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Tamoxifen and Raloxifene Differ in Their Functional Interactions with Aspartate 351 of Estrogen Receptor α

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ABSTRACT

The bulky side chains of antiestrogens hinder folding of the ligand binding domain (LBD) of estrogen receptors (ERs) into a transcriptionally active conformation. The presence of a tertiary amine in the side chain of raloxifene, which interacts with a negatively charged residue in helix H3 of the ER LBD [Asp351 in human (h)ERα], is important for antiestrogenicity in animal and cellular models. To better understand the molecular basis of the differential activity of tamoxifen and raloxifene, we have examined the influence of tertiary amine substituents and of mutations at position 351 in hERα on the activity profiles of tamoxifen derivatives. Results obtained in several cellular model systems suggest that the degree of antagonist activity of tamoxifen derivatives does not strictly correlate with the basicity of the side chain but depends on an optimal spatial relationship between the tertiary amine of these antiestrogens and the negative charge at position 351. Although altering the position of the negative charge at residue 351 (mutation D351E) had little effect on transcriptional activity in the presence of tamoxifen, it drastically increased the partial agonist activity of a tamoxifen derivative with improved antagonist activity as well as that of raloxifene. Our results suggest that contrary to raloxifene, tamoxifen and most of its derivatives do not interact with Asp351 in an optimal manner, although this can be improved by modifying tertiary amine substituents.

Antiestrogens are synthetic estrogen analogs used clinically in breast cancer treatment to inhibit the proliferative action of estrogens (MacGregor and Jordan, 1998; Katzenellenbogen et al., 2000; Clarke et al., 2001). They compete with estrogens for binding to estrogen receptors ERα and ERβ (Green and Chambon, 1988; Kuiper et al., 1996). These ligand-dependent transcription factors, members of the nuclear receptor superfamily, contain two transcriptional activation functions flanking a DNA binding domain (Robinson-Rechavi et al., 2003). The C-terminal activation function (AF2), which is part of the ligand binding domain (LBD), recruits coactivators in the presence of estrogens, but not of antiestrogens (Rosenfeld and Glass, 2001; Belandia and Parker, 2003). The N-terminal activation function (AF1) cooperates with AF2 for transcriptional activation in the presence of agonists and has been suggested to be critical for the partial agonist activity of antiestrogens (Berry et al., 1990; Tzukerman et al., 1994).

Antiestrogens contain a bulky side chain attached to a steroid or steroid-like skeleton. Pioneering studies demonstrated the importance of the side chain for the antagonist activity of antiestrogens using uterotrophic assays in immature rats. A tamoxifen derivative lacking its alkylaminoethoxy side chain was fully estrogenic in this assay (Jordan and Gosden, 1982). Crystallographic studies of the estrogen receptor LBD complexed to the antiestrogens tamoxifen or raloxifene have demonstrated that the bulky side chain of antiestrogens cannot be accommodated within the LBD. The resulting structures differ from that observed in the presence

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ABBREVIATIONS: ER, estrogen receptor; AF, activation function; LBD, ligand binding domain; H12, helix 12; SERM, selective estrogen receptor modulator; h, human; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; OHT, 4-hydroxytamoxifen; ICI 164,384, *N-n*-butyl-*N*-methyl-11-(3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl)undecanamide; ICI 182,780, faslodex.

of estrogen by the position of the C-terminal helix 12 (H12), which in the presence of agonist sits above the ligand binding cavity and contributes to coactivator recruitment (Brzozowski et al., 1997; Shiau et al., 1998). In the antagonist-bound structures, the side chains of antiestrogens force H12 to reposition over the coactivator binding groove, preventing recruitment of coactivators.

The length and composition of the side chain can differ markedly between antiestrogens. Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, which behave as tissue-specific partial agonists, have a shorter side chain than the full antiestrogens ICI 164,384 and ICI 182,780. Tamoxifen and raloxifene are estrogen agonists in bone, and tamoxifen but not raloxifene is estrogenic in the uterus of ovariectomized rats or mice (Grese et al., 1997). Both SERMs contain alkylaminoethoxy side chains, with different tertiary amine substituents. Replacement of the nitrogen in raloxifene with a carbon or a nonbasic nitrogen atom abolished the capacity of raloxifene derivatives to antagonize estrogen-induced increases in uterine wet weight (Grese et al., 1997) and resulted in increased ER-dependent transcription in stably transfected MDA-MB-231 cells (Liu et al., 2002). In tamoxifen derivatives, conversion to a nonbasic aromatic amine also abolished antagonist activity in a rat uterotrophic assay (Robertson et al., 1982), consistent with a possible role of the tertiary amine in the antagonist activity of tamoxifen.

The crystal structure of the ERα LBD complexed with raloxifene indicated that the tertiary amine in the raloxifene side chain can establish a hydrogen bond with the carboxyl group of amino acid Asp351 (Brzozowski et al., 1997). For tamoxifen, the distance between the two groups was compatible with an electrostatic interaction (Shiau et al., 1998). The functional importance of Asp351 in the antiestrogenic activity of SERMs is supported by the demonstration that a D351Y mutation, characterized in a tamoxifen-stimulated breast tumor (Wolf and Jordan, 1994), results in increased agonist activity of raloxifene on ERα-dependent transcription (Levenson and Jordan, 1998). Mutation D351E had a similar effect (Liu et al., 2002). Conversely, mutating Asp351 into hydrophobic residues (Ala, Val, and Gly) preserved the antiestrogenicity of tamoxifen in HeLa cells (Anghel et al., 2000) and reduced its partial agonist activity in MDA-MB-231 cells (MacGregor Schafer et al., 2000). It has been proposed that neutralization of the charge of Asp351 is important for the antiestrogenicity of SERMs (Liu et al., 2002).

To address whether differential interaction with Asp351 may be responsible for the different degree of agonist activity of tamoxifen and raloxifene, we examine here the importance of this interaction in a series of tamoxifen derivatives on growth of ERα-positive MCF7 cells, on alkaline phosphatase activity in uterine Ishikawa cells, and on transcriptional activity of hERα in HeLa and HepG2 cells. Our results suggest that tamoxifen and raloxifene interact differentially with Asp351 as a result of differences in side-chain structure/conformation. The activity of tamoxifen seems mostly independent of interaction with Asp351 in the cellular systems tested, but the lower agonist activity of raloxifene or of a tamoxifen derivative with improved antagonist properties are dependent on interaction with Asp351 and on the AF1.

