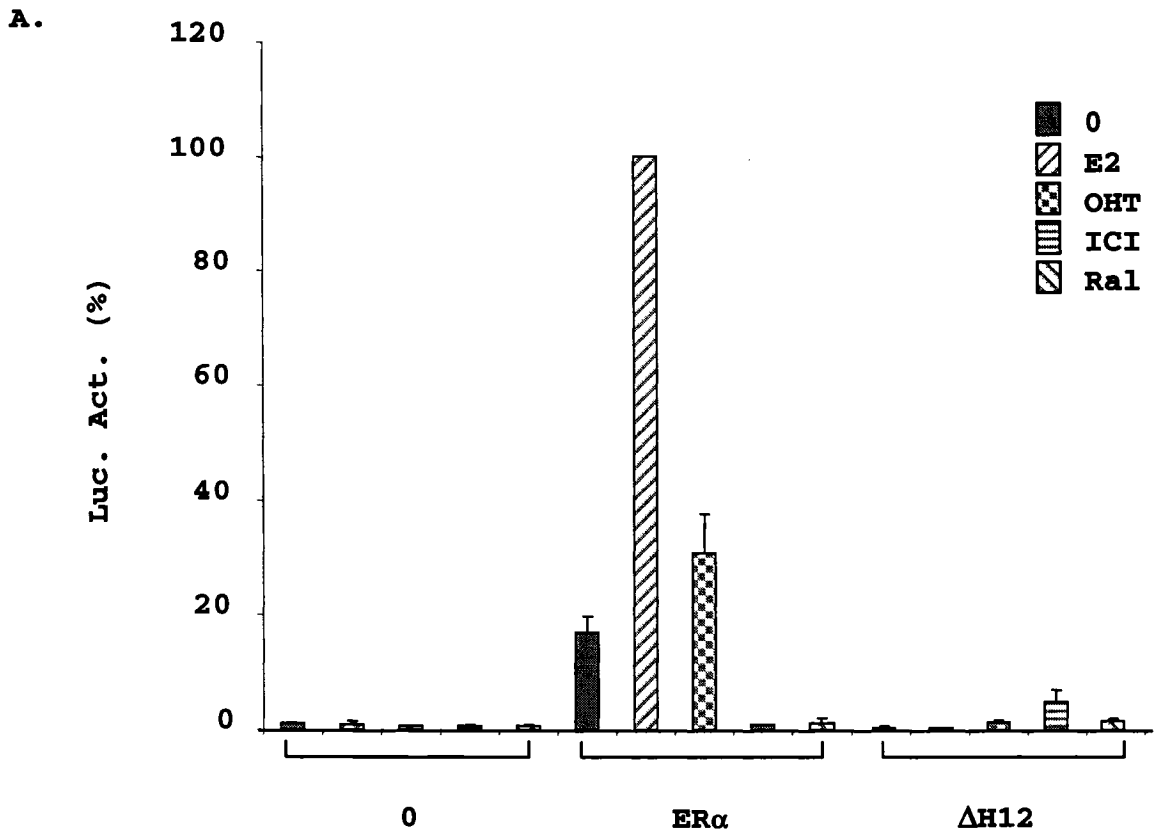

A.
120
100
80
60
40
20
0
Luc. Act. (%)
0
E2
OHT
ICI
Ral
0
ERα
ΔH12
B.
HSB
Laemmli
HepG2
0
E2
OHT
ICI
Ral
ERα
ΔH12
C.
HSB
HSB
HeLa
MCF7
0
E2
OHT
ICI
Ral
ERα

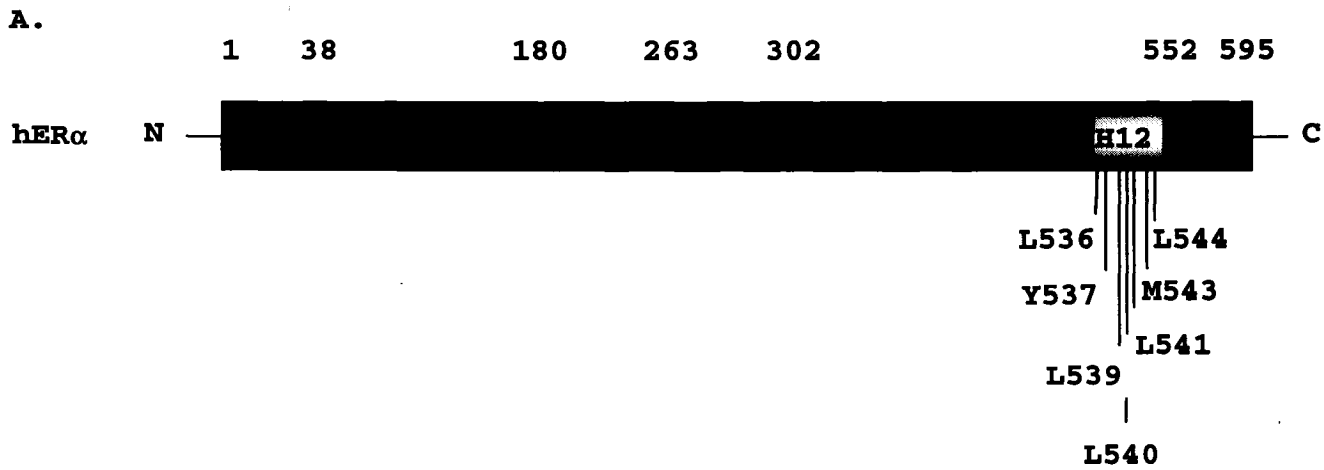
Figure 4.2: Conservation of hydrophobic amino acids in helix H12 in the nuclear receptor superfamily.

A. The domain organization characteristic of nuclear receptor is shown, and amino acids at the interdomain boundaries in ERα are indicated. The hydrophobic amino acids in helix H12 of the ligand binding domain are also highlighted.

B. Alignment of H12 and flanking sequences (residues 535-545 in ERα) in the nuclear receptor superfamily. Conserved hydrophobic amino acids are underlined (h: human; r: rat; m: murine).

C. Positioning of the hydrophobic residues in Helix 12. Nonpolar (yellow), polar and uncharged (green), or acidic (red) residues are placed on an alpha helical wheel (generated using software at http://cti.itc.virginia.edu/~cmg/Demo/contents.html)

Figure 4.2



B.

	H12
536-537	H12
hERα	-VVPLYDLLLEMLDAHR-
hERβ	-VVPVYDLLLEMLNAHV-
hERRγ	-KVPMHKLFLEMLEAKV-
hRXR	-DTPIDTFLMEMLEAPH-
hPR	-SVEFPEMMSEVTAAQL-
hAR	-SVDFPEMMAEIISVQV-
hGR	-SIEFPEMLAEIITNQI-
hMR	-KVEFPAMLVEIISDQL-
hPPAR	-DAALHPLLQEIYRDMY-
hCAR	-LSAMMPLLQEICS----
hLXR	-DKKLPPLLSEIWDVHE-
hFXR	-DHKFTPLLCEIWDVQ--
hVDR	-SMKLTPLVLEVFGNEI-
hRARγ	-PGPMPPLIREMLENPE-
mROR	-NTLFPPLYKELFNPDC-
rTRα	-TELFPPLFLEVFEDQE-

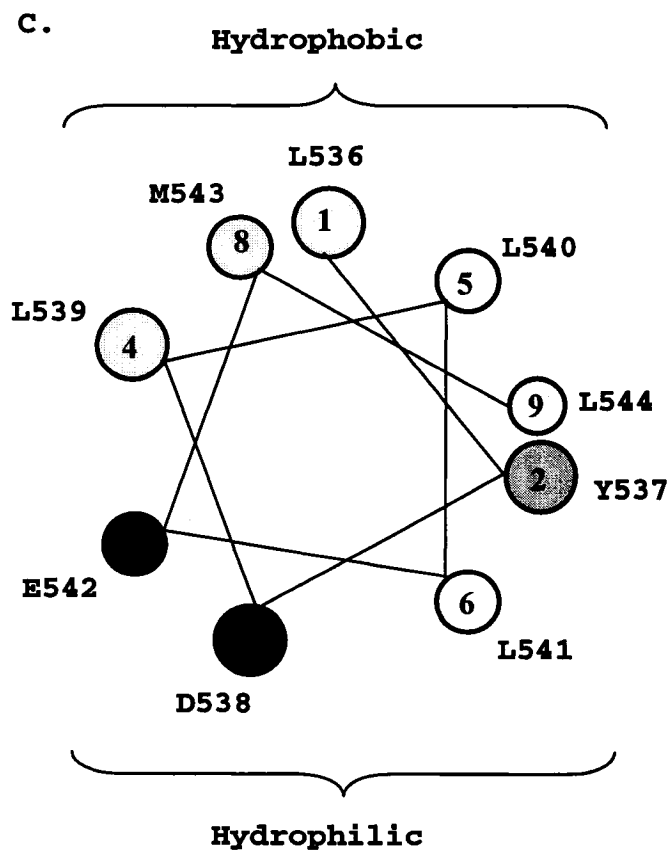


Figure 4.3: The long hydrophobic amino acids of H12 modulate receptor solubility and activity in an antiestrogen-specific manner.

A. HepG2 cells were electroporated with the ERE3-TATA-Luc reporter, the internal control plasmid CMV-β-gal and expression vectors for wild-type ERα or mutants affected in helix 12 hydrophobic residues. Cells were treated with hormones and harvested as described in Fig. 4.1A. Relative luciferase activity is shown. Significant differences between response to ligand and basal level was determined by a one-way anova (* = $p \leq 0.05$; ** = $p \leq 0.001$)

B. After transient transfection with expression vectors for wild-type or mutant ERα, HepG2 cells were cultured in steroid-free medium for 36 hours and subsequently treated with hormones as in Fig. 1B. High-salt buffer extracts (HSB) (50 μg) and Laemmli sample buffer extracts were analyzed by SDS-PAGE and Western analysis using anti-ERα antibody B10 as described in Fig. 4.1 A-B.

Figure 4.3

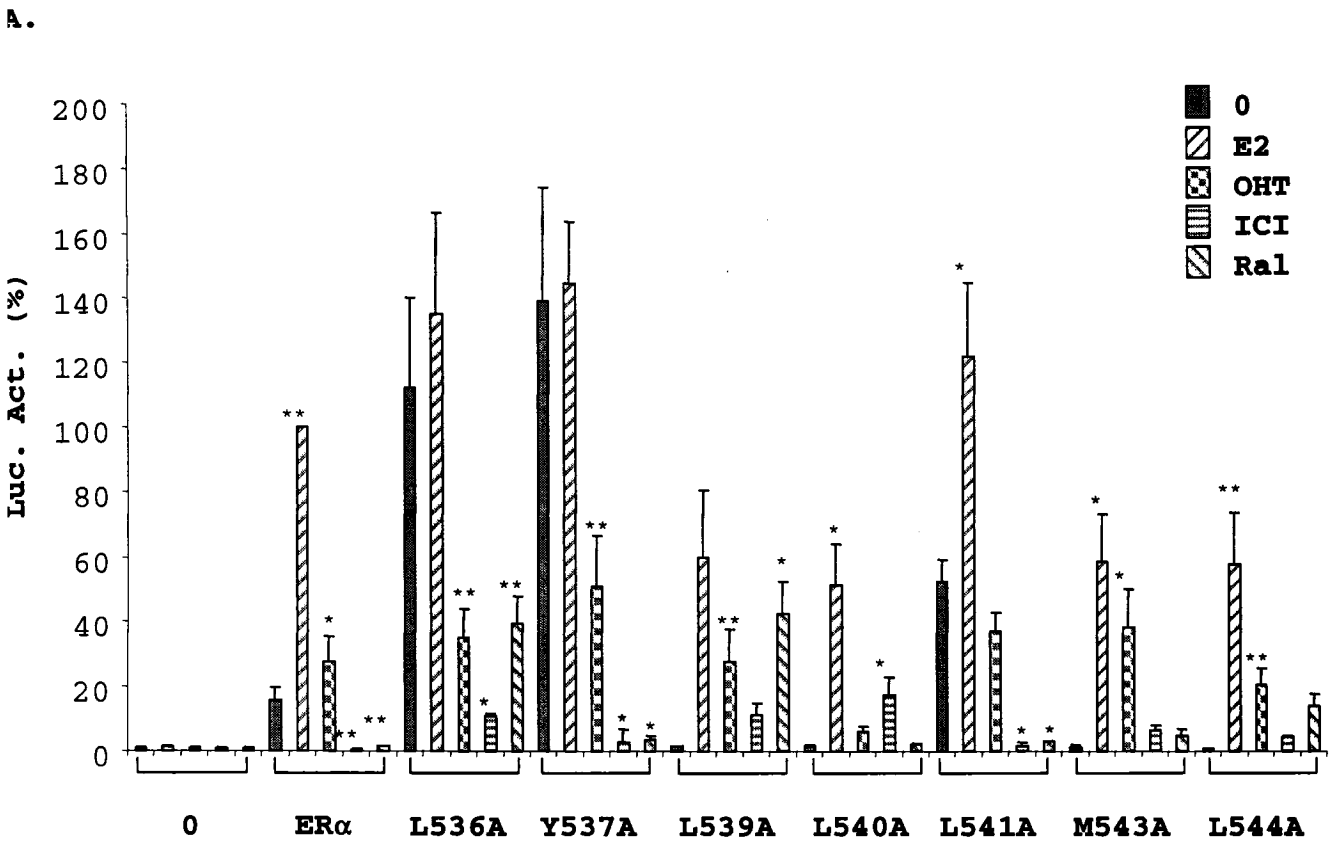

A.
Luc. Act. (%)
200
180
160
140
120
100
80
60
40
20
0
0
E2
OHT
ICI
Ral
0
ERα
L536A
Y537A
L539A
L540A
L541A
M543A
L544A

Figure 4.3

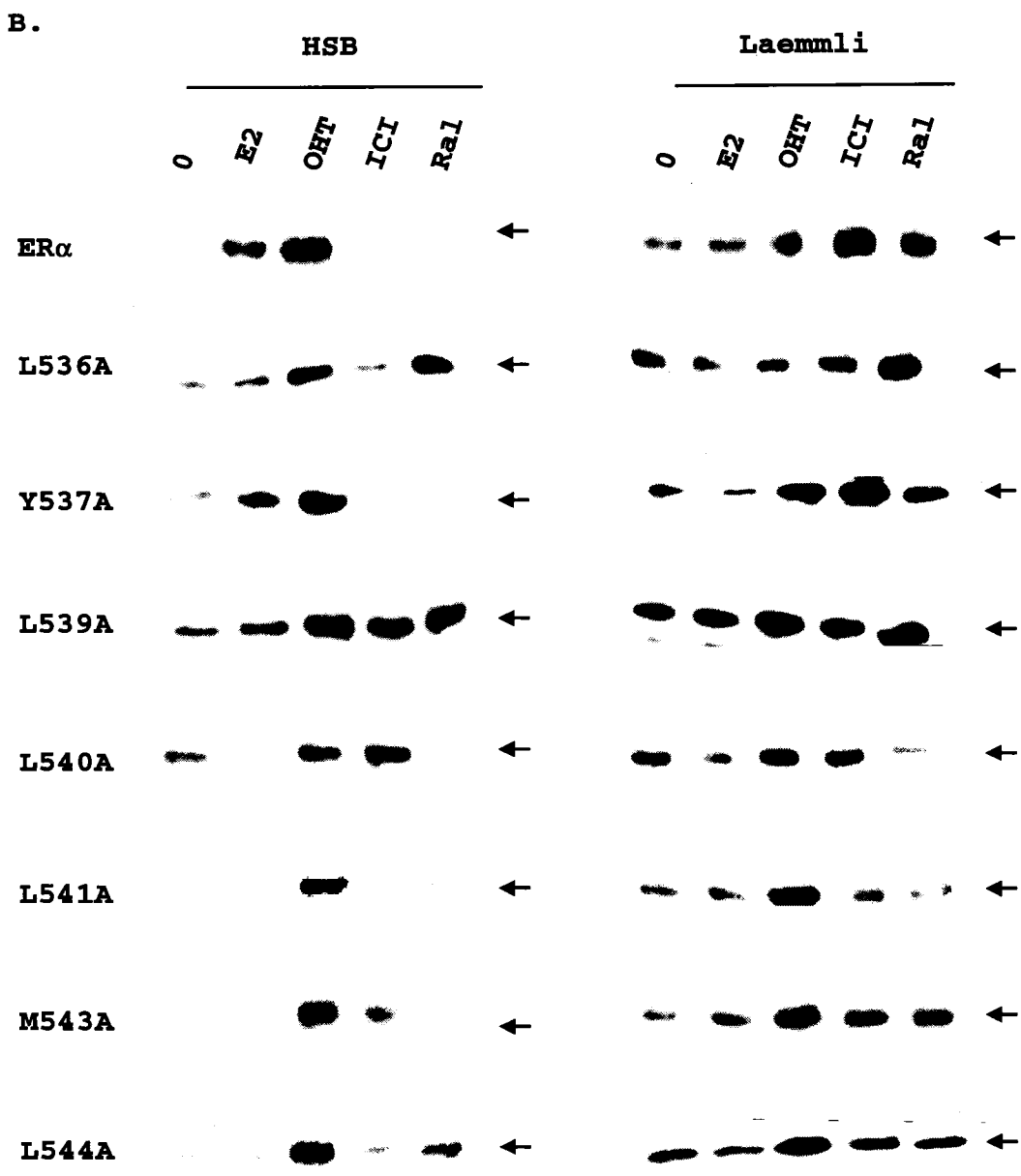

B.
HSB
Laemmli
0
E2
OHT
ICI
Ral
ERα
L536A
Y537A
L539A
L540A
L541A
M543A
L544A

Figure 4.4: Structural constraints exerted by the side chains of Ral and ICI on positioning of H12 on top of the ligand binding pocket.

The ERα-E2 complex (68) (**A**), was superimposed with that obtained in the presence of tamoxifen (56) (**B**), raloxifene (55) (**C**) or ICI164,384 (57) (**D**) using the Lsq-man module of the O package. The side chain of Y537 does not result in steric hindrance with the antiestrogen side chains, while that of amino acids L536 or L540 result in steric clash with the side chain of OHT and Ral/ICI, respectively.

Figure 4.4

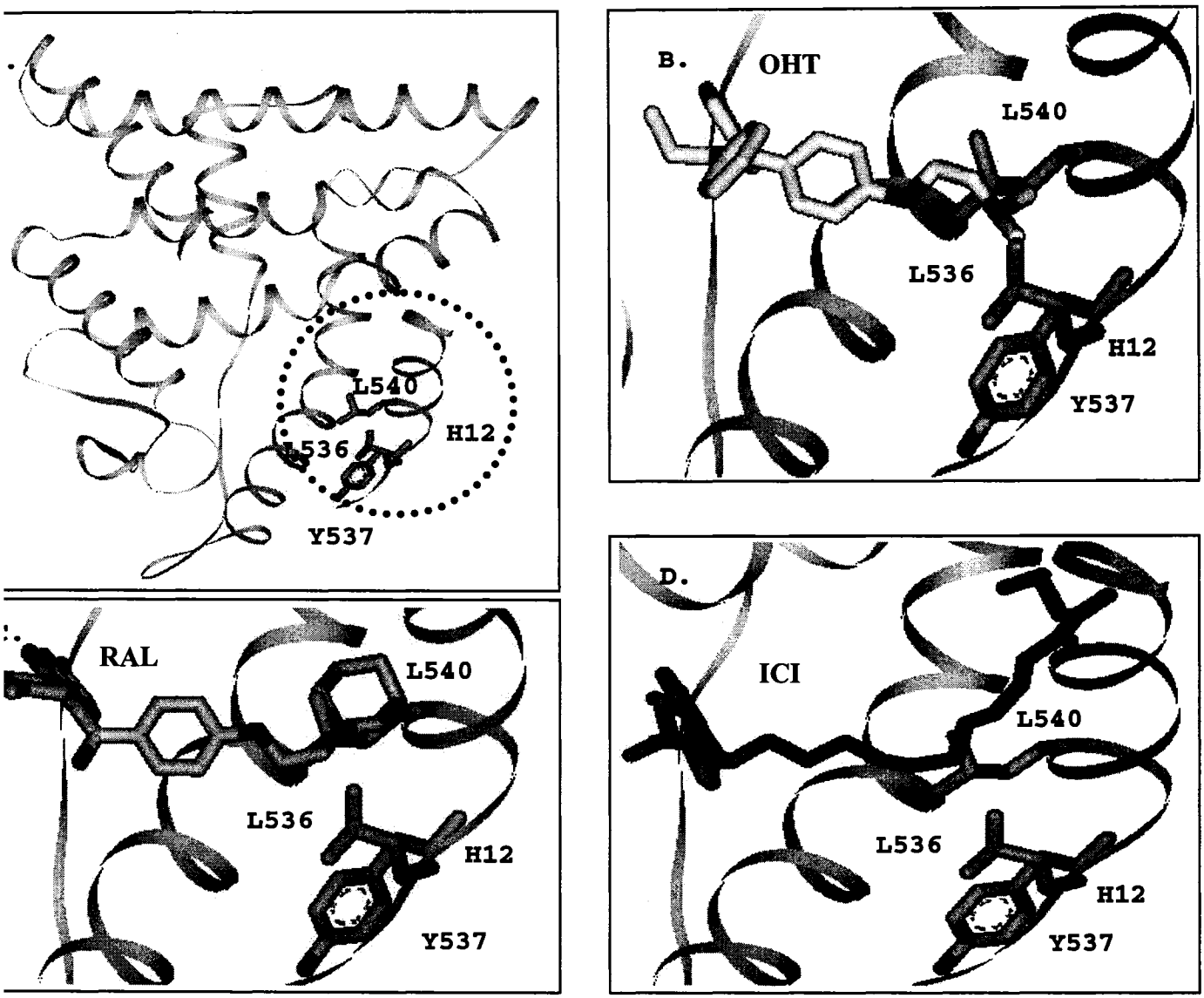

L540
H12
L536
Y537
B.
OHT
L540
L536
H12
Y537
RAL
L540
L536
H12
Y537
D.
ICI
L540
L536
H12
Y537

Figure 4.5: Structural constraints exerted by the side chains of Ral and ICI182,780 on positioning of H12 in the coactivator binding groove. The structure of the ERα-OHT complex (56) (**A-B**) was superimposed with that obtained in the presence of raloxifene (55) (**C**) or ICI164,384 (57) (**D**) as in Fig. 4.4. The side chains of L536 and L540, which generate steric hindrance with the ICI164,384 side chain, are shown.

Figure 4.5

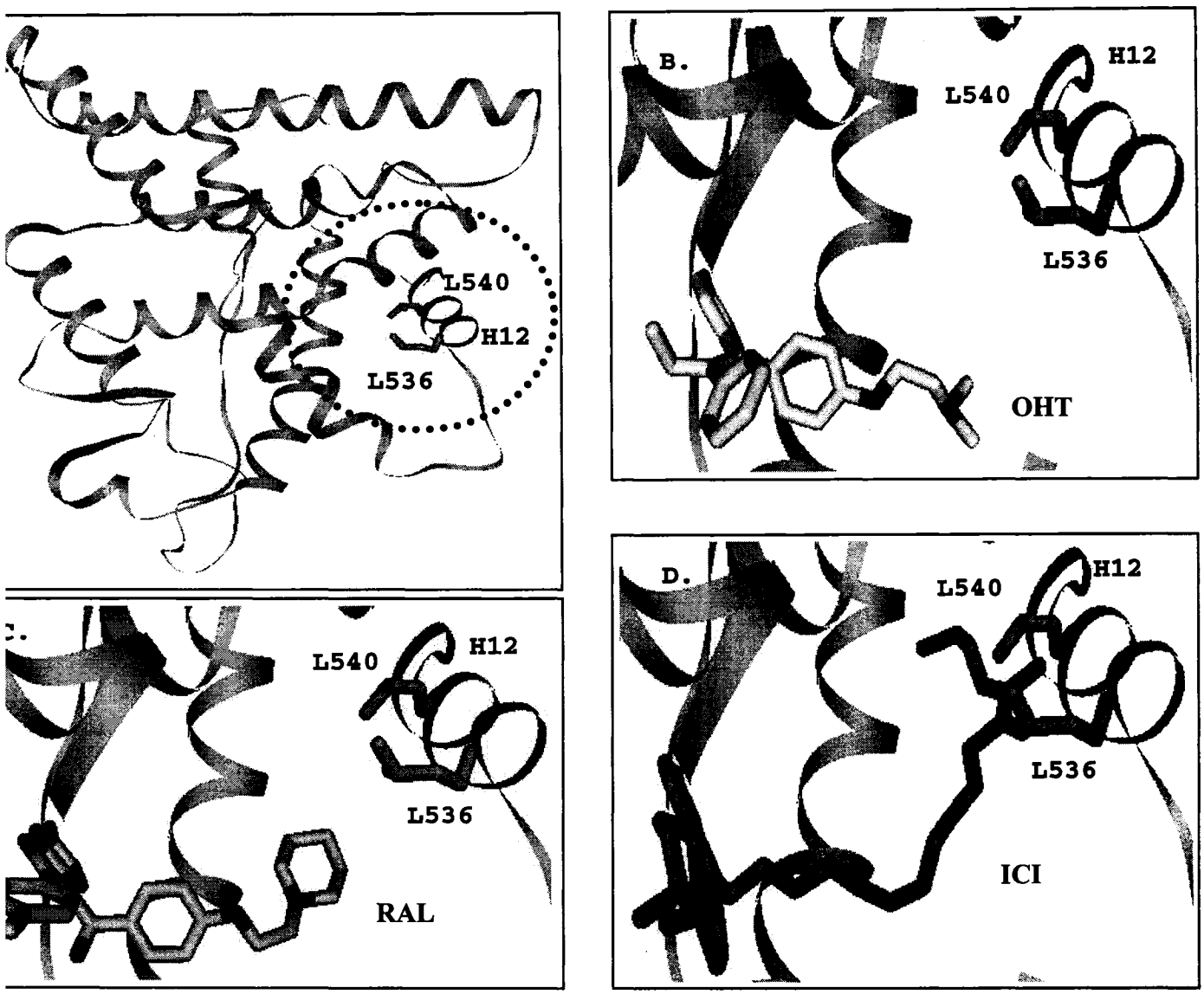

L540
H12
L536
B.
H12
L540
L536
OHT
C.
L540
H12
L536
RAL
D.
L540
H12
L536
ICI

Figure 4.6: Lack of AF2 activity for ERα mutants increasing transcriptional activity in presence of Ral or ICI182,780.

HeLa cells were electroporated with the 5x GAL4-tata-Luc reporter, the internal control plasmid CMV-β-gal, and expression vectors for the GAL4-pep α/β I fusion protein and for full-length wild-type ERα or mutants of H12 fused to VP16. Cells were treated with hormones and harvested as described in Fig. 4.1A. Relative luciferase activity is shown.

Figure 4.6

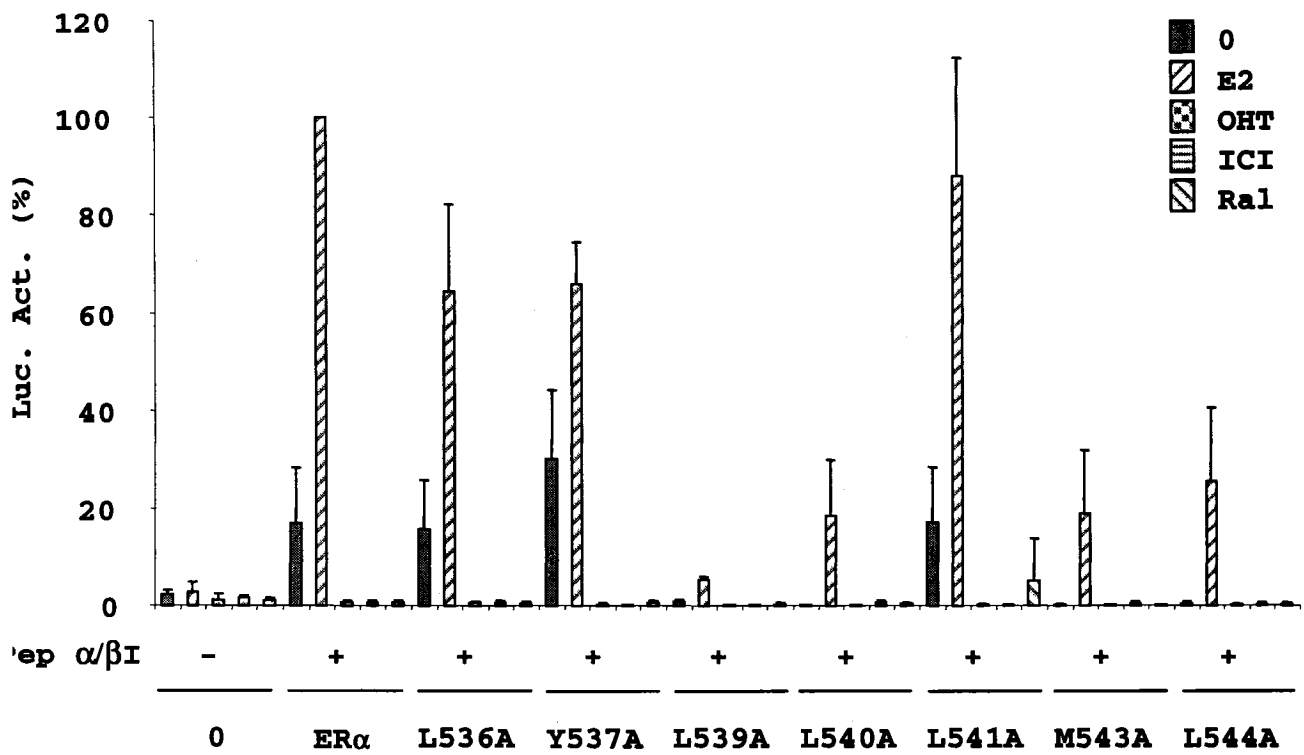

120
100
80
60
40
20
0
Luc. Act. (%)
0
E2
OHT
ICI
Ral
'ep α/βI
-
+
+
+
+
+
+
+
+
0
ERα
L536A
Y537A
L539A
L540A
L541A
M543A
L544A

Figure 4.7: Increased solubility of ERα protein in high salt buffer is insufficient to confer increased agonist activity to total antiestrogens. **A-B** Transcriptional activity (**A.**) and steady state protein levels (**B.**) of the wt ERα or of mutants D351E, D351Y, D351G or D351V were measured as in Fig 4.1.