Materials and Methods

Reagents and Hormones. 17β-Estradiol and tamoxifen were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). [2,4,6,7-^{3}H]Estradiol was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). ICI 182,780 and raloxifene were purchased from Tocris Cookson Inc. (Ellisville, MO) and Sigma-Aldrich Canada Ltd., respectively. Cell culture media were purchased from Wisent Inc. (St-Bruno, QC, Canada), and fetal bovine serum was purchased from Sigma-Aldrich Canada Ltd.

Cell Culture and Transient Transfection Assays. HeLa and HepG2 cells were maintained in DMEM supplemented with 5 or 10% fetal bovine serum, respectively, and switched 3 days before initiating experiments to medium without phenol red supplemented with charcoal-stripped serum. For chloramphenicol acetyl transferase (CAT) assays, transient transfections were carried out in HeLa cells in 10-cm plates by the calcium phosphate coprecipitation method as described previously (Anghel et al., 2000). DNA mixes contained typically 0.5 μg of expression vector for wild-type ERα (pSG5-HEG0) or for ERα mutants at position 351, together with 2 μg of reporter vector (ERE3-TATA-CAT/EBV; Anghel et al., 2000) and 2 μg of internal control plasmid pCMV-βGal, and they were supplemented to 15 μg in total with pBluescribe-M13+ (Stratagene, La Jolla, CA). Hormones were added 18 to 20 h after transfection, after removing the calcium phosphate precipitates. For competition assays with ERα, estradiol (0.01 or 0.03 nM) and serially diluted antiestrogens were premixed in 1 ml of medium before 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 (250 mM). CAT activity was measured after standardization for β-galactosidase activity. IC_{50} values were calculated using the Prism software (ver. 3; GraphPad Software, Inc., San Diego, CA).

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 Gene Pulser II apparatus (Bio-Rad, Mississauga, ON, Canada). DNA mixes contained typically 1 μg of expression vector for wild-type ERα (pSG5-HEG0) or for ERα mutants at position 351 (Anghel et al., 2000), together with 2 μg of reporter vector (pERE3-TATA-Luc), and 2 μg of internal control plasmid pCMV-βGal, and they were supplemented to 40 μg in total with salmon sperm DNA (Invitrogen Canada Inc., Burlington, ON, Canada). Cells were plated in six-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 (100 mM Tris-HCl, pH 7.9, 0.5% Nonidet P-40, and 50 mM dithiothreitol). Luciferase activities were measured in presence of luciferin substrate with a Fusion universal microplate analyser (PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada), and β-galactosidase activities were measured at 420 nM with a SpectraMax 190 (Molecular Devices, Sunnyvale, CA). Luciferase activities were normalized for β-galactosidase activities.

For Western blot assays, HeLa cells were transfected with 20 μg of pSG5-HEG0 expression vector supplemented to 80 μg in total with salmon sperm DNA (Invitrogen Canada Inc.) using the electroporation technique (10^7 cells; 0.24 kV; 950 μF in a Bio-Rad Gene Pulser II apparatus). Hormones were added 24 h after transfection and incubated for 16 h. Whole cell extracts were analyzed by 8% SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and incubated with the anti-hERα mouse monoclonal antibody B10 (obtained from Prof. P. Chambon, Institut Clinique de la Souris, Illkirch, France). Complexes were revealed by enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences) 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 six-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 was measured using a detergent-compatible protein assay (Bio-Rad).

Alkaline Phosphatase Assays. Ishikawa cells were maintained in α-minimum Eagle's medium supplemented with 5% fetal bovine serum. 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 plates (2.5×10^4 cells/well). Treatments were performed in triplicate for 48 h, after which cells were washed in phosphate-buffered saline twice, frozen at −80°C for 15 min, and incubated with 50 μl of reaction buffer (5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$, and 1 M diethanolamine, pH 9.8). Plates were incubated at room temperature until production of a yellow color, and levels of *p*-nitrophenol 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 used to electroporate 10^7 cells contained 20 μg of expression vector for wild-type hERα (pSG5-HEG0) or for ERα mutants at position 351 (Anghel et al., 2000), supplemented to 80 μg in total with salmon sperm DNA. Cells were plated in six-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; GE Healthcare) for 2 h at 37°C. To determine nonspecific binding, levels of bound ^{3}H-labeled estradiol were measured in the presence of 500-fold excess of nonlabeled estradiol. Specific binding was obtained by subtracting nonspecific binding from total levels of bound ^{3}H-labeled estradiol. For competition binding assays, cells were incubated with 0.5 nM ^{3}H-labeled estradiol with increasing concentrations of antiestrogens (0.05 nM–3 μM). Cells were incubated for 2 h at 37°C, and radioactivity was quantified after extraction by scintillation counting. The K_d values for estradiol and the IC_{50} values for antiestrogens were calculated using GraphPad Prism software.

Results

Interaction with Asp351 Minimally Affects the Affinity of Tamoxifen Derivatives for Estrogen Receptor α. SERMs, but not full antiestrogens, contain a tertiary amine in their side chains that interact with amino acid Asp351 in hERα. Structure-function analyses (Grese et al., 1997; Liu et al., 2002) have pointed to the importance of this group in modulating the antagonist/agonist properties of raloxifene. In tamoxifen derivatives, antagonist activity was observed in a uterotrophic assay in immature rats (Robertson et al., 1982) when the tertiary amine was replaced by a hydroxyl group but not when it was part of a pyrrole group (compound **14**; Fig. 1). Because the hydroxyl but not the pyrrole group can engage in hydrogen bond interactions, these results were compatible with a requirement to establish a hydrogen bond with Asp351 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 tamoxifen derivatives in the inhibition of human ERα transcriptional activation properties, we measured the relative IC_{50} values in the inhibition of estradiol-dependent reporter gene expression in HeLa cells for a series of tamoxifen derivatives with decreasing basicity of the tertiary amine (Fig. 1). The most significant effect was a 4.5-fold increase in the IC_{50} value for the nonbasic, aromatic compound **14** (Table 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 (Robertson et al., 1982). A

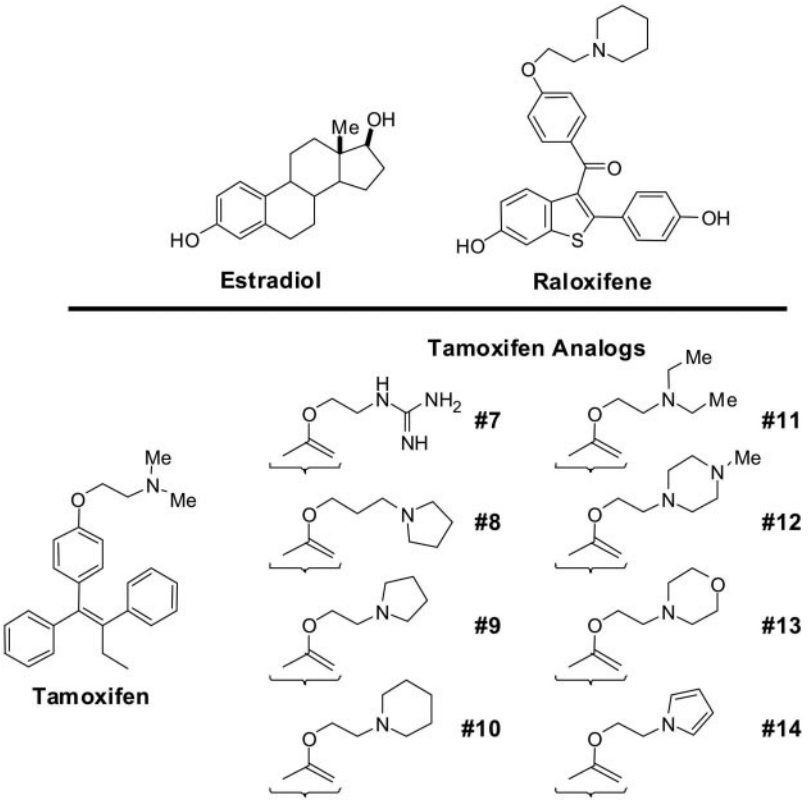

Fig. 1. Tamoxifen derivatives. The structures of estradiol, raloxifene, tamoxifen, and tamoxifen derivatives used in this study are shown. The triphenylethylene skeleton is identical in all tamoxifen derivatives.

2.3-fold increase in the IC_{50} for compound **13**, which has the second lowest pK_a in this series of derivatives (8.7), was also observed (Table 1). These results suggest that the basicity of the tertiary amine affects the affinity of interaction with human ERα minimally.

To further substantiate this conclusion, we compared IC_{50} values for tamoxifen, compounds **9** and **14**, in both transactivation and hormone binding assays with the wild-type receptor and mutants at position 351. Mutations D351A, D351E, D351V, and D351Y were found to have little effect on affinity for estradiol (Fig. 2A), and IC_{50} values for tamoxifen and compound **9** were only minimally affected (≤4-fold) by mutations D351A and D351V both in competitive transactivation assays (Fig. 2, B–D, light columns) and in hormone binding assays (dark columns). The only substantial effect of D351A or D351V mutations was the ~20-fold increase in binding of compound **14**, which had lower affinity for the wild-type receptor than tamoxifen and other derivatives (Table 1). This suggests that Asp351 destabilizes the aromatic side chain of derivative **14**, which may form productive contacts in the absence of an acidic residue at position 351. A small, but reproducible increase in IC_{50} values (2- to 4-fold) observed for compound **9** with mutants D351A or D351V could also indicate a role for Asp351 in stabilizing binding of this compound.

A Tamoxifen Derivative with a Tertiary Amine That Cannot Engage in Interaction with Asp351 Retains Antiestrogenicity in MCF7, Ishikawa, and HeLa Cells. To further investigate whether the nature of the amine group in the antiestrogen side chain affects the antagonist/agonist activity of tamoxifen derivatives, we characterized the activity of compound **14** and other tamoxifen derivatives (Fig. 1) in different human cell lines. In ERα-positive MCF7 human breast tumor cells, tamoxifen and one of its derivatives with a basic side chain, compound **9** (pyrrolidine derivative), fully suppressed the stimulatory effect of 0.1 nM 17β-estradiol on cellular growth (Fig. 3A). In this system, compound **14** also fully suppressed estradiol-induced proliferation, albeit at higher concentrations, returning growth rates to those observed in the absence of ligand. Although tamoxifen and compound **9** had growth-suppressive effects at the maximal concentrations tested, the effect of compound **14** at 2 μM was comparable with those observed with tamoxifen at 1 μM and with compound **9** at 100 nM. Thus, whether the lack of a full suppressive effect on growth of compound **14** reflects its lower affinity for ERα or the absence of specific growth-suppressive properties remains unclear. However, these results indicate that 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. 3B), although competition was only partial as a result of the higher concentrations of estradiol used in this assay (10 nM). It is noteworthy that compound **14** alone did not have increased basal activity compared with tamoxifen and compound **9** in Ishikawa cells at all concentrations tested (Fig. 3C). Finally, we also tested the capacity of tamoxifen, compounds **9** and **14**, to modulate expression of a minimal ERE3-TATA-Luc reporter vector in transiently transfected HeLa cells expressing hERα (Fig. 4, A–C). There was no dose-dependent increase in transcriptional activity with any of the ligands including compound **14**, whereas full competi-