Figure 4.7

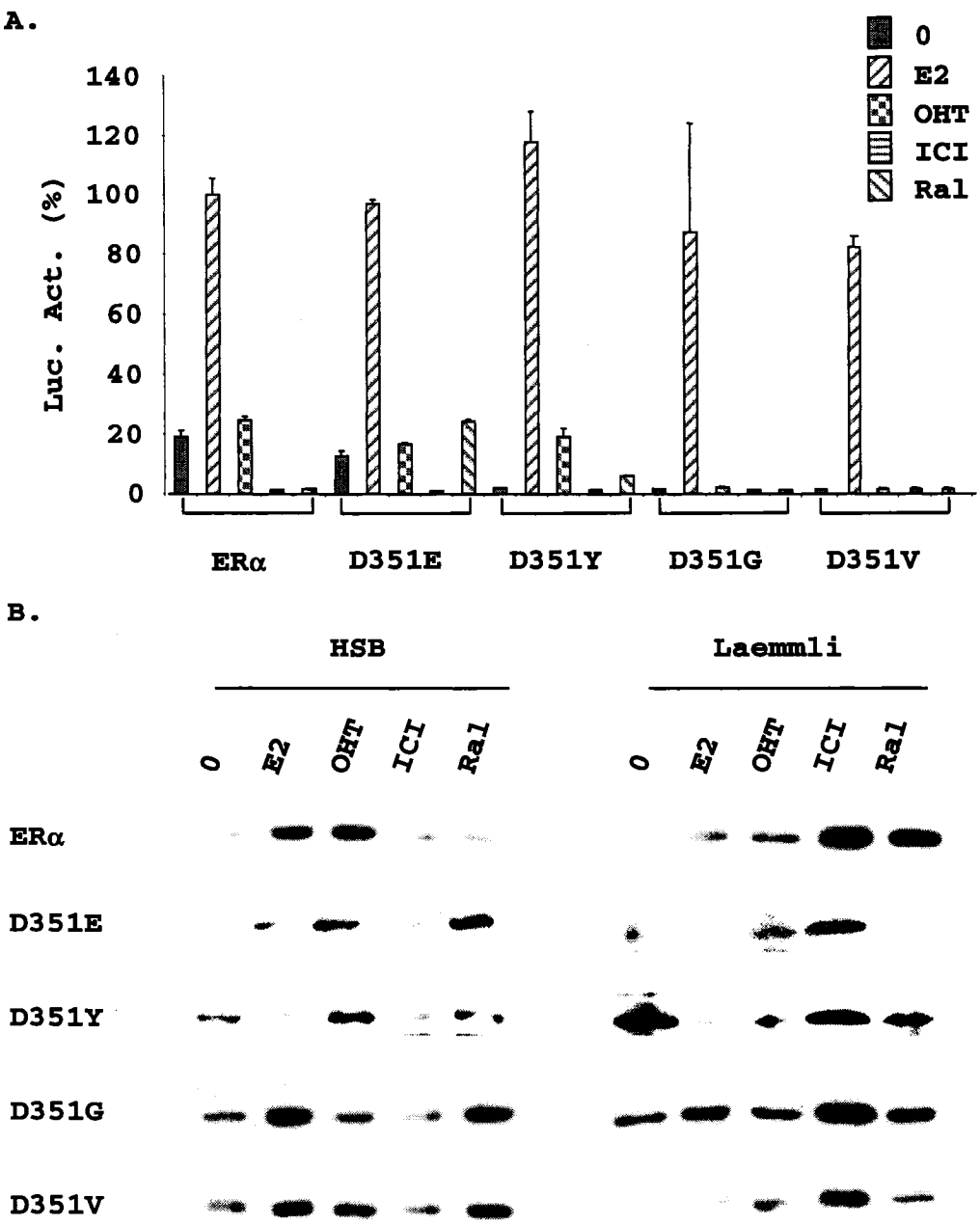
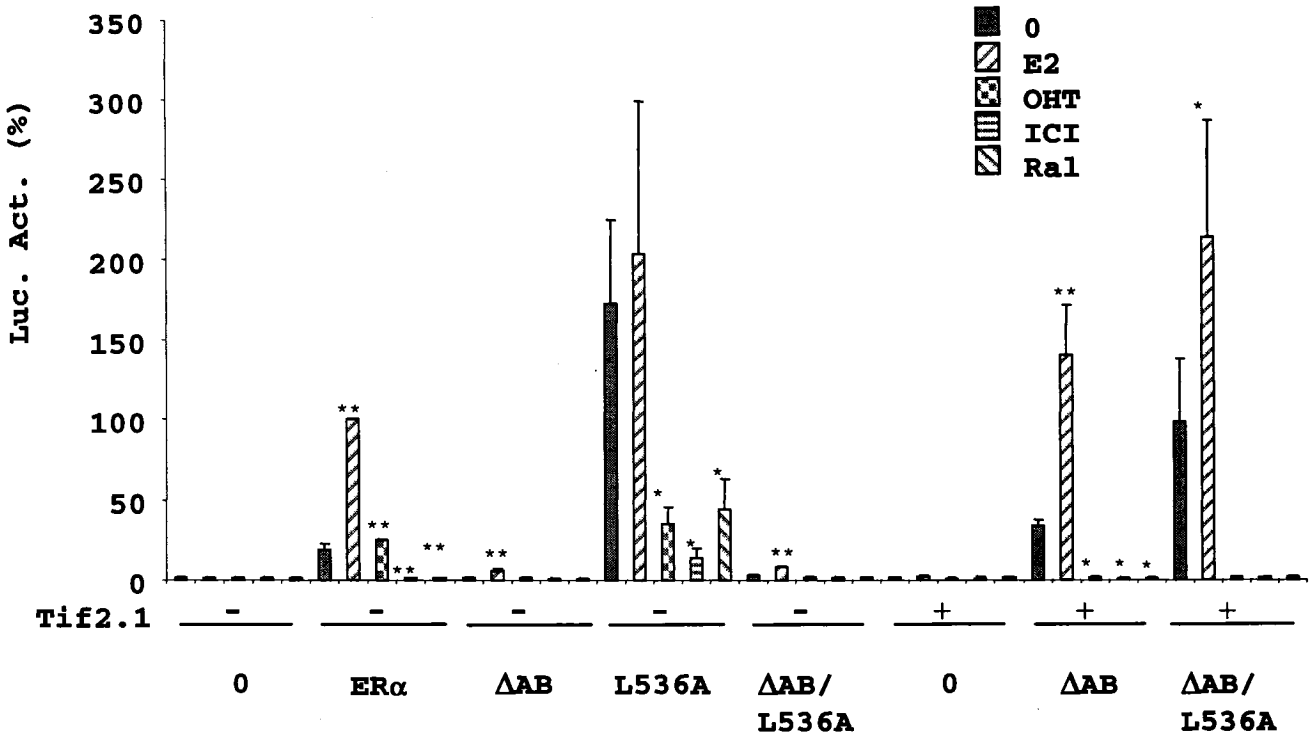

A.
0
E2
OHT
ICI
Ral
Luc. Act. (%)
140
120
100
80
60
40
20
0
ERα
D351E
D351Y
D351G
D351V
B.
HSB
Laemmli
0
E2
OHT
ICI
Ral
ERα
D351E
D351Y
D351G
D351V

Figure 4.8: The agonist activity of total antiestrogens requires AF-1 activity.

Transient transfection analysis of the transcriptional activity of the wt ERα, or the mutant L536A and of derivatives thereof carrying deletions of the AB region in the presence or not of overexpressed TIF2.1 (4 μg) was performed as in Fig. 4.1A. Relative luciferase activity is shown. Significant differences between response to ligand and basal level was determined by a one-way anova (* = $p \leq 0.05$; ** = $p \leq 0.001$)

Figure 4.8


Luc. Act. (%)
350
300
250
200
150
100
50
0
0
E2
OHT
ICI
Ral
Tif2.1
-
-
-
-
-
+
+
+
0
ERα
ΔAB
L536A
ΔAB/
L536A
0
ΔAB
ΔAB/
L536A

Connecting statement: bridging chapter 4 and 5

In chapters 3 and 4 we focused our efforts on identifying key structural elements relevant to the development of novel antiestrogens. We identified the importance of specific residues in H12 and the rest of the ligand-binding domain for the efficient inactivation of the receptor by antiestrogens. We also established that sequestration of the receptor in an insoluble fraction contributes to its inactivation. In chapter 5 we show that treatment with full antiestrogens can lead to receptor degradation or accumulation in an insoluble fraction depending on the cellular model. In ERα-positive MCF7 cells, treatment with proteasome inhibitors did not lead to increased transcriptional activity in the presence of full antiestrogens, while reducing activity in the presence of ligand and partial antiestrogens in a manner that did not correlate with effects on receptor levels. However, ERα accumulated in an insoluble fraction in MCF7 cells treated with proteasome inhibitors, as observed in HepG2 cells in the absence of proteasome inhibitors (Chapter 4). We next tested the hypothesis that accumulation of the receptor in an insoluble fraction is dependent on its ubiquitination, and on lack of degradation by the proteasome pathway. In 293T cells transfected with an expression vector for ERα, ubiquitination of the receptor in the presence of ICI182,780 and raloxifene was suggested by the presence of a ladder of bands corresponding to higher molecular weight than the expected 66 kDa form. Ubiquitination in the presence of these ligands was confirmed and could also be detected in the presence of OHT, in the absence of ligand and in the presence of E2 by cotransfection with a GFP-ubiquitin fusion protein. We further demonstrate that the C-terminal half of the receptor is sufficient for ubiquitination of the receptor and initiated site directed mutagenesis of all lysine residues in the ligand-binding domain to identify sites of ubiquitination in the presence of different ligands. Characterization of mutants that cannot be ubiquitinated should increase our understanding of the mechanisms of receptor degradation in the presence of agonists

versus full antagonists, and clarify the role of receptor degradation in its transcriptional activity in the presence these ligands. These studies should indicate whether induction of receptor degradation/insolubility is a good assay for development of full antiestrogens. In addition, they are of particular importance for breast cancer treatment since positivity for estrogen receptor is often assessed only at the protein level, and may be masked by a high turn-over ratio, with poorly understood implications for receptor activity.

Chapter 5: Both agonist and antagonist-dependent ERα ubiquitination and degradation are dependent on C-terminal lysine residues.

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Running title: Molecular determinants for ubiquitination of the estrogen receptor alpha.

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5.1) Abstract

The estrogen receptor alpha (ERα) undergoes degradation through the ubiquitin/proteasome degradation pathway following treatment with either agonists or full antagonists. Whether rapid receptor degradation in the presence of full antagonists contributes to their antiestrogenicity remains unclear. Here we show that proteasome inhibitors do not lead to increased ERα transcriptional activity in the presence of full antagonists, but inhibit transcription in the presence of 17β-estradiol or tamoxifen. However, receptors accumulated in an insoluble form in the presence of full antiestrogens and proteasome inhibitors, providing a potential explanation for the lack of increase in transcriptional activity. Treatment with ICI182,780 or raloxifene (Ral) in transiently transfected 293T cells resulted in accumulation of ubiquitinated forms of the receptor in an insoluble fraction even in the absence of proteasome inhibitors. Ubiquitination of the receptor in the absence of ligand or in the presence of 4-hydroxytamoxifen or estradiol could also be detected by cotransfection with a GFP-ubiquitin fusion protein. The C-terminal half of the receptor, but not the N-terminal half, was sufficient for ubiquitination, suggesting that some of the lysine (Lys) residues contained in the DNA- or ligand-binding domains are targets of ubiquitination. In order to assess whether the same residues are targeted in the presence of agonists versus full antagonists, we initiated a systematic mutagenesis of each Lys residue in the ligand-binding domain into arginines. None of the Lys mutants tested prevented ubiquitination or transcriptional repression in the presence of full antiestrogens.

5.2) Introduction

Breast cancer, characterized by the uncontrolled proliferation of mammary gland epithelial cells, is the most frequently diagnosed type of cancer (30% of all cancers diagnosed) and is the second highest cause of cancer-related deaths among women in North America (15%). High levels of circulating estrogens are correlated with an increased risk of breast cancer development. The proliferative effects of estrogen, such as 17β-estradiol (E2), in mammary tissue are primarily mediated by the estrogen receptor alpha (ERα). ERα is a ligand-inducible transcription factor member of the nuclear hormone receptor (NHR) family (1-4). It contains two transcriptional activation functions flanking a central DNA binding domain (DBD). The DBD, located in the homology region C (5), mediates binding to the palindromic estrogen response elements (EREs) (6-9). ERα ligand-dependent activation involves recruitment of coactivators to the C-terminal coactivator-binding groove (CBG), which is part of the ligand-binding domain (LBD) harboring the activation function (AF2) (10-17). The ligand-independent activation function (AF1) synergizes with AF2 for coactivator recruitment in a cell type- and promoter-dependent manner (18).

Following ligand binding, ERα recruits a series of coactivator complexes responsible for histone acetylation, chromatin remodeling, and enhanced recruitment of the basal transcription machinery (11, 15-17, 19, 20). Components of the ubiquitination/proteasome degradation pathways are also recruited on estrogen target promoters in a manner that appears to coincide with the end of transcription cycles (21). Inhibition of the proteasone degradation pathway abolishes estrogen induced-expression of reporter vectors in several cell lines in the first 6-8 hours (22-24).This may be due to the need to liberate DNA and allow for a second round of expression, as suggested/demonstrated with other transcription factors (25-33). However, degradation can have the reverse effect, acting in a

negative feedback loop for other transcription factors, such as the glucocorticoid receptor (GR) (34, 35). This also appears to be occurring for ERα after the first 6-8 hours of transcription (24). In addition, the role of estrogen receptor degradation in regulation of its activity is complex since full antiestrogens such as ICI182,780, RU58,668 and GW7604, but not partial antiestrogens such as tamoxifen (Tam), induce rapid degradation of the receptor. In addition, ERα is ubiquitinated *in vitro* and *in vivo* in the absence or presence of estrogen or antiestrogens (36-38).

The covalent attachment of ubiquitin, a highly conserved 8.6 kDa protein present both in the nucleus and cytoplasm, occurs on lysine (Lys) residues within target proteins. This requires an ubiquitin-activating enzyme (e1), an ubiquitin-conjugating enzyme (e2) and an ubiquitin-protein ligase (e3) for the activation and transfer of ubiquitin on a target protein (39, 40). Monoubiquitination, the attachment of a single ubiquitin to a Lys or to multiple Lys on the target protein, plays a role in endocytosis and transcriptional activation (41). More commonly, additional ubiquitins are added to the ubiquitin bound to the target protein generating a polyubiquitin chain. This polyubiquitination can occur either on Lys29, 48 or 63 of ubiquitin. Lys63 polyubiquitin chains direct the action of the tagged-protein to non-proteolytic signaling such as DNA repair (42-44) while Lys29 and Lys48 polyubiquitin chains are recognized by the 26S proteasome facilitating the entry of the target protein in the core of the proteasome where it is digested by proteases (45). Not surprisingly, unregulated ubiquitin-dependent proteolysis has been implicated as a causative factor in cancer (46-51).

To better understand the role of receptor degradation in the mechanisms of action of agonists and antagonists, we investigated the sites of ERα ubiquitination in the presence of either type of ligand. Our results demonstrate that inhibition of the 26S proteasome reduces the agonist activity of E2 as well as the partial-agonist activity of OHT, without increasing activity in the presence of full antiestrogens. In 293T cells, ERα

ubiquitination was inversely correlated with its transcriptional activity when analysed in presence of different ligands and mapped to the C-terminal ligand-binding domain of the receptor.

5.3) Results

26S proteasome inhibitors repress ERα transactivation in presence of full or partial-agonists and do not increase activity in the presence of full antiestrogens.

ERα expression levels are reduced following estrogen treatment in a manner that is counteracted by incubation with proteasome inhibitors, and this increased turn-over appears necessary for early estradiol-induced transcriptional activity (22-24). Here we assessed the effect of proteasome inhibitors on both receptor levels and activity in the presence of partial and full antiestrogens in MCF7 breast cancer cells expressing endogenous ERα (Fig. 5.1A) or in transiently transfected HeLa cells (Fig. 5.1B). 4-hydroxytamoxifen (OHT) increased receptor levels in high salt buffer extracts compared to extracts from non-treated cells, and coincubation with proteasome inhibitor MG132 (10 μM) did not increase receptor levels (Fig. 5.1C-D). However, expression of the ERE3-TATA-Luc reporter vector in the presence of OHT was inhibited by MG132 (Fig. 5.1A-B), No significant effects of MG132 were observed in reporter gene expression in the presence of ICI182,780 or raloxifene (Ral) either in MCF7 or in Hela cells, although receptor levels in high salt extracts were increased in the presence of Ral and ICI182,780 in MCF7 cells, and in the presence of Ral in HeLa cells. In conclusion, MG132 had a general repressive effect on receptor transcription that appears independent from its effect on receptor turn-over rate.

It is also noteworthy that at the same concentration of MG132, receptor levels were still drastically reduced by ICI182,780 in both cell lines even though levels of receptor in the presence of E2 were completely restored. This suggests that different mechanisms underly receptor down-regulation in both cases. Indeed, as previously observed (52), receptor levels were fully recovered in Laemmli extracts of MCF7 cells treated with

MG132 and ICI182,780, suggesting accumulation of non-functional receptor in an insoluble fraction

The C-terminal half of ERα is sufficient for its ubiquitination

To further investigate the molecular mechanisms of receptor downregulation in the presence of agonists and antagonists, we sought to map the determinants responsible for receptor ubiquitination in the presence of different ligands. ERα ubiquitination in the presence of various ligands has previously been reported (36-38). A smear of ERα-specific bands migrating at a higher molecular weight in presence of proteasome inhibitors has been observed in MCF7 cells (38), and ERα copurified on a nickel column when cotransfected in HeLa cells with a 6His-tagged ubiquitin construct (37). In 293T cells transiently transfected with an expression vector for ERα, high-molecular weight forms of ERα could be observed in the presence of ICI182,780 and Ral even without incubation with proteasome inhibitors. To monitor more directly ubiquitination of the receptor, we cotransfected an expression vector for a GFP^2-Ubiquitin (GFP^2-Ubi) fusion proteins (53) together with the pSG5-HEG0 vector in 293T cells. In order to prevent polyubiquitination and therefore allow for the detection of a strong ubiquitination of the receptor, Lys 48 and 63 of the ubiquitin protein part of the GFP^2-Ubi construct were mutated to alanine residues. As a negative control we used the GFP^2-Ubi_{AA} construct, where the two C-terminal glycine residues 75 and 76 essential for a functional ubiquitin were mutated to alanine residues (54). Western blots of total extracts from 293T cells transiently expressing wild-type ERα and GFP^2-Ubi, treated with E2, OHT, ICI182,780 or Ral revealed an additional band migrating at 109 kDa, corresponding to mono-GFP^2-Ubi (43 kDa) conjugated ERα (66 kDa) (Fig. 5.2B). This band was not observed in the absence of transfected ERα (Fig. 5.2C lane 6, 7 and 8) or in the presence of cotransfected GFP^2-Ubi_{AA} (Fig. 5.2D). Althought the relative protein

level of non-ubiquitinated ERα was equivalent under all treatments, the ubiquitinated form was the most abundant in presence of ICI182,780 and gradually less abundant in presence of Ral, no ligand, OHT and E2 (Fig. 5.2B).

In order to identify the domains of ERα required for its degradation, HeLa cells were transfected with ERα deletion mutants (Fig. 5.3A). The HE15 (ERαΔEF) mutant encoding the first 281 amino acids of the ERα was no longer degraded following either agonist or antagonist treatment (Fig. 5.3B). However, the HEG19 (ERαΔAB) deletion mutant, encoding the C-terminus of ERα, was still downregulated under E2 as well as in the presence of Ral or ICI182,780 (Fig. 5.3B). Surprisingly, ERαΔAB protein level was reduced under OHT. This indicates that the C-terminus of ERα habors the essential residues for its down-regulation.

Ubiquitination assays revealled that ubiquitination of ERα still occurred on the ERαΔAB mutant while it was not detected for the ERαΔEF mutant (Fig 5.3C and D). Surprisingly, ERα ubiquitination in presence of E2 or OHT was equivalent to the basal ubiquitination (Fig. 5.3C). This suggests that ERα N-terminus restrains wild-type ERα ubiquitination in presence of E2 (Fig. 5.3B). As well, these results indicate that the site(s) of ubiquitin attachment are located in the C-terminus of ERα.

Role of Lys residues in the ERα ligand-binding domain in receptor ubiquitination.

Since ubquitination occurs on lysine (Lys) residues and since ERα C-terminus is essential for its degradation we decided to systematically mutagenize Lys residues in the ligand binding domain into arginine (Arg), an amino acid that carries the same charge as Lys but canot be ubiquitinated, in order to characterize their individual roles in ERα degradation in the presence of different ligands (Fig. 5.4A). Ubiquitination assays demonstrated a lack of effect of mutations K449R, K467R, K472R, K481R, K492R and K529R on ubiquitination of the receptor (Fig 5.4B).

Furthermore, transactivation assays demonstrated that none of the Lys to Arg mutations tested, including K362R and K520R, could antagonize estrogen induced transcriptional activity of ERα, or derepress transcription in the presence of ICI182,780 or Ral, although effects on basal transcription (without ligand) could be observed (Fig 5.4C). Note however that not all lysine residues have been tested to date.

5.4) Materials and methods

Plasmids and reagents - 17β-estradiol (E2), 4-hydroxytamoxifen (OHT) and raloxifene (Ral) were purchased from Sigma (Sigma, Oakville, ON, Canada) and ICI182,780 (ICI) was purchased from Tocris Cookson Ltd (Ballwin, MO, USA). MG132 was purchased from EMD Biosciences, Calbiochem (EMD Biosciences, La Jolla, CA, USA). pSG5-ERα, pSG5-HE15 (ERαΔEF) and pSG5-HEG19 (ERαΔAB) were kind gifts from Prof. P Chambon (55, 56). The Lysine to Arginine mutations at positions 362, 449, 467, 472, 481, 492, 520 and 529 were introduced by site-directed mutagenesis using polymerase chain reaction (PCR) amplification of the ERα cDNA (the sequence of oligonucleotides used for mutagenesis is available upon request). Expression plasmids for ERα mutants were generated by subcloning 792 base pair HindIII/BamHI PCR fragments into the pSG5-ERα expression vector. Clones for each mutant were characterized by restriction digest and sequencing. The GFP^2-Ubi and GFP^2-Ubi_{AA} expression vectors (pGFP2-C1 from Perkin-Elmer Biosignal) were kind gifts from Dr. Michel Bouvier.

Cell culture and luciferase assays - HeLa cells were maintained in DMEM (Wisent, St-Bruno, QC, Canada) supplemented with 5% fetal bovine serum (FBS, Sigma, Oakville, ON, Canada). MCF7 cells were maintained in α-MEM (Wisent, St-Bruno, QC, Canada) supplemented with 10% FBS. 293T cells were maintained in DMEM supplemented with 10% FBS. Three days before experiments HeLa cells were switched to phenol red-free DMEM medium containing 5% charcoal-stripped serum while MCF7 and 293T cells were switched to phenol red-free DMEM medium containing 10% charcoal-stripped FBS.

For luciferase assays, HeLa cells were electroporated (0.24 kV, 950 μF in a Biorad Gene Pulser II apparatus) and plated in 6 well plates ($8x10^5$ cells/well). Typically, a DNA mix contained 1 μg expression vector, 2 μg ERE3-TATA-Luc reporter vector, 2 μg internal control pCMV-βGal and 35

μg carrier salmon sperm DNA (Invitrogen, Burlington, ON, Canada). 17β-estradiol (E2, 25 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI182,780 (ICI, 100 nM) raloxifene (Ral, 100 nM) or vehicle (ethanol) were added immediately after electroporation. Cells were harvested 48 h later in lysis buffer (Tris-HCl 100mM pH 7.9, 0.5% NP40, 1mM DTT). For proteasome inhibition, HeLa cells were pretreated for 1 h with MG132 (10 μM) the day after transfection and subsequently treated with E2 (25 nM), OHT (100 nM), ICI (100 nM) or Ral (100 nM) for 5 h. Luciferase activity was measured in the presence of luciferin with a Fusion Universal Microplate Analyser (Perkin-Elmer, Woodbridge, ON, Canada) and was normalized for β-galactosidase activity, measured at 420 nm with a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). Each transfection was carried out in duplicates and repeated at least three times. MCF7 cells were also electroporated (0.25 kV, 975 μF), with the exception that the DNA mix did not contain the ERα expression vector, and plated in 6 well plates ($1x10^6$ cells/well). The following procedures were carried out as in HeLa cell.

Western analysis of receptor levels - For western blotting, HeLa cells were transiently transfected by electroporation ($5x10^6$ cells) with 10 μg of pSG5 expression vectors containing wild-type or mutant ERα cDNAs and 30 μg carrier salmon sperm DNA (Invitrogen, Burlington, ON, Canada) and plated in 10 cm plates. Cells were treated with E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM), Ral (100 nM) or vehicle for 5 hours. Cells were harvested in ice-cold PBS, and whole cells extracts were prepared from half the cells by three freeze-thaw cycles in high salt buffer as previously described (57). To detect ubiquitination, 293T cells ($1x10^6$ cells) were cotransfected with 5μg of GFP^2-ubi or the negative control GFP^2-ubi_{AA} expression vectors (53) and 15 μg of wild-type or lysine mutant ERα by the calcium phosphate technique and plated in 10 cm plates. 293T cells were treated as HeLa cells, harvested in phosphate buffered saline (PBS)

1X supplemented with 20 mM N-ethylmaleimide (NEM), and lysed inLaemmli buffer (58).

For western blotting of endogenous ERα in MCF7 cells, cells were seeded in 10 cm plates ($4x10^6$ cells/plate). The following day, cells were pretreated for 1 hour with MG132 (10 μM) or vehicle (DMSO). E2 (25 nM), OHT (100 nM), ICI182,780 (100 nM) or Ral (100 nM) were then added for 5 hours. Cells were harvested in ice-cold PBS, and whole cells extracts were prepared as described for HeLa cells.

Whole cell extracts from ERα expressing cells (HeLa, 293T or MCF7 cells) were analyzed following electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (7,5% acrylamide), transfer onto nitrocellulose, and incubation of membranes with an anti-ERα mouse monoclonal antibody (B10 or F3A6, kind gift from Prof. P. Chambon). Complexes were revealed by ECL (NEN Life Science Products) as recommended by the manufacturer. Equal loading was confirmed through an anti-β-actin Western blot.

5.5) Discussion

Degradation of ERα and or of several other nuclear receptors by the ubiquitin-proteasome pathway has been suggested to be required for efficient transcriptional activation (22, 23, 25-33). The transcriptional activation properties of ERα are indeed compromised in the presence of proteasome inhibitors or in cell lines that are deficient for proteasome function (21). However, proteasome inhibitors could have a general effect on many proteins involved in regulation of gene expression (24). Furthermore, some reports suggest that ERα transactivation can occur independently of ERα degradation (52, 59). For instance, treatment for 24 hours with forskolin (FSK) can prevent E2 induced degradation of the receptor and allows for its transcriptional activation in pituitary cells, a process dependent on the protein Kinase A (PKA) pathway (59). Similarly, PKA, MAPK and phosphatidyl-inositol-3 kinase (PI3K) were shown to impede the proteasome dependent degradation of ERα in presence of E2 in MCF7 cells without affecting its transcriptional activation after 18 hours of incubation (52). However, the discrepancy between these reports and previous reports demonstrating the importance of ERα degradation for its efficient transcriptional activation may be due to different experimental conditions. For instance, Fan and colleagues have demonstrated that a functional ubiquitin-proteasome pathway is required in the first 6-8 hours following induction with estrogens for efficient transactivation on an minimal promoter, while it acts to dampen estrogenic stimulation in the longer term (24). However, whether degradation of ERα itself is important is not clear. Although a number of proteins part of the ubiquitin-proteasome degradation pathway have been characterized as coactivator of the ERα none require their degradation function for this purpose (36, 60-63). In addition, different antiestrogens repress ERα transcription while having variable effects on ERα turnover. Here we have investigated the effect of proteasome inhibition on transcriptional activation in the presence of agonists and antagonists in MCF7 cells and in transiently transfected

HeLa cells. Degradation of endogenous receptor by full antiestrogens in breast carcinoma MCF7 cells may contribute to total antagonism, while receptor stabilization by OHT may facilitate its partial agonist activity. Thus, it may be expected that proteasome inhibitors would not affect transcription in the presence of OHT, while gains in partial agonist activity may be observed in the presence of full antiestrogens. Contrary to these expectations, we observe that transcriptional activity in the presence of OHT is repressed by MG132, while transcription in the presence of full antiestrogens is not affected. Lack of gain in transcriptional activity in the presence of full antiestrogens may be explained by differential partitioning of the receptor in high salt buffer and in detergent-containing Laemmli buffer, suggesting accumulation of the receptor in an insoluble fraction. However, transcriptional repression in the presence of OHT, which occurs independently from an increase in receptor levels, strongly suggests that proteasome inhibitors affect targets other than the receptor. This may be specific to transcriptional activation in the presence of antiestrogens, which recruit corepressors with differential affinities. Potential stabilization of corepressors may decrease the agonist activity of OHT, which has a lower capacity to recruit corepressors compared to full antiestrogens. On the other hand, a general effect of proteasome inhibitors on luciferase and β-galacotsidase gene expression has been observed in transfected cells (64), raising the possibility that stimulatory effects of proteasome inhibitors may be masked by a destabilizing effect on luciferase protein. These experiments therefore will be repeated using different reporter vectors and endogenous estrogen target genes.