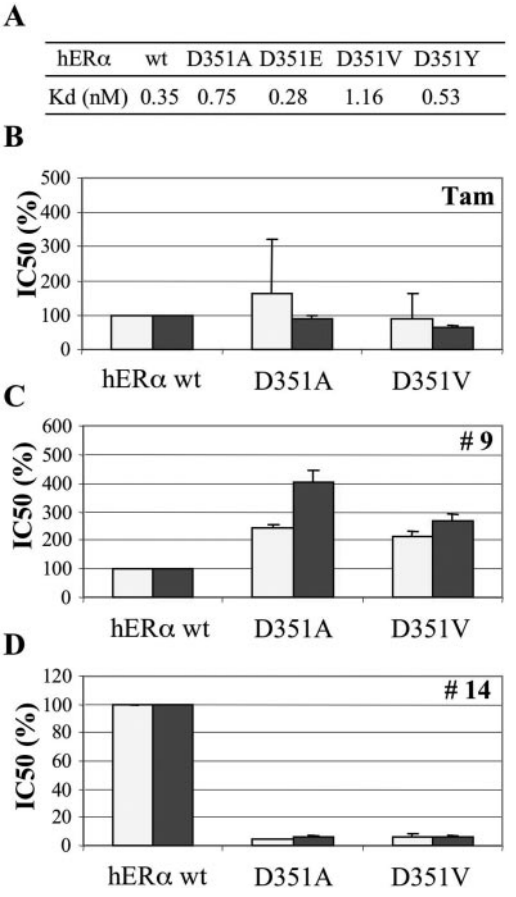

Fig. 2. Effect of mutations of amino acid Asp351 of hERα on the binding affinity of tamoxifen derivatives. A, K_d values of wild-type or mutant hERα for estradiol were calculated from saturation binding assays. HeLa cells were transiently cotransfected with expression vectors for wild-type hERα (pSG5-HEG0) or mutants D351A, D351V, D351E, or D351Y (20 μg/10-cm plate). Cells were treated 18–20 h after transfection with increasing concentrations of tritiated estradiol (0.03–16 nM), and harvested 2 h later. B to D, IC_{50} values for tamoxifen, derivative **9** and **14**, respectively, in competitive transactivation assays (light columns) or in competitive hormone binding assays (dark columns). For each compound, IC_{50} values are expressed as relative values for the mutant versus wild-type receptor. Error bars indicate the standard deviation between three different independent experiments.

TABLE 1

The basicity of the antiestrogen side chains has only a minor impact on affinity for hERα

pK_a values correspond to reported values for the corresponding secondary amine (Robertson et al., 1982). IC_{50} values (expressed as a percentage of the IC_{50} value measured for tamoxifen) measured in competitive transactivation assays or hormone binding assays in HeLa cells transfected with the wild-type hERα are the average of three or four independent experiments, respectively, and are reproducible within ± 30%.

	7	8	9	10	11	Tam	12	13	14
pK_a	13.6	11.3	11.3	11.2	10.9	10.8	9.8	8.7	3.8
IC_{50} (transactivation)	80	120	40	40	110	100	40	230	450
IC_{50} (hormone binding)			34			100			410

Tam, tamoxifen.

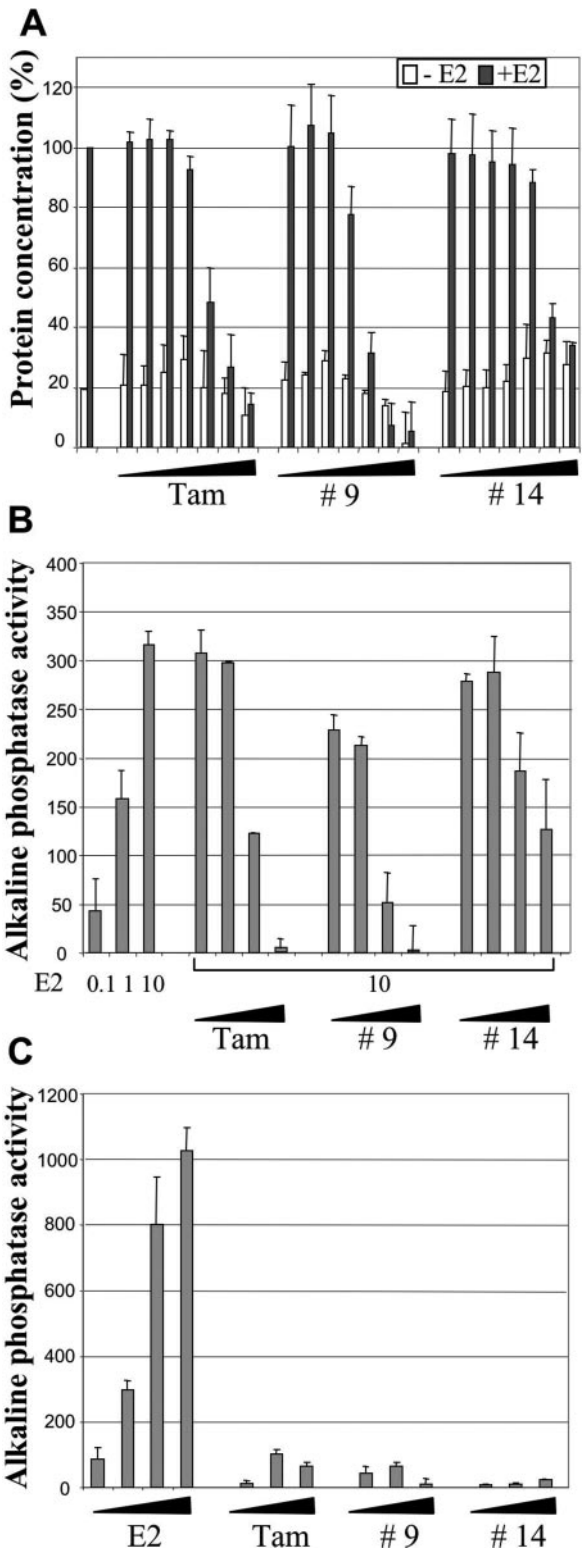

Fig. 3. Agonist/antagonist activities of tamoxifen derivatives in MCF7 and Ishikawa cells. A, repression of estradiol-induced growth of MCF7 cells by tamoxifen derivatives. MCF7 cells were seeded at 3×10^4 cells/well in six-well plates in phenol red-free DMEM and incubated in the presence or absence of 0.1 nM estradiol, either alone or premixed with increasing concentrations of tamoxifen, 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 S.D. between three different experiments with triplicate measurements for each point. B, antagonist activity of tamoxifen 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 tamoxifen, derivative **9** or **14** (1, 10, 100, or 1000 nM) for 48 h. Cells were then lysed in the plate, and alkaline phosphatase activity was assayed using production of *p*-nitrophenol in a colorimetric assay. This experiment was reproduced three times with similar results. A typical experiment is shown. Error bars represent the standard S.D. between triplicate samples. C, agonist activity of tamoxifen derivatives in Ishikawa cells. Cells were incubated with increasing concentrations of estradiol (1, 10, 100, or 1000 nM) or tamoxifen, derivative **9** or **14** (10, 100, or 1000 nM) for 48 h, and alkaline phosphatase activity was assayed as described above.

tion of estrogen-induced activity was observed with all compounds, although with different potencies. Likewise, 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 tamoxifen to engage in interaction with Asp351 is not required for its antiestrogenic properties in these experimental systems.