To better understand the role of receptor degradation in the effects of agonists/antagonists on receptor activity, more specific approaches need to be developed. Inhibition of receptor ubiquitination through site-directed mutagenesis would provide assays to investigate the role of receptor turnover rates in the modulation of its transcriptional activation properties by different ligands. Receptor ubiquitination in the presence of

ICI182,780 and Ral was readily observable in transiently transfected 293T cells, and monoubiquitination could be observed upon cotransfection with the GFP^2-Ubi vector in the absence of ligand or the presence of E2 and OHT. Surprisingly given the effect of E2 on receptor degradtion in other cell lines, monoubiquitination in the presence of E2 was not stronger than in the presence of OHT or in the absence of ligand. This experiment will be repeated in the presence of proteasome inhibitors to exclude effects due to receptor degradation in the presence of E2. However, this does not appear likely as no reduction in the non-ubiquitinated form the receptor was apparent in the presence of E2 compared to its absence. Therefore, 293T cells may turn out to be model where E2 does not induce significant receptor degradation, in spite of the receptor being fully transcriptionally active in the presence of this ligand (see Fig. 5.3A). This would further confirm that receptor degradation is not essential to its transcriptional activation properties.

Ubiquitination of ERα was mapped to its C-terminal half, suggesting that lysine (Lys) residues that are targeted for ubiquitination are located in this portion of the receptor. Additional receptor mutants eliminating region C and/or D of the receptor are under construction to further pinpoint the location of these residues. Note that deletions within the ligand-binding domain itself lead to loss of three-dimensional structure and will not be tested for that reason. Point mutagenesis of all Lys residues in the ligand-binding domain has been initiated. Our preliminary results indicate that all residues tested so far do not affect ubiquitination or transcriptional activation of the receptor in the presence of agonists or antagonists, although some effects were observed in the absence of ligand. This may be due to a destabilizing effect in the absence of ligand, since it is known that binding of any ligand stabilizes the LBD conformation (65). However, several Lys residues in the LBD domain remain to be tested, and we cannot exclude the involvement of Lys residues in regions C-D. Identification of Lys residues targeted for ubiquitination should indicate

whether different ligands modulate the rate of receptor ubiquitination through differential accessibility of a critical Lys residue, or lead to ubiquitination of different residues. Moreover, obtaining receptor mutants that are not ubiquitinated in the presence and/or absence of ligand should confirm that ERα degradation is ubiquitination-dependent in the presence of all ligands, and clarify which role the ubiquitin/proteasome pathway plays in modulating the functional properties of estrogen receptors.

5.6) Acknowledgements

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[1]The abbreviations used are: AF1, activation function 1, AF2, activatio function 2; AR, Androgen receptor; CBG, coactivator-binding groove; DBD, DNA-binding domain; e1, ubiquitin-activating enzyme; e2, ubiquitin-conjugating enzyme; E2, 17β-estradiol; e3, ubiquitin-protein ligase; ERα, estrogen receptor alpha; ERE, estrogen response element; GFP^2-Ubi: Green fluorescent $protein^2$-ubiquitin; GR, Glucocorticoid receptor; LBD, ligand-binding domain; NHR, nuclear hormone receptor; PR, progesterone receptor; RAR, retinoic acid receptor; OHT, 4-hydroxytamoxifen; Ral, raloxifene;

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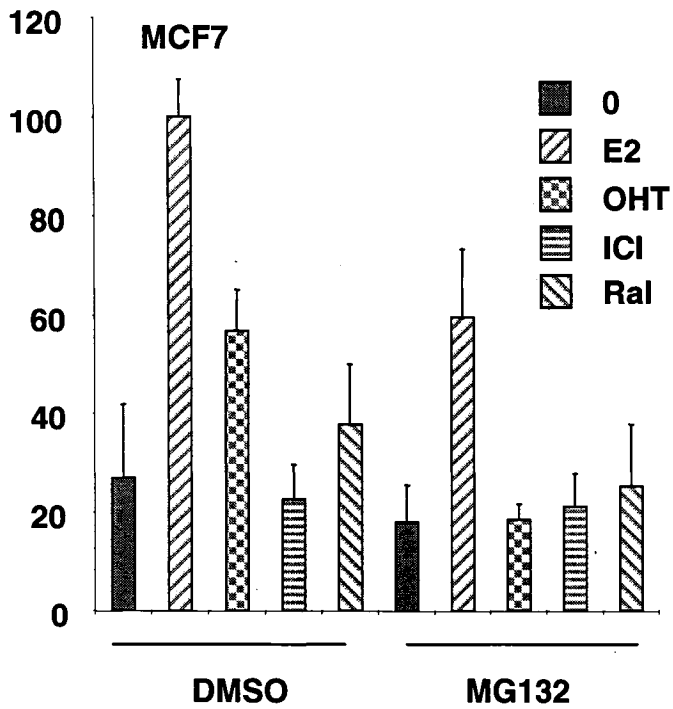
5.8) Figure legends

Figure 5.1: Efficient ERα transactivation requires degradation from the 26S proteasome.

A: MCF7 cells were pretreated for 1 hour with or without MG132 (10μM) 24 hours following their transient transfection with the ERE3-TATA-Luc reporter and the CMV-β-galactosidase constructs. Induction with vehicle (0), 17β-estradiol (E2, 25nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI182,780 (ICI, 100 nM) or raloxifene (Ral, 100 nM) for 5 h. Luciferase activity and β-galactosidase activity were quantified, and relative luciferase activity was calculated. Results from at least three experiments performed in duplicates are shown. The error bars reflect the standard deviation between independent experiments.
B: HeLa cells transiently transfected with wild-type ERα, ERE3-TATA-Luc reporter and CMV-β-galactosidase expression constructs were treated and analysed as in A.
C: Expression levels of endogenous ERα in MCF7 cells following 5 hours of treatment with vehicle (0), E2 (25nM), OHT (100 nM), ICI (100 nM) or Ral (100 nM) with or without 30 min pre-treatment with MG132 (10 μM) was determined by western blot using the anti-ERα antibody B10. Extraction was performed using a high-salt buffer (HSB) or a Laemmli buffer. A representative from two independent experiments is shown.
D: Expression levels of transfected ERα in HeLa cells were determined as in C.

Figure 5.1

A.


120
100
80
60
40
20
0
MCF7
0
E2
OHT
ICI
Ral
DMSO
MG132

B.

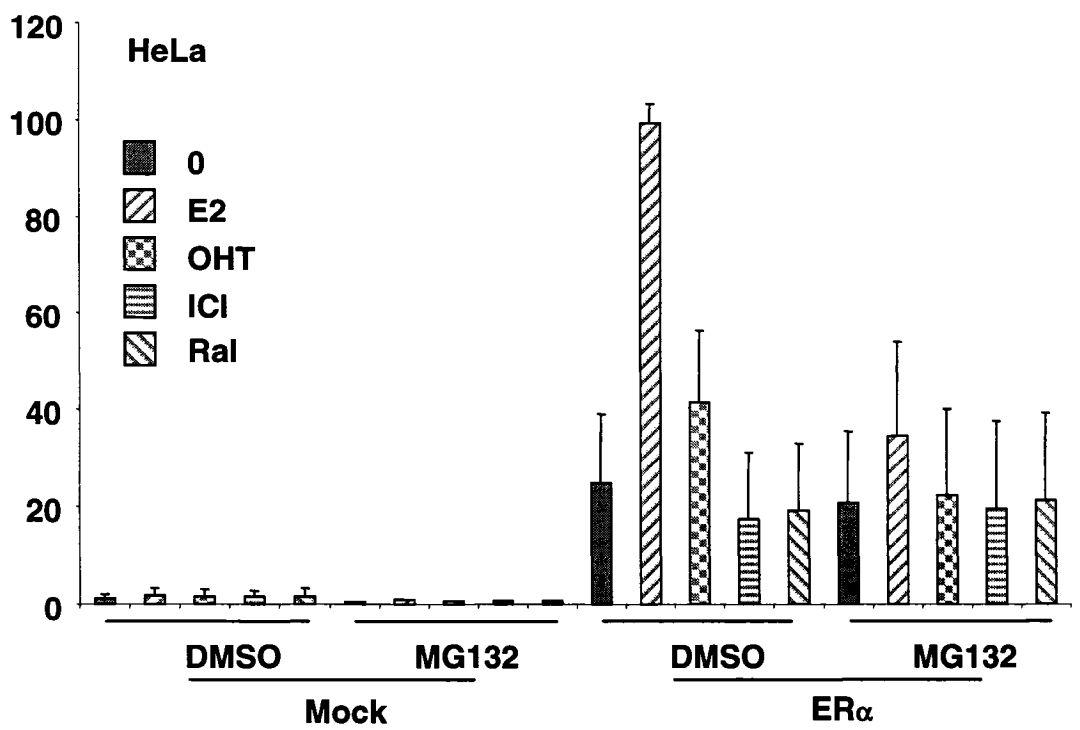

120
100
80
60
40
20
0
HeLa
0
E2
OHT
ICI
Ral
DMSO
MG132
DMSO
MG132
Mock
ERα

Figure 5.1

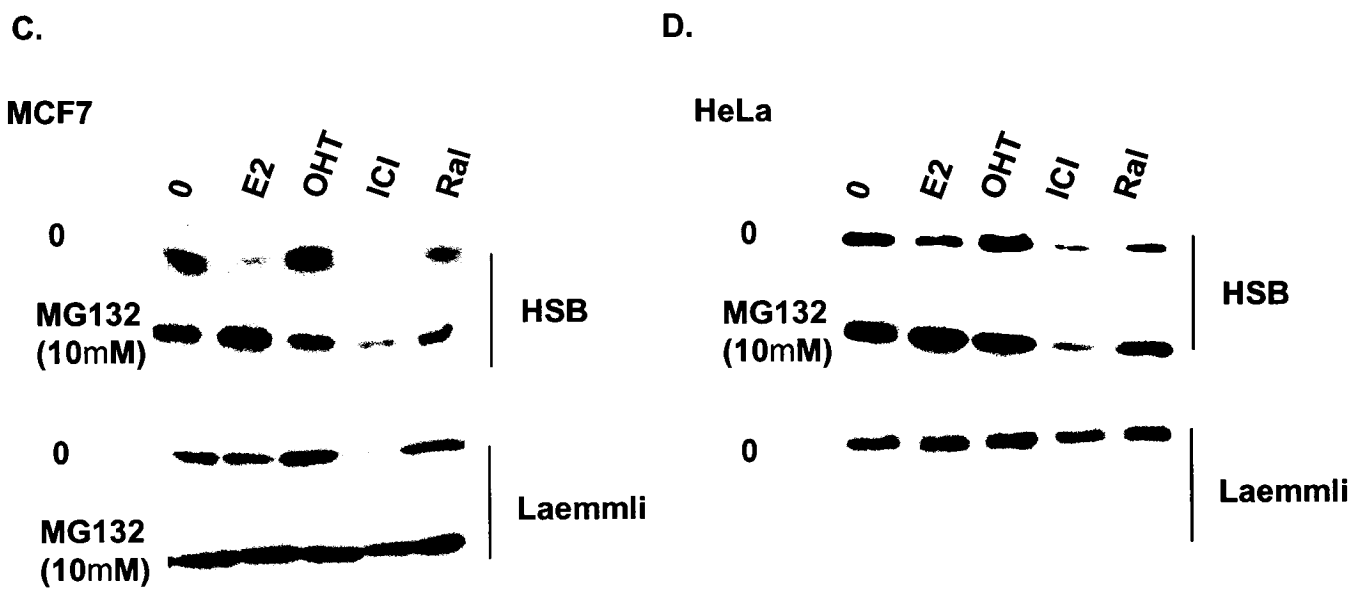

C.
MCF7
0
E2
OHT
ICI
Ral
0
MG132
(10mM)
HSB
0
MG132
(10mM)
Laemmli
D.
HeLa
0
E2
OHT
ICI
Ral
0
MG132
(10mM)
HSB
0
Laemmli

Figure 5.2: ERα ligand dependent transactivation and ubiquitination.

A: ERα-negative 293T cells were transiently transfected with the CMV-β-galactosidase, the ERE3-TATA-Luc reporter constructs and the empty vectors (mock) or the expression vector for wild-type ERα. Induction was carried out for 5 hours as in figure 5.1A. Results from three independent experiments performed in duplicates are shown.

B: 293T cells were transiently transfected with the expression vectors for wild-type ERα and GFP^2-Ubi and treated with vehicle (0), estrogen (E2, 25 nM) 4-hydroxytamoxifen (OHT, 10 nM), ICI182,780 (ICI, 10 nM) or raloxifene (Ral, 10 nM) for 5 hours. Western blot analysis on total cell extracts using an anti-ERα antibody (B10) revealed the non-ubiquitinated ERα (open arrow) and the GFP^2-Ubi conjugated ERα in presence of all ligands (closed arrow). A representative result from three independent experiments is shown.

C: Cells were transfected with the empty vector (mock), the expression vector for wild-type ERα, the GFP^2-Ubi or the GFP^2-Ubi_{AA} expression vector and treated as in B.

D: The negative control GFP^2-Ubi_{AA} expression vector was cotransfected with the wild-type ERα expression vector and cells were treated as in B.

Figure 5.2

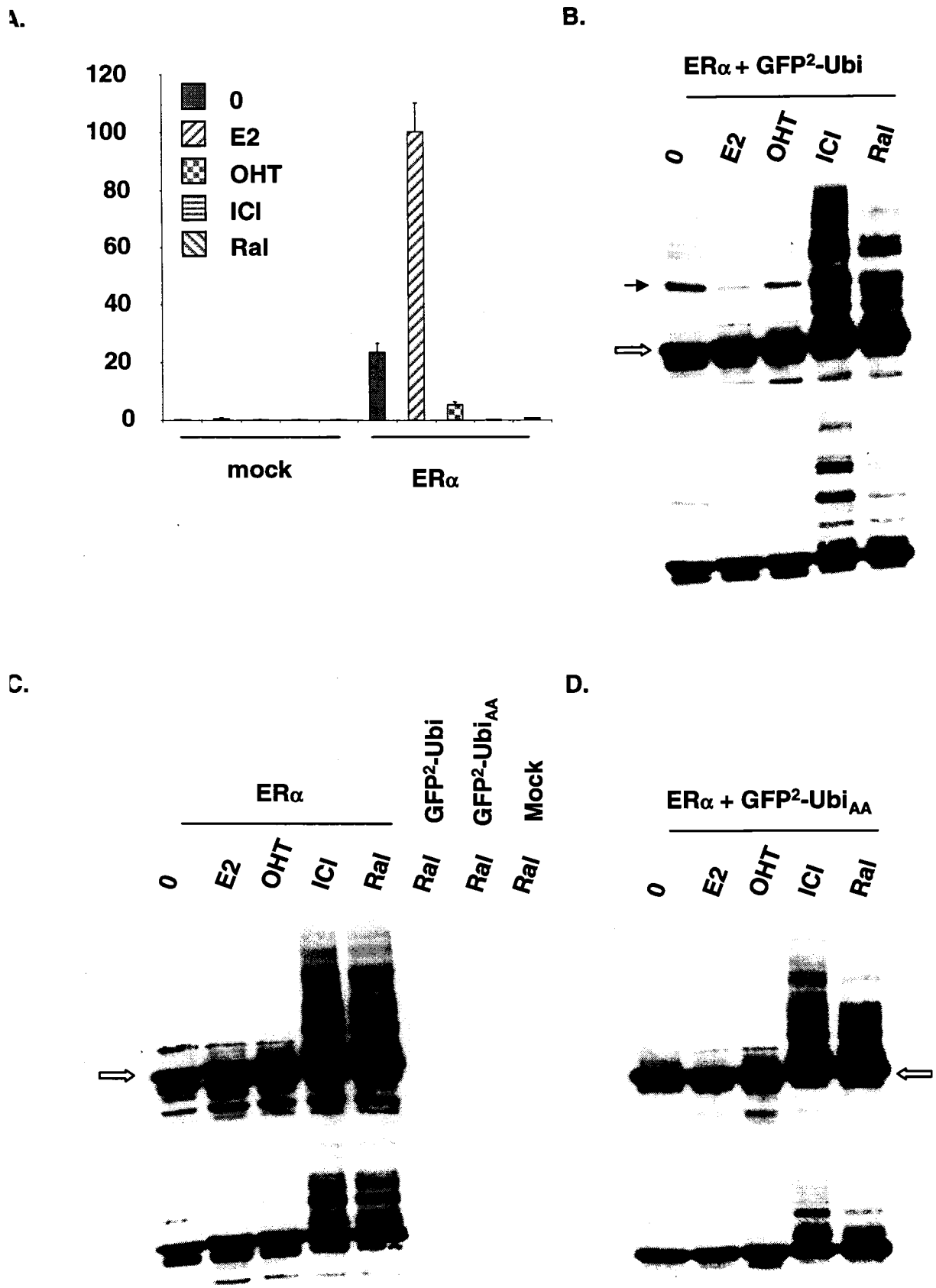

A.
120
100
80
60
40
20
0
0
E2
OHT
ICI
Ral
mock
ERα
B.
ERα + GFP²-Ubi
0
E2
OHT
ICI
Ral
C.
ERα
GFP²-Ubi
GFP²-Ubi$_{AA}$
Mock
0
E2
OHT
ICI
Ral
Ral
Ral
Ral
D.
ERα + GFP²-Ubi$_{AA}$
0
E2
OHT
ICI
Ral

Figure 5.3: Establishing the regions of ERα required for its degradation.

A: Schematic representation of the 595 amino acid (A.A.) long wild-type ERα. The ERαΔEF (HE15) mutant is deleted of the last 314 A.A. while the ERαΔAB (HEG19) mutant is deleted of the first 178 A.A.

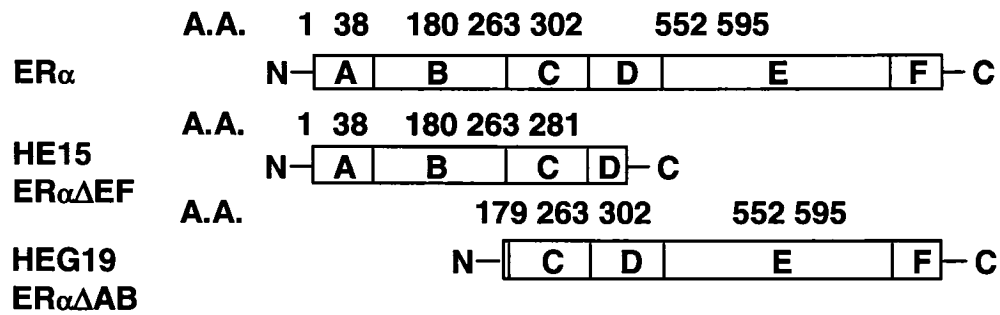
B: HeLa cells were transiently transfected with the wild-type ERα or the mutants ERαΔEF or ERαΔAB. ERα protein level was determined as in Fig. 5.1C following overnight treatment.

C: 293T cells were transiently cotransfected with the empty vectors (mock) or ERαΔAB and/or either GFP^2-Ubi or GFP^2-Ubi_{AA}. Western blot analysis using an anti-ERα antibody (F3A6) on total cell extract reveals the non-ubiquitinated ERα (open arrow) and a GFP^2-Ubi conjugated ERα (closed arrow) following treatment with vehicle (0), estrogen (E2, 25 nM) 4-hydroxytamoxifen (OHT, 10 nM), ICI182,780 (ICI, 10 nM) or raloxifene (Ral, 10 nM) for 5 hours. A non-specific band was also revealed with the F3A6 anti-ERα antibody (asterisk).

D: Cells were transfected with the ERαΔEF expression vector and or either GFP^2-Ubi or GFP^2-Ubi_{AA} and treated as in C. Western blot was performed using the B10 anti-ERα antibody.

Figure 5.3

A.


A.A. 1 38 180 263 302 552 595
ERα
N A B C D E F C
A.A. 1 38 180 263 281
HE15
ERαΔEF
N A B C D C
A.A. 179 263 302 552 595
HEG19
ERαΔAB
N C D E F C

B.

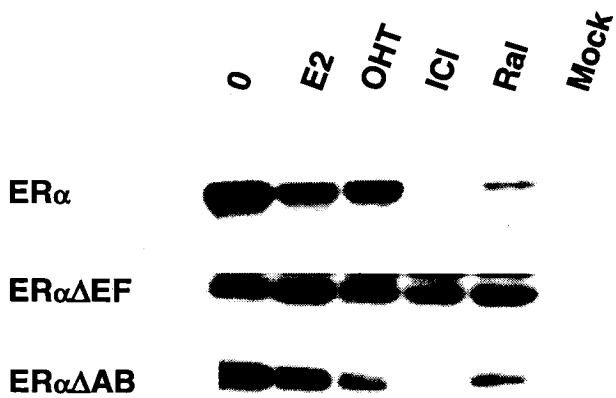

0
E2
OHT
ICI
Ral
Mock
ERα
ERαΔEF
ERαΔAB

Figure 5.3

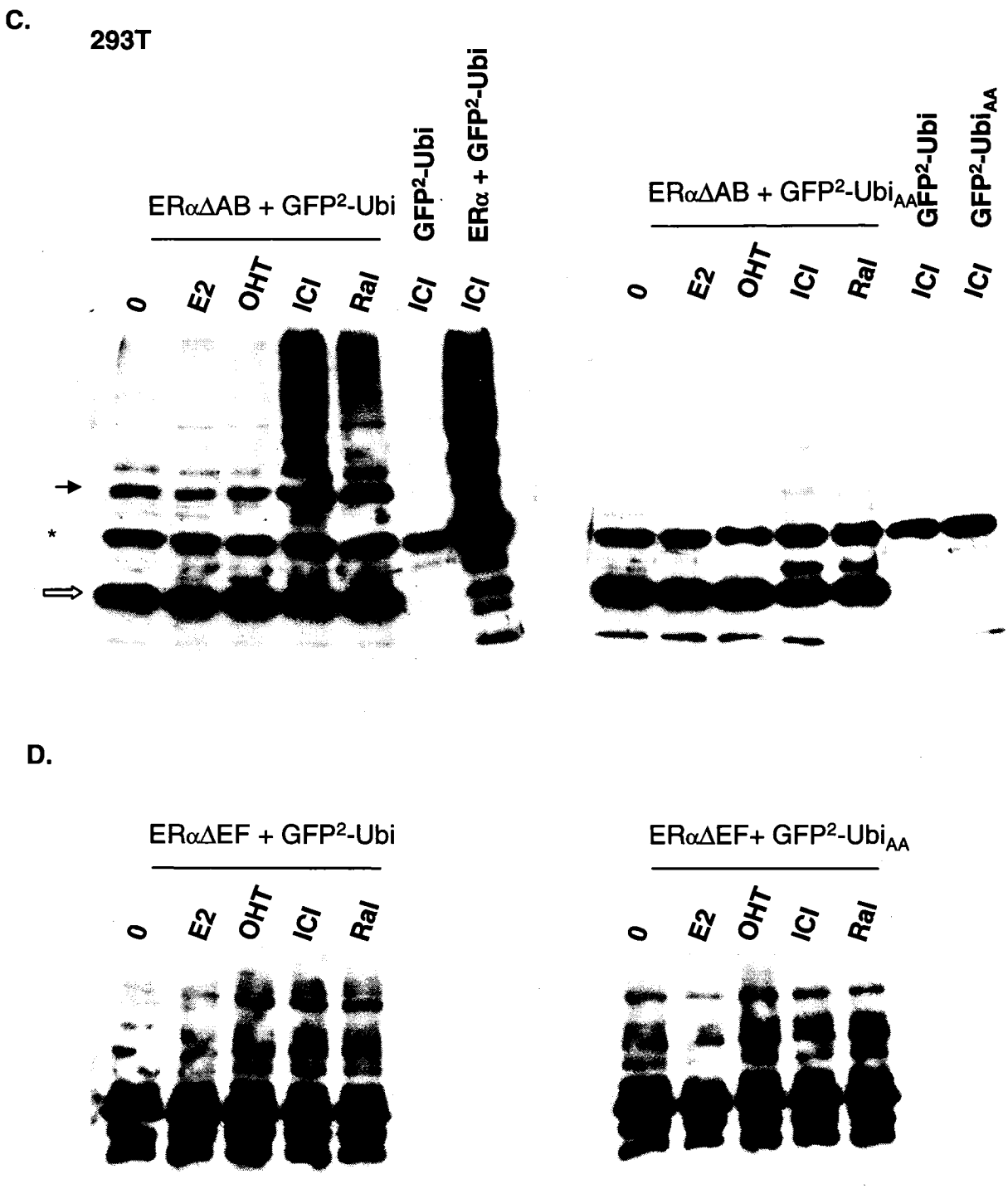

C.
293T
ERαΔAB + GFP²-Ubi
GFP²-Ubi
ERα + GFP²-Ubi
0
E2
OHT
ICI
Ral
ICI
ICI
ERαΔAB + GFP²-Ubi$_{AA}$
GFP²-Ubi
GFP²-Ubi$_{AA}$
0
E2
OHT
ICI
Ral
ICI
ICI
D.
ERαΔEF + GFP²-Ubi
0
E2
OHT
ICI
Ral
ERαΔEF+ GFP²-Ubi$_{AA}$
0
E2
OHT
ICI
Ral

Figure 5.4: Identification of the potential ubiquitination sites on ERα.

A: Schematic representation of the position of all lysine (K) residues in ERα C-terminus (K302, 303, 362, 401, 416, 449, 467, 472, 481, 492, 520, 529, 531 and 581).

B: 293T cells transiently expressing the lysine to arginine ERα mutants and either the GFP^2-Ubi or GFP^2-Ubi_{AA} were treated for 5 hours with vehicle (0), 17β-estrogen (E2, 25 nM), 4-hydroxytamoxifen (OHT, 10 nM), ICI182,780 (ICI, 10 nM) or raloxifene (Ral, 10 nM) and analysed as in Fig. 5.2B. Each mutant was tested a single time.

C: Lysine (K) to Arginine (R) ERα mutant expression vectors were transiently cotransfected with the ERE3-TATA-Luc and CMV-β-galactosidase constructs in 293T cells. Cells were treated as in Fig. 5.2A.

Figure 5.4

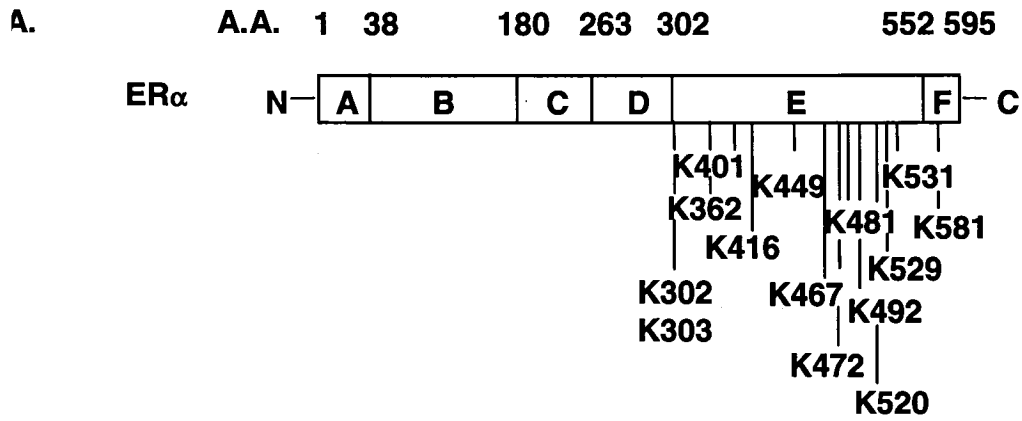

A.
A.A. 1 38 180 263 302 552 595
ERα
N
A
B
C
D
E
F
C
K401
K362
K416
K449
K481
K531
K581
K529
K302
K303
K467
K492
K472
K520

Figure 5.4

3.

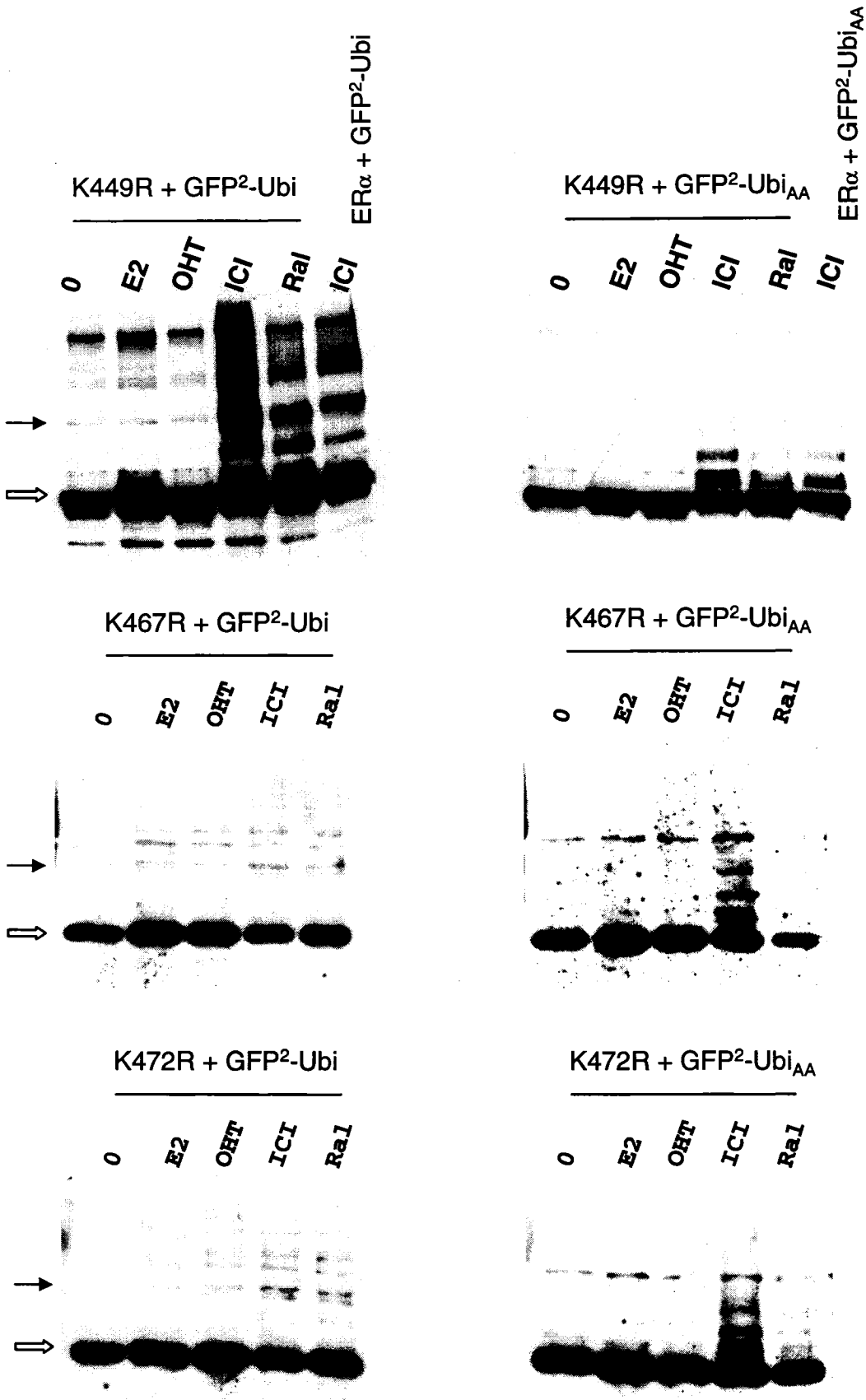

ERα + GFP2-Ubi
K449R + GFP2-Ubi
0
E2
OHT
ICI
Ral
ICI
ERα + GFP2-UbiAA
K449R + GFP2-UbiAA
0
E2
OHT
ICI
Ral
ICI
K467R + GFP2-Ubi
0
E2
OHT
ICI
Ral
K467R + GFP2-UbiAA
0
E2
OHT
ICI
Ral
K472R + GFP2-Ubi
0
E2
OHT
ICI
Ral
K472R + GFP2-UbiAA
0
E2
OHT
ICI
Ral

Figure 5.4

3.