The Agonist Activity of Tamoxifen Derivatives in HepG2 Cells Does Not Correlate with the Degree of Basicity of Their Side Chains. 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 (McDonnell et al., 1995). Transcriptional activity of ERα was markedly different in the presence of tamoxifen and of raloxifene (Fig. 5). Most tamoxifen derivatives were also partially permissive for transcriptional activity, whereas compound **9** completely repressed transcription. No correlation could be observed between the levels of receptor activity and the basicity of the tertiary amine in the side chain of tamoxifen derivatives (which decreases from compounds **7** to **14**). Variations in IC_{50} values between the different compounds did not influence these results because saturating concentrations of antiestrogens were used in this assay (Fig. 6). Note also that compounds with similar IC_{50} values led to variable levels of receptor activity (compounds **9**, **10**, and **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 tamoxifen derivatives, compound **9,** like raloxifene, almost fully repressed basal activity (Fig. 5). This repression occurred in a dose-dependent manner and was almost as effective as that achieved by ICI 182,780 at saturation (Figs. 5 and 6B). Compound **9** also had a greater efficacy than OHT in the repression of estradiol-mediated reporter gene expression, although with lower potency compared with raloxifene and OHT (Fig. 6A, hERα). 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. 1), both led to intermediate levels of activity between compound **9** and tamoxifen. These results suggest that the specific structure of the side chain of compound **9** is responsible for its capacity to fully repress ERα-dependent transcription.

Full antiestrogens, such as ICI 182,780, that completely antagonize estrogen transactivation in HepG2 cells (Figs. 5 and 6B) induce degradation of ERα (Dauvois et al., 1992; Wijayaratne et al., 1999). 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 tamoxifen and compound **9** (Fig. 7), suggesting that the en-

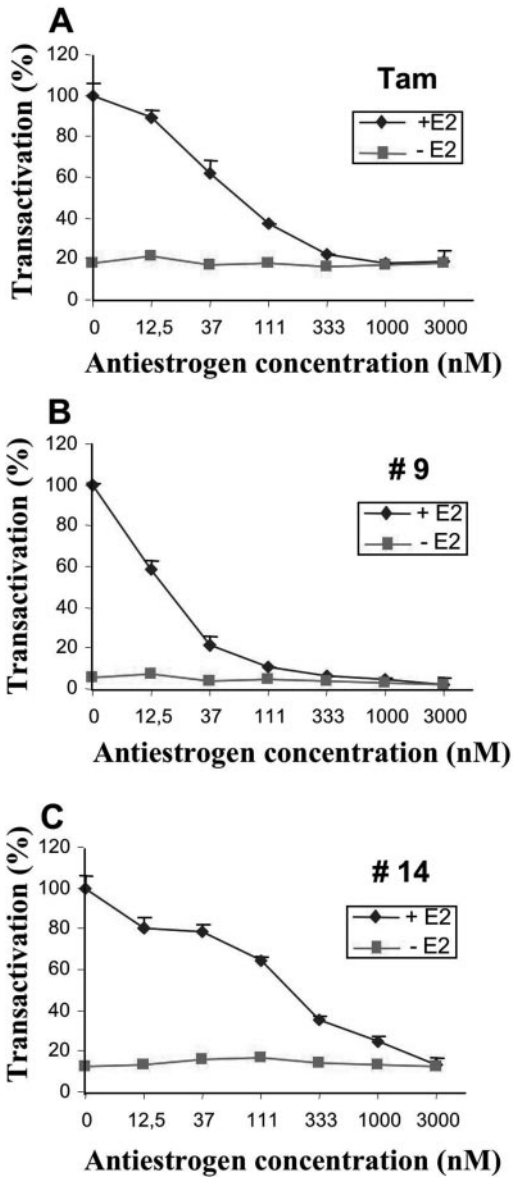

Fig. 4. Antagonist activity of tamoxifen derivatives on hERα-dependent transcription in HeLa cells. A to C, an expression vector for hERα (pSG5-HEG0) was transiently cotransfected into HeLa cells along with the reporter vector ERE3-TATA-CAT/EBV and the internal control vector pCMV-βGal. CAT activity was measured in extracts from cells treated for 48 h with increasing concentrations of tamoxifen (A), derivative **9** (B), or derivative **14** (C) administered either alone or premixed with 0.03 nM estradiol.

hanced 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 Tamoxifen Derivatives. Because abolition of the charge at position 351 has been shown to suppress the agonist activity of tamoxifen on estrogen target gene transcription in MDA-MB-231 cells (MacGregor Schafer et al., 2000; Liu et al., 2001, 2002), we examined the effect of mutations D351A and D351V on receptor activity in the presence of tamoxifen or of its derivatives in transfected HepG2 cells. Loss of activity was observed with these mutants in the absence but not in the presence of hormone (Fig. 5), consistent with our previous report in HeLa cells (Anghel et al., 2000). The lack of basal activity of these mutants is probably due to a role of the charge of Asp351 in maintaining an active conformation in the absence of hormone. Potential stabilizing interactions include hydrogen bonds involving Asp351 and the peptide backbone at the N terminus of H12 in its agonist conformation. Mutations D351A or D351V also abolished transcriptional activity in the presence of tamoxifen and all tamoxifen derivatives with agonist activity (Fig. 5). Thus the presence of a negative charge at position 351 seems important for the partial agonist activity of all tamoxifen derivatives in HepG2 cells.