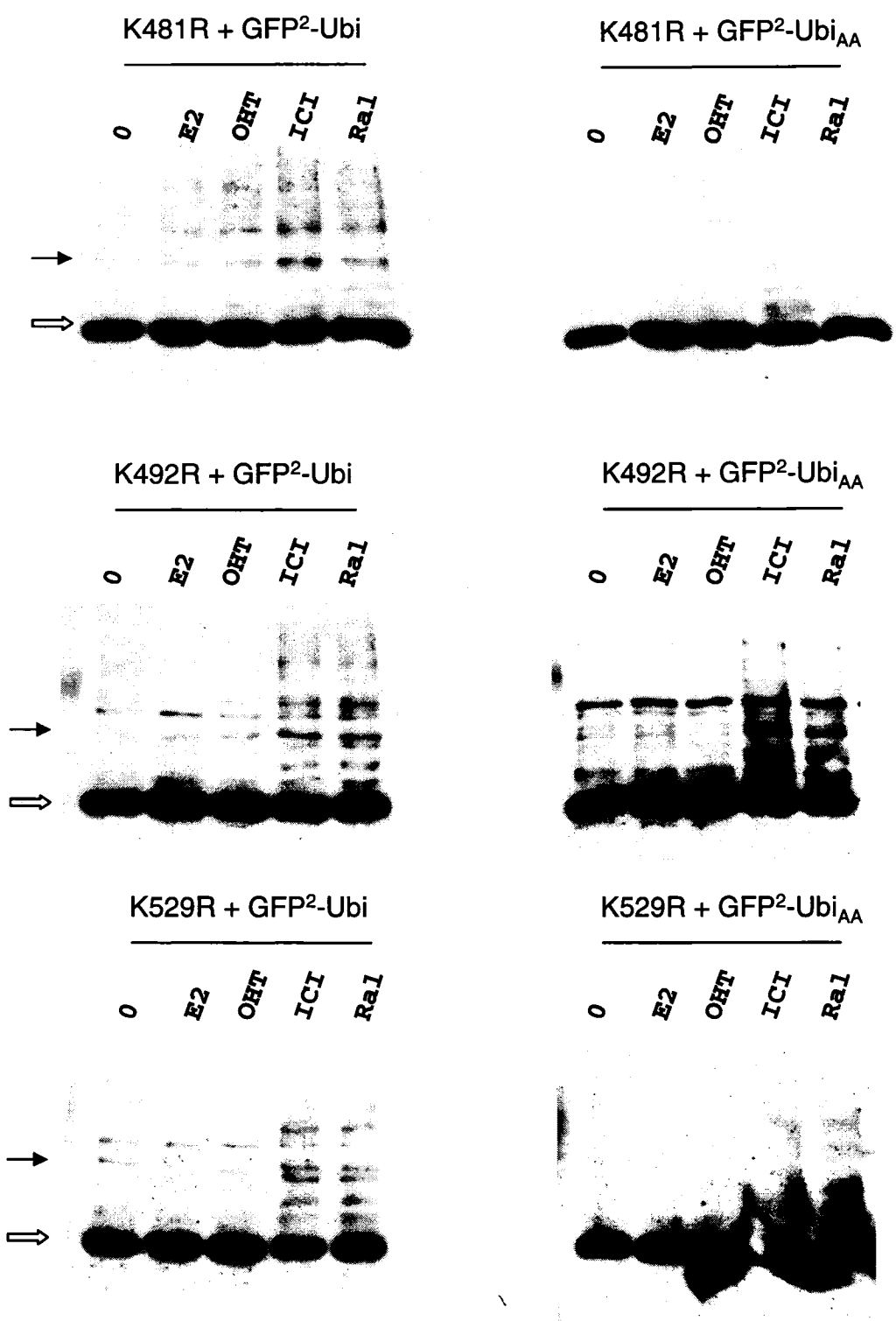
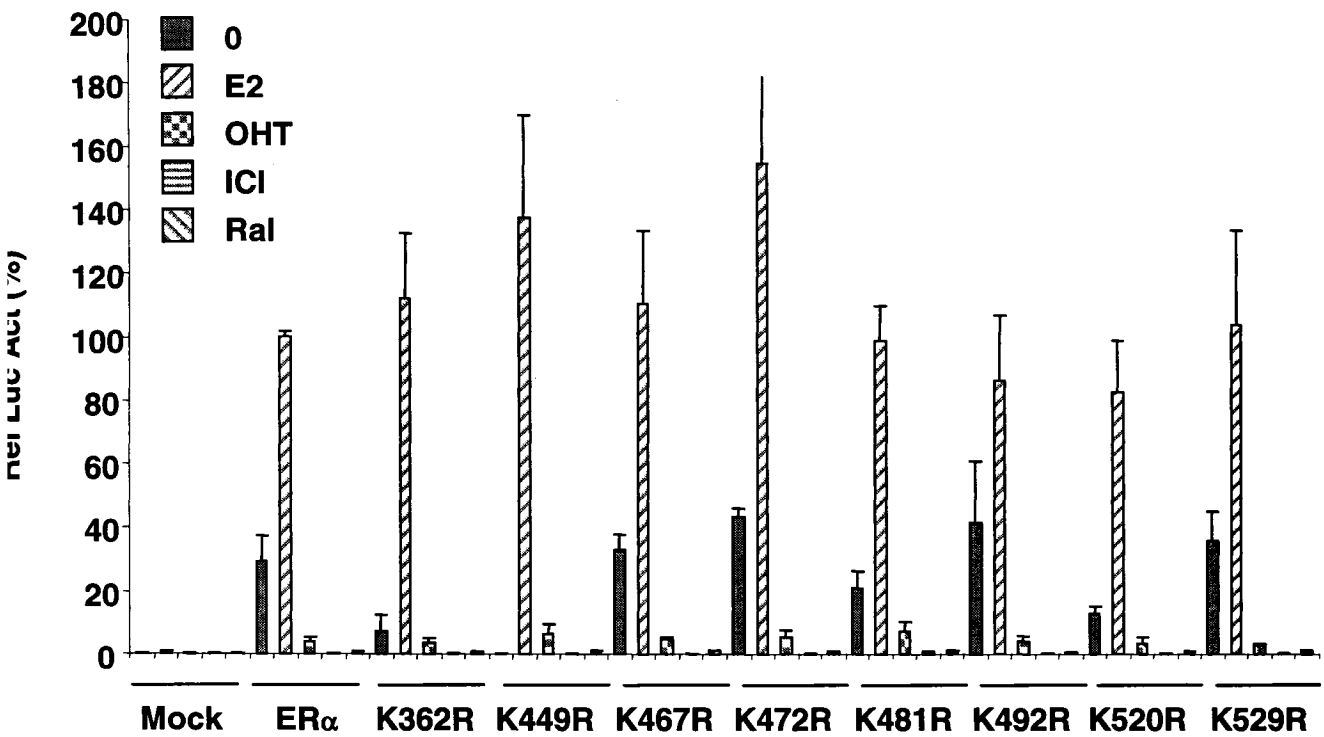

K481R + GFP2-Ubi
0
E2
OHT
ICI
Ral
K481R + GFP2-UbiAA
0
E2
OHT
ICI
Ral
K492R + GFP2-Ubi
0
E2
OHT
ICI
Ral
K492R + GFP2-UbiAA
0
E2
OHT
ICI
Ral
K529R + GFP2-Ubi
0
E2
OHT
ICI
Ral
K529R + GFP2-UbiAA
0
E2
OHT
ICI
Ral

Figure 5.4

C.


200
180
160
140
120
100
80
60
40
20
0
0
E2
OHT
ICI
Ral
Mock
ERα
K362R
K449R
K467R
K472R
K481R
K492R
K520R
K529R

Chapter 6: GENERAL DISCUSSION AND PERSPECTIVES:

The implication of the estrogen receptor alpha (ERα) in numerous physiological functions including reproduction and in the onset and progression of breast cancer has led to the development of biological compounds that modulate its activity. Antiestrogens, primarily developed as anti-fertility agents, were revealed as useful tools for breast cancer treatment at all stages. However, the first antiestrogen widely used in breast cancer treatment, tamoxifen, turned out to have tissue-specific partial agonist activity. This included an undesirable increase in the incidence of uterine cancer, while other effects such as stabilization of bone mass were beneficial, leading to the development of compounds that can differentially modulate the activity of the receptor in different tissues. The results presented in this dissertation were directed at further understanding the mechanisms of ERα inactivation by different classes of antiestrogens. The impact of specific structural determinants in the receptor ligand binding domain on receptor expression levels and transcriptional activity were investigated in order to contribute to the development of novel therapeutic drugs and to the identification of additional mechanisms for therapeutic inactivation of ERα.. This general discussion intends to focus on matters that were not covered in the discussions of each individual manuscript. In addition, potential new approaches to further our understanding of estrogen signaling will be presented.

6.1) Regulating ERα activity through intramolecular interactions:

Intramolecular interactions have long been recognized for their regulatory role in ERα activation. Indeed, crystal structure studies with ERs highlighted the importance of the interaction between helix 12 (H12)

and the ligand-binding pocket of ERα for recruitment of coactivator molecules and of the alternative interaction between H12 and the coactivator binding groove (CBG) to repress activation function 2 (AF2) (1-3). Following work on the trout ERα, Metivier and colleagues suggested that the unliganded receptor is transcriptionally repressed through interaction between a potential helix in the A region of the receptor and the C-terminal CBG (4, 5). This model suggests that this intramolecular interaction represses AF2 through occupation of the CBG and also prevents coactivator access to AF1 (5). Repression of AF1 can be relieved following binding to antiestrogens such as 4-hydroxytamoxifen (OHT), which induces positioning of H12 on the CBG (3), displacing the N-terminal helix and liberating AF1. On the other hand, coactivators are still prevented from interacting with the CBG and AF2 remains inactive (3). Therefore, OHT would generate an AF1 active/AF2 inactive conformation. In the presence of agonists, H12 lies above the ligand-binding pocket and contributes to recruitment of coactivators in the CBG (1, 3). This would prevent the N-terminal helix from interacting at this site, liberating AF1 for recruitment of coactivators including the p160 family members, which possess an AF1-interacting region in addition to LXXLL motifs. Under these conditions synergy between AF1 and AF2 can be observed and the receptor is fully active (6).

In the third chapter we highlight the importance of having a negatively charged amino acid (D or E) at position 351 to maintain an AF1 active/AF2 inactive conformation in presence of the OHT precursor tamoxifen (Tam). Interestingly, raloxifene (Ral) is not an agonist under the same conditions even though the crystal structure of Ral-bound ERα is similar to that of Tam's active metabolite OHT-bound ERα (1, 3). Our results suggest that the tertiary amine on the side chain of Ral, but not that in the side chain of Tam, is optimally positioned for interaction with D351 (1). Ral can be converted to a Tam-like compound by replacing D351 with E, which possesses a longer side chain. We propose that having an E in

position 351 favors the Tam-like activity profile by relieving interactions with Ral's side chain and facilitating AF1 liberation. Note however, that contrary to what was observed in the absence of hormone, deletion of the N-terminal A region did not increase activity in the presence of Ral, suggesting that AF1 repression by Ral is not due simply to occupation of the CBG by the A region. As Ral is more appealing than Tam due to the lack of increase of uterine cancer incidence in patients treated with this compound, our research highlights the potential importance of developing SERMs that contain a tertiary amine in their side chain capable of interacting with D351 of ERα.

In the fourth chapter we highlight the importance of specific residues at the N-terminus of H12 in modulating the antagonist activity of antiestrogens. This research indicates that compounds with side chains resulting in steric clashes with specific long hydrophobic residues in H12 lead to accumulation of the receptor in an insoluble fraction in HepG2 cells. Interestingly, Ral behaves partially as a full antiestrogen in these cells, and leds to a partial exclusion of ERα from the soluble fraction when compared to OHT or ICI182,780. Its lower agonist activity compared to OHT may therefore be derived from intermediary levels of inactivation of the receptor through this mechanism. In support of this hypothesis, specific mutations leading to increased ERα levels in the soluble fraction in the presence of Ral (ERα L536A and L539A) also demonstrated an increased transactivation on a minimal promoter. Whether this observation with transiently transfected reporter vectors is also relevant for the regulation of endogenous ERα target genes remains to be determined. One approach to elucidate this question would be to generate cell lines stably expressing the L536A or L539A mutants in ERα-negative cells such as the breast carcinoma cell line MDA-MB231.The pattern of gene expression following Ral treatment could be investigated in these cells through gene microarray experiments. If our hypothesis suggesting that the differential agonist-profiles of Ral and Tam depends on their

differential stabilisation of ERα in the soluble fraction is reflecting reality, we should observe similar patterns of gene regulation in presence of Ral and Tam in the cells lines expressing mutant receptors. Similarly, it would be possible to identify whether the same coactivator proteins contribute to the agonist activity of Ral and Tam in these cell lines through chromatin immunoprecipitation assays.

Another interesting aspect that our research reveals is the apparent preponderant role of the AF1 function in the partial-agonist activity of antiestrogens. Indeed, the AF1 function is essential for the agonist response of ERα on a minimal promoter in presence of Tam or OHT (Chapter 3). We report in chapter 4 that the activation of L536A or L539A in presence of Ral or ICI182,780 is also dependent on the AF1 function. Since modeling revealed that the H12 mutations that led to partial agonist responses to ICI182,780 or Ral may relieve steric hindrance between these antiestrogen side chains and H12 that are not observed with OHT, it is tempting to speculate that the resulting H12 mutants may adopt a conformation similar to that of OHT-bound receptor. Numerous techniques have been developed to investigate the conformation of ERα in presence of ligands including fluorescence anisotropy assays (7). This approach allows to determine the helical conformation of the end of helix 11 (H11), which is directly affected by the positioning of H12. It has previously been reported that OHT induces a shortening of H11 necessary for H12 displacement over the CBG while it does not occur in the presence of ICI182,780 or 17β-estrogen (E2) (7). Therefore, if AF1 activation in ERα mutants transcriptionally activated by Ral and ICI182,780 is dependent on the positioning of H12 within the CBG like in the presence of OHT, we would expect to detect similar levels of fluorescence anisotropy at the end of H11 in these mutants when bound to either OHT, ICI182,780 or Ral, but not E2 .

6.2) Relationship between accumulation of ERα in an insoluble fraction and transcriptional inactivation:

In chapter 4 we demonstrated that the antagonist properties of full-antiestrogens, such as ICI182,780 and to a lesser degree Ral, were partly dependent on their capacity to remove ERα from the soluble fraction. Interestingly, in cells expressing endogenous ERα, no accumulation of the receptor is detected in the insoluble fraction while in transiently transfected cells expressing exogènous ERα, the receptor accumulates in the insoluble fraction. This may be an artifact of protein overexpression resulting in saturation of the capacity of the proteasome to clear badly conformed proteins, as previously described (8). Our results indicate that endogenous ERα in MCF7 cells undergoes rapid proteasome degradation in the presence of full antiestrogens and that accumulation in the insoluble fraction can be achieved following treatment with the proteasome inhibitor MG132. Noteworthy, this did not affect the antagonist actions of full-antiestrogens and therefore suggests that receptor degradation is not solely responsible for clearance of the receptor from the soluble fraction. This is of importance in breast cancer treatment as our results indicate that antiestrogens can still inactivate the receptor in tumors with defects in the proteasome degradation pathway, both by accumulation of the receptor in an insoluble fraction and by blocking transactivation surfaces in the A/B (AF1) and E (AF2) regions of ERα. The mechanisms governing this accumulation are still obscure. Our hypothesis suggesting that ERα accumulation in presence of full antiestrogens is derived from proteasome saturation in transiently transfected ERα-negative cells implies that ERα degradation in presence of ICI182,780 may be observed at low concentration of transfected ERα protein. Similarly, transfection in MCF7 cells of aggregating molecules which saturate the proteasome as described by Bennet and collegues (8) should prevent ERα degradation and lead to its accumulation in the insoluble fraction in the presence of

ICI182,780. Additional research should also involve characterizing the cellular distribution of the accumulated ERα. This could be accomplished through immunocytochemistry using antibodies raised against ERα in transiently transfected HepG2 cells treated with ICI182,780. Formation of perinuclear aggregates of receptor has been described in the presence of ICI182,780 in transfected cells (9-12). In addition overexpression of heat shock proteins (Hsps) is known to increase proteasomal degradation in HepG2 cells and can revert the formation of nuclear inclusions of aggregated proteins (13). These proteins contribute to the proper folding of novel and misfolded proteins acting as a molecular chaperone (14). It would therefore be relevant to test whether overexpression of Hsps prevent ERα aggregation in presence of ICI182,780 and Ral in transiently transfected HepG2 cells. (15). Results obtained with the classical cellular model of ERα-positive breast cancers, the MCF7 cells, indicate that ERα accumulation does not occur normally but is an artifact of overexpression of ERα in transfected cells. To clarify whether this is physiologically relevant it would be appropriate to sample tumor breast tissue of patients treated with ICI182,780 and examine whether aggregation of ERα can be detected. Finally, it would be of interest to investigate whether cotreatment of MCF7 cells with full antiestrogens and proteasome inhibitors or inhibitors of hsp activity may lead to cellular death through toxic accumulation of receptor aggregates

6.3) Modulation of ERα ubiquitination:

It has previously been reported that optimal ERα transactivation activity is dependent on its degradation by the ubiquitin-proteasome pathway (16). More recently, this process was described as required in the first 6-8 hours of induction with hormone, but as a dampening mechanism afterwards (17). The signal for degradation through the 26S proteasome is the attachment of a polyubiquitin chain to lysine residues on target

proteins. ERα ubiquitination was previously reported to occur following agonist or antagonist treatment (18-20). ERα ubiquitination was initially suggested from the observation of a ladder of bands migrating at a higher molecular weight than ERα in presence of proteasome inhibitors. Wijayaratne and collegues have provided the only direct demonstration to date of ERα ubiquitination (17). They demonstrated that ERα can be purified through His-affinity columns when cotransfected with a 6His-tagged ubiquitin expression vector. Here, we also demonstrated ubiquitination of the receptor in the absence or presence of ligand through another assay that detects the initial addition of a single ubiquitin residue. This is possible as the assay employs a GFP^2-ubi fusion protein where the lysine 48 and 63, which allow for polyubiquitination, are mutated to alanine (21). The ultimate aim of our study was to compare the mechanisms of ubiquitination in the presence of different ligands and to investigate directly the relationship between ubiquitination and transactivation by ERα in presence of antiestrogens. Our preliminary results reveal that site(s) of ubiquitin attachment lie in the C-terminus of the receptor. The specific Lys residues targeted for ubiquitin attachment are presently being investigated through an arginine scanning mutagenesis. Mutants in ubiquitinated Lys residues will provide tools to assess the consequences of inhibiting receptor turn-over induced by different ligands on ERα functional properties including nuclear localization, interaction with DNA, recruitment of coactivators and transcriptional activation.

It will also be of interest to identify the specific ubiquitin-protein ligase(s) involved in ERα ubiquitination in the presence of different ligands. We could use RNAi to prevent the expression of potential ubiquitin-protein ligases and investigate the impact of their absence in the cell on ERα ubiquitination using the GFP^2-Ubi fusion protein described in chapter 5. A high throughput RNAi approach, based on a whole-genome RNAi screen as described in (22), could be used to identify proteins contributing to ubiquitination. We would expect that preventing the expression of key

proteins involved in ERα ubiquitination should decrease the amount of GFP^2-Ubi-conjugated ERα.

Other post-translational modifications can impact the process of protein ubiquitination. For instance, several transcription factors, including GR, AR, PR, p53, c-Jun and c-Myb can be covalently modified by SUMO-1, a small ubiquitin-like modifiers, through the process of sumoylation. (23-28). This modification also occurring on Lys regulates protein localisation and activity (For review see (29)). As ubiquitin residues cannot access lysines, the target protein is protected from degradation by the proteasome. Although ERα sumoylation has yet to be described, modification of lysine residues by other small ubiquitin-like proteins has been reported. Indeed, ERα can be neddylated. This modification enhances ERα turnover by the 26S proteasome (30), but increases ERα-mediated transcription (31). The impact of neddylation on ERα ubiquitination is still not defined. Neddylation of ERα is directed by the ubiquitin-activating enzyme (Uba) 3 (30). Repressing the expression of Uba3 using RNAi would allow us to establish the role of neddylation on ERα ubiquitination following ligand treatment in the ubiquitination assay described in chapter 5.

Other post-translational modifications not occurring on Lys can also impact ERα ubiquitination. Such modifications include phosphorylation. This has been described for the NR to progesterone (PR), androgen (AR) and retinoic acid (RAR) (32-34). Not surprisingly, deregulation of ubiquitin-dependent proteolysis has been implicated as a causative factor in cancer (35-40). It would therefore be interesting to investigate the implication of post-translational modifications on ERα ubiquitination and degradation. For instance, numerous phosphorylation sites on ERα have been identified and mutants for these sites have been generated. Using the ubiquitination assays described in chapter 5, we could investigate the impact of each phosphorylation site on ERα ubiquitination using the mutants S104A,

S106A, S108A, S118A, S167A, T311A and Y537A in the ubiquitination assay described in chapter 5.

6.4) Bioavailability of antiestrogens:

Although tamoxifen (Tam) has been the treatment of choice for breast cancer since the early 1970's, its side effects on the incidence of uterine cancer are undesirable. Newer compounds such as raloxifene (Ral) also act as Selective Estrogen Receptor Modulator (SERM), but do not have agonist activity in the uterus. Unfortunately, a major drawback for the widespread use of Ral relates to its low bioavailability. Tam has a bioavailability of approximately 100%, whereas that of Ral is only 2% (41, 42) getting rapidly excreted (43). Indeed, elevated doses of this compound (300 mg/day) weakly inhibit tumor progression in advanced breast cancer patients (44). However, at five times lower doses (60 mg/day) the estrogenic actions of Ral on the skeletal system significantly reduce osteoporosis (45) (46). In order to circumvent the low bioavailability of Ral researchers have been developing a nanoparticule based delivery approach. Initial developments have actually involved covalently attaching Tam to nanoparticles (47-49). Within 6 hours following intravenous injection of Tam-bound nanoparticules in nude athymic mice bearing a human breast carcinoma xenograft from MDA-MB231 cellls, more than 26% of the total nanoparticules could be recovered in the tumors (49). Although promising, this approach is still in its infancy and still needs to be tested with Ral.

An alternative approach has been to identify novel antiestrogens with Ral-like charateristics. In the third chapter of this dissertation we present data that identifies the compound **9** as having a Ral-like activity profile in mammary and endometrial cell lines. Future studies should establish its activity profile on other estrogen target tissues, namely the skeletal, cardiovascular and central nervous systems. This could readily

be done in cell culture assays through the classical testing systems that have been available for Ral. For instance, initial work presented in chapter 3 highlights the antagonist potential of compound **9** on both breast and endometrial cells. Studies of growth advantage on osteoclast versus osteoblast could also be performed in *in vitro* cultures or in animal models, such as the ERα knockout mice (αERKO) as previously described (50). As well, the neurotrophic effects of compound **9** could be tested in tissue explants derived from the hypothalamus and preoptic area of developing mice (51). In order to establish the bioavailibility of this compound we would measure its blood levels following oral administration in wild-type mice, while its toxicity could be determined following oral administration of increasing doses. The actual efficacy of compound **9** in tumor treatment would need to be tested *in vivo* in mouse models, such as nude mice injected with transformed MCF7 breast cancer cells. We would expect that compound **9** be capable of further reducing tumour growth compared to Tam-treated littermates.

6.5) Role for aromatase inhibitors:

Aromatase inhibitors provide an alternative to antiestrogens in the inhibition of the estrogen signalling pathway. Aromatase inhibitors antagonise the production of estrogens from their androgenic precursors, whether synthesis takes place in the ovary (premenopausal women) or in peripheral tissues (post-menopausal women). Aromatase inhibitors are currently used for adjuvant treatment of breast cancer, and may be tested soon for breast cancer prevention in comparison with SERMs. However, long-term treatment with aromatase inhibitors may be undesirable due to the negative impact of estrogen depletion on the skeletal and nervous systems.

Selective Tissue Estrogenic Activity Regulators (STEAR) are compounds that get converted into active estrogenic metabolites in target

tissues. They have the advantage of acting locally as agonists on some estrogen target tissues. The best-studied family member is tibolone, which is as efficient as estrogens in promoting beneficial effects on the skeletal and central nervous systems without stimulating the proliferation of the endometrium and the breast epithelium (52-54). A report indicates that aromatase activity is not required for the conversion of tibolone to some of its active metabolites (54). However, this still needs to be further investigated as it has never been tested *in vivo*. It would be of interest to test whether tibolone maintains beneficial effects on the skeletal as well as the central nervous system in aromatase knockout mice models. In parallel, the capacity of tibolone to restore bone mass during treatment with aromatase inhibitors can be assessed in normal mice. Finally, the lack of negative effect of tibolone on the antiproliferative effects of aromatase inhibitors should be tested in an *in vivo* model for the effect of aromatase inhibitors (nude mice injected with aromatase-overexpressing MCF7 cells). If tibolone has no effect on the aromatase inhibitor action then we would expect the reduction in tumor growth to be equivalent in animal co-treated with tibolone and aromatase inhibitors or treated with the aromatase inhibitors alone.

6.6) Novel estrogen receptor:

Recently the GPR30 transmembrane protein, member of the G-protein coupled receptor (GPCR), was identified as a membrane receptor for estrogens (55, 56). Interestingly this receptor also binds the antiestrogens Tam and ICI182,780 and this allows for its activation as observed in the presence of E2. GPR30 may thus mediate agonist activity of these compounds on some aspects of estrogen function that have not been investigated thus far (55). It would therefore be of interest to further characterize the role of this receptor in breast tumor cell growth. Due to its capacity to respond to antiestrogens as well as to estrogens this GPR30

receptor may play a role in acquired tolerance to antiestrogen treatment, highlighting the advantage of aromatase inhibitors instead of antiestrogens in the treatment of breast cancer. This new estrogen receptor appears to be involved in the initial rapid response to estrogen that characterizes the non-genomic response. In order to clarify the importance of this signalling pathway in tumor cell proliferation, we propose to test the expression of the GPR30 receptor in cells where estrogen has growth-stimulatory properties and to assess whether RNAi-mediated inhibition of GPR30 expression affects cell growth. It would also be of interest to identify genes whose expression is affected following GPR30 activation. This could be done through gene array analysis using cDNA derived from the ERα (-), ERβ (-) GPR30 (+) cell line human SKBR3 cells (55, 57-59). Ultimately, generation of GPR30 knockout mice will allow determining the full spectrum of action of this receptor.

6.7) Concluding statement

Studies described here have furthered the knowledge concerning the mechanisms governing ERα inactivation by antiestrogens. Overall, we have demonstrated the importance of specific interactions between the antiestrogen side chain and residues in ERα LBD to repress receptor-mediated transcriptional activation, highlighting direct targets for future antiestrogen development. In addition, the work presented in this thesis demonstrates that inhibition of proteasome-mediated degradation of the receptor in the presence of full antiestrogens does not lead to increased agonist activity, suggesting that antiestrogens would still inactivate the ERα in tumors in which the proteasome degradation pathway has been inactivated either following cotreatment with proteasome inhibitors or as a result of acquired modification in the course of cancer progression. Our work also demonstrates that full antiestrogens induce the accumulation of the receptor in an insoluble fraction in MCF7 cells treated with proteasome

inhibitors or in transiently transfected cells. Finally, ubiquitination of the receptor was detected in the absence or presence of ligand and involved lysine residues in the C-terminus of the receptor. Continuation of these studies will clarify the mechanisms of proteasome-induced degradation of estrogen receptors in the presence of agonists and antagonists and the role of receptor degradation in the mechanisms of action of full antiestrogens. These studies therefore proposed alternative research avenues to complement the present armamatarium available to treat breast cancer patients.

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From: "Xavier Mascle" <xavier.mascle@UMontreal.CA> Add to Address Book

To: "mathieu lupien"

Subject: permission de reproduction

I grant Mathieu Lupien permission to reproduce the manuscript entitled "Both agonist and antagonist dependent ERa ubiquitination and degradation is dependent on C-terminal lysine residues" in his doctoral dissertation.

Xavier MASCLE

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From: "Muriel Aubry" <muriel.aubry@umontreal.ca> Add to Address Book

To: "'mathieu lupien'"

Subject: RE: permission de reproduction

Date: Thu, 2 Jun 2005 16:32:48 -0400

Bonjour Mathieu,
Bien sûr, tu peux reproduire le manuscrit traitant de l'ubiquitination du
récepteur des estrogènes auquel Xavier a contribué. Félicitation pour le
dépôt prochain de ta thèse.

Muriel Aubry, Ph.D.

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