Mutation D351E and D351Y Restore Receptor Activity in the Presence of Compound 9 and Raloxifene but Only Minimally Affect Activity with Tamoxifen. 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 raloxifene in MDA-MB-231 cells (MacGregor Schafer et al., 2000; Liu et al., 2001, 2002). The D351E mutant was also stimulated by raloxifene in our HepG2 assay (Fig. 5). Furthermore, transactivation levels in the presence of compound **9** were also increased by this mutation, reaching levels observed with tamoxifen (Fig. 5). Note that derivative **9** and raloxifene can compete out 0.5 nM estradiol under the same experimental conditions with the wild-type (Fig. 6B) and the mutant receptor (data not shown), indicating full occupancy of the wild-type and mutant receptors at maximal concentrations. It is remarkable that mutant D351E had similar levels

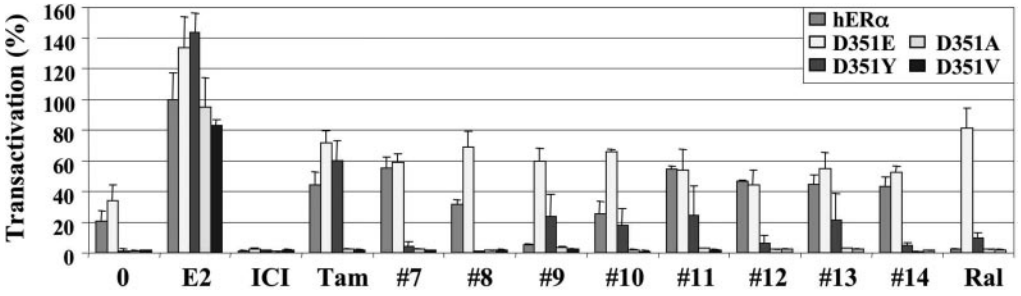

Fig. 5. Mutation D351E increases the agonist activity of tamoxifen derivative **9** and of raloxifene to levels observed with tamoxifen in HepG2 cells. Expression vectors for wild-type hERα (pSG5-HEG0) or Asp351 mutants (pSG5-HEG0-D351E, Y, A, or V) were transiently cotransfected into HepG2 cells along with the reporter vector pERE3-TATA-Luc and the internal control vector pCMV-βGal. Luciferase activity was measured in extracts from cells treated for 48 h with vehicle, 25 nM estradiol, 10 nM ICI 182,780, 100 nM raloxifene, and 1000 nM tamoxifen or its derivatives. This experiment was performed two times with similar results. Error bars indicate the S.D. between triplicate samples.

of transcriptional activity in the presence of saturating concentrations of all tamoxifen derivatives tested in HepG2 cells (Fig. 5) However, this mutation did not lead to major changes in the activity of the receptor in the presence of tamoxifen or OHT in HepG2 and HeLa cells (Fig. 5; data not shown). These results are compatible with the conclusion 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 raloxifene or compound **9**, but not of OHT or tamoxifen, 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, this mutation was reported to increase markedly the agonist activity of raloxifene 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 (Liu et al., 2001, 2002). In HepG2 cells, we observed only a minimal increase in the receptor activity in the presence of raloxifene (Fig. 5). A slightly more pronounced increase in the transcriptional activity was observed with compound **9**. Overall, the levels of receptor activity observed with mutant D351Y at micromolar concentrations of the tamoxifen 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 tamoxifen (Fig. 5). The variable degree of activity observed with this mutant in the presence of the different tamoxifen 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 tamoxifen derivatives on ERα-dependent transcription in HepG2 cells. Furthermore, a precise relative positioning of the negative charge of Asp351 and of the positive charge of the tertiary amine seems required for maximal suppression of receptor activity observed with compound **9** and raloxifene, whereas the activity in the presence of tamoxifen seems 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 tamoxifen has been associated with its capacity to transactivate through the AF1 (Berry et al., 1990; Tzukerman et al., 1994). We have investigated the implication of this activation function in mediating the agonist activity of SERMs with Asp351 mutants. We transiently transfected HepG2 cells with the full-length receptor, with Asp351 mutants (D351A, -E, or -Y), or with derivatives thereof truncated in the AB region containing AF1 (ΔAB). Deletion of the AB region eliminates AF1 activity and inactivates the wild-type receptor on minimal reporter vectors (data not shown). Transcriptional activity can be rescued by cotransfection of the core domain of coactivator TIF2 (TIF2.1), a member of the p160 family of coactivators (Voegel et al., 1998), in the presence of estradiol and in the absence of ligand, but not in the presence of OHT (Fig. 8). Deletion of the AF1 function abrogated activity of the D351E and D351Y mutants in the presence of raloxifene as well as in the presence of OHT (Fig. 8). These results indicate a common mechanism of activation for raloxifene with Asp351 mutants and for tamoxifen with the wild-type 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 raloxifene 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 wild-type or D351E

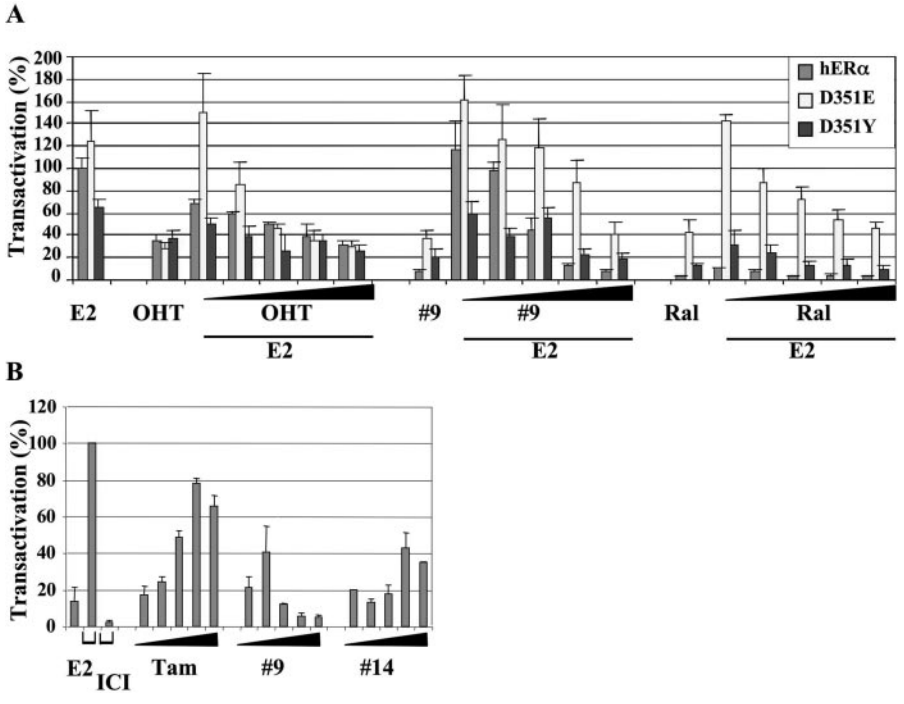

Fig. 6. Agonist activity of tamoxifen, derivative **9**, and derivative **14** on ERα-mediated transcription in HepG2 cells. A, competition assays performed as in Fig. 3 but using HepG2 cells transiently cotransfected with expression vectors for wild-type hERα (pSG5-HEG0) or Asp351 mutants (D351E or D351Y), reporter vector pERE3-TATA-Luc, and the internal control vector pCMV-βGal. Increasing concentrations of tamoxifen, raloxifene, or compound **9** were added either alone (1000 nM) or premixed at different concentrations (0.1, 1, 10, 100, and 1000 nM) with 0.5 nM estradiol before addition to cells in culture. Error bars indicate the S.D. between two different experiments, each performed in duplicate. B, an expression vector for hERα (pSG5-HEG0) was transiently cotransfected into HepG2 cells along with the reporter vector pERE3-TATA-Luc and the internal control vector pCMV-βGal. Luciferase activity was measured in extracts from cells treated for 48 h with vehicle, 25 nM estradiol, 100 nM ICI 182,780, or increasing concentrations of tamoxifen, derivative **9** or derivative **14** (0.1, 1, 10, 100, or 1000 nM, respectively). Error bars indicate the S.D. between triplicate samples. This experiment was performed three times with similar results.

mutant receptor with the VP16 transcriptional activation function generated a constitutively active receptor, which was also fully active in the presence of OHT, compound **9** or raloxifene, but not in the presence of ICI 182,780 (Fig. 9). These results indicate that an exogenous activation domain can restore transcriptional activity in the presence of SERMs with low degree of agonist activity (raloxifene and compound **9**). The presence of the VP16 activating region also derepressed transcriptional activity of the D351A mutant in the presence of OHT, and to a lesser extent, raloxifene and compound **9**. Together, these results suggest that transcriptional repression by SERMs interacting optimally with Asp351 or by removal of the negative charge at position 351 occurs through a mechanism specific to activation function AF1.

Discussion

Antiestrogenicity requires high-affinity binding to estrogen receptors and low intrinsic estrogenic activity. Early evidence indicated that antiestrogenicity of SERMs is dependent on the presence of the side chain and on the position of the tertiary amine in this side chain (Lednicer et al., 1967; Jordan and Gosden, 1982). Interaction between the tertiary amine in the side chain of SERMs and amino acid Asp351 of hERα was subsequently demonstrated by crystallographic analysis of the structures of estrogen receptor LBD complexes to OHT or raloxifene (Brzozowski et al., 1997; Shiau et al., 1998). Characterization of estrogen receptor Asp351 mutants indicated an important role of this amino acid in the balance between the antagonist/agonist activity of raloxifene, mutations D351Y and D351E increasing the partial agonist activity of raloxifene (Levenson and Jordan, 1998; MacGregor Schafer et al., 2000; Liu et al., 2002). Our previous observation that a pyrrole derivative of tamoxifen, which contains a nonbasic tertiary amine, did not antagonize estrogen activity in a uterotrophic assay (Robertson et al., 1982), was compatible with a role of the tertiary amine in the antagonist properties of tamoxifen derivatives. However, our previous study of Asp351 mutations in HeLa cells did not reveal an increase in OHT agonist activity when mutations D351E and D351Y were introduced at position 351. To reconcile these apparently conflicting observations, we characterized the affinity and antagonist properties of tamoxifen derivatives in various estrogen-responsive cellular model systems.

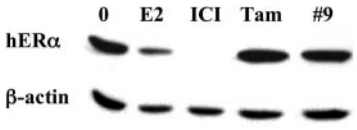

Fig. 7. Compound **9** does not induce degradation of hERα. An expression vector for wild-type ERα was electroporated in HeLa cells (pSG5-HEG0) and hormonal treatment (16 h, 25 nM estradiol, or 100 nM antiestrogens) was performed 24 h after electroporation. ERα and β-actin protein levels were assessed by Western blot analysis using antibody B10.

Our results indicate that the tertiary amine contributes to the affinity of receptor/antiestrogen interaction in a minor way compared with the 4-hydroxyl group (~50-fold difference in affinity between tamoxifen and OHT in our assays). The affinity of the pyrrole derivative was only 4-fold lower than that of tamoxifen, a smaller reduction compared with our previous observations in a rat uterine cytosolic assays (Robertson et al., 1982). However, its interaction with ERα mutants in which Asp351 was replaced by small hydrophobic amino acids was markedly increased (~20-fold), suggesting that Asp351 restricts stabilizing interactions between the aromatic side chain of compound **14** and amino acids of the ligand binding domain.

When tested in cultured human cells, the pyrrole derivative antagonized estrogenic action and did not display increased agonist activity compared with tamoxifen in three different assays: 1) estradiol-dependent proliferation of breast MCF7 cells, 2) estradiol-induced alkaline phosphatase activity in uterine Ishikawa cells, and 3) estradiol transactivation of an ERE3-TATA-CAT reporter plasmid in transiently transfected HeLa cells. Furthermore, the pyrrole derivative repressed recruitment of LXXLL motifs by ERα to the same extent as tamoxifen in a bioluminescence resonance energy transfer experiment (data not shown). Therefore, it seems that neither the charge of the tertiary amine nor its capacity to engage in hydrogen bonds is by itself crucial to the levels of antagonist activity obtained with tamoxifen in these cell models. Weaker affinity for rat estrogen receptors compared with human receptors and/or metabolism of this compound in vivo may explain the lack of antagonist activity observed previously in uterotrophic assays, especially in view of the fact that this compound did not display marked agonist activity even at the highest dose. It remains possible that differences in the pharmacokinetic properties of this compound in the two experimental systems contribute to the different outcome of competition assays.

Our study with tamoxifen derivatives, which indicates that modulation of the basicity of the tertiary amine does not

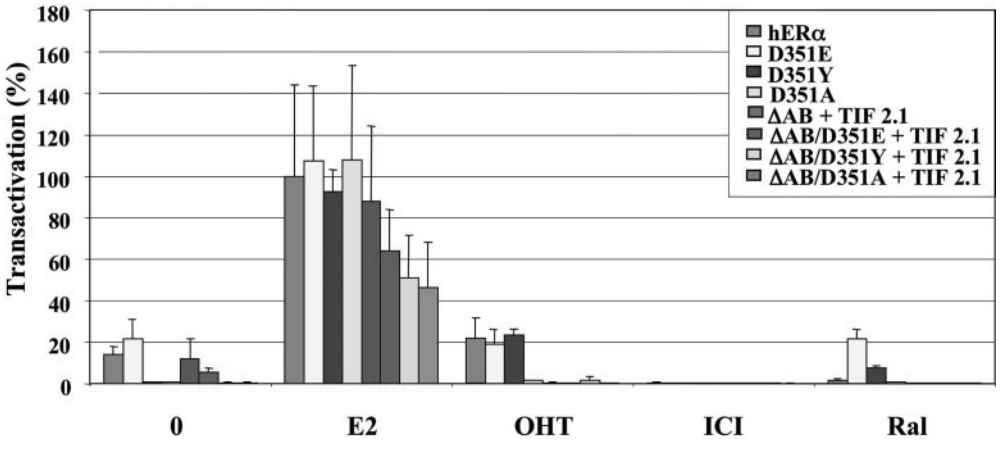

Fig. 8. AF-1 activity is required for the agonist activity of SERMs. Transient transfection analysis in HepG2 cells of the transcriptional activity of the wild-type ERα, the D351E, -Y, and -A mutants, or derivatives thereof deleted of the AB region in the absence or presence of overexpressed TIF2.1 was performed as in Fig. 5 except that the expression vector for TIF2.1 (4 μg/10-cm plate) was cotransfected with other expression vectors. Relative luciferase activity is shown.

increase the agonist activity of tamoxifen in several cell systems, contrasts with results obtained with raloxifene derivatives. Conversion of the piperidine group of raloxifene into a cyclohexane generated a derivative with estrogenic activity in stably transfected MDA-MB-231 cells (Liu et al., 2002). In addition, mutations D351E, and to a lesser extent D351Y, increased the agonist activity of raloxifene on transforming growth factor-α expression in stably transfected MDA-MB-231 cells. Together, these results indicated an important role of interaction between the tertiary amine and Asp351 in repressing activity in the presence of raloxifene. The fact that mutation D351Y was initially isolated from an MCF7 xenograft tumor grown in athymic mice in the presence of tamoxifen (Wolf and Jordan, 1994) suggested that Asp351 may play a similar role with tamoxifen. However, our results with tamoxifen derivatives indicate that the antagonist activity of tamoxifen is much less sensitive than that of raloxifene to disruption of the interaction between Asp351 and the tertiary amine in the antiestrogen side chain. Nevertheless, we note that the transcriptional profiles of estrogen receptor mutants in the presence of raloxifene or tamoxifen derivatives in HepG2 cells reported here are compatible with observations in the MDA-MB-231 system (Liu et al., 2002). In both systems, a higher transcriptional activity was observed in the presence of tamoxifen versus raloxifene, with little further enhancement by mutations D351E or D351Y but a repressive effect of mutation D351A, indicating a role of amino acid Asp351 in transcriptional activity in the presence of tamoxifen. The relatively small effect of mutation D351Y in increasing the agonist activity of raloxifene in HepG2 cells compared with the effects of this mutation on transforming growth factor-α expression in MDA-MB-231 cells 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 site sequences on coactivator recruitment profiles have been described previously (Loven et al., 2001; Hall et al., 2002).

Characterization of raloxifene derivatives has implicated the hinge region between the side chain and the steroid-like skeleton as a main determinant for the differential uterotrophic activity of tamoxifen and raloxifene, but in addition conversion of the piperidine ring of raloxifene into a dimethylamine increased agonist activity (Grese et al., 1997). Our results indicate that, conversely, the agonist activity of tamoxifen can be lowered by modification of the structure of its side chain. Compared with tamoxifen, the pyrrolidine derivative of tamoxifen had reduced agonist activity in the HepG2 system and was a slightly more potent compound in all assays. Contrary to what is observed with full antiestrogens such as ICI 182,780 (Dauvois et al., 1992; Wijayaratne et al., 1999), 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. This decreased level of agonist activity was highly dependent on the precise position of the cyclic tertiary amine relative to the charge at position Asp351. Indeed, either addition of a carbon in the side chain or in the ring, or replacement of Asp351 by glutamic acid, or, to a lesser extent, by tyrosine, led to increased agonist activity. This suggests that the tertiary amine in the side chain of derivative **9** is more optimally positioned than that of tamoxifen or of other tamoxifen derivatives for antagonist activity. The similar levels of activity of all tamoxifen derivatives with mutant D351E further suggest that their variable degree of agonist activity with the wild-type receptor result from their different capacity to mask the charge of Asp351. Thus, our results indicate that interaction of tamoxifen with Asp351 is not optimal but can be improved by modifying tertiary amine substituents.

The observation that tamoxifen and its derivatives are antagonists even in the absence of interaction with Asp351 is probably explained by the steric clash generated by the antiestrogen side chain with helix 12 of the ERα LBD in the agonist position, preventing recruitment of coactivators through the AF2 function. Indeed, deletion of the AF1 region completely suppresses the partial agonist activity of tamoxifen derivatives, whereas addition of an exogenous activation domain relieves transcriptional repression by raloxifene and compound **9** and allows activity in the absence of a negative charge at position 351 in the presence of OHT, or to a lesser extent, raloxifene or compound **9**. Although we cannot exclude the possibility that addition of the VP16 domain may prevent interaction between Asp351 and the side chains of raloxifene or compound **9**, these results suggest that the repressive effect of charge neutralization at position 351 is specific to the AF1 transcriptional activation function of ERα. Further understanding of the mechanisms of partial agonist activity of antiestrogens will thus necessitate the characterization of the protein surface(s) engaging in interaction with the ligand binding domain of ERα in the presence of SERMs in an Asp351-modulated manner.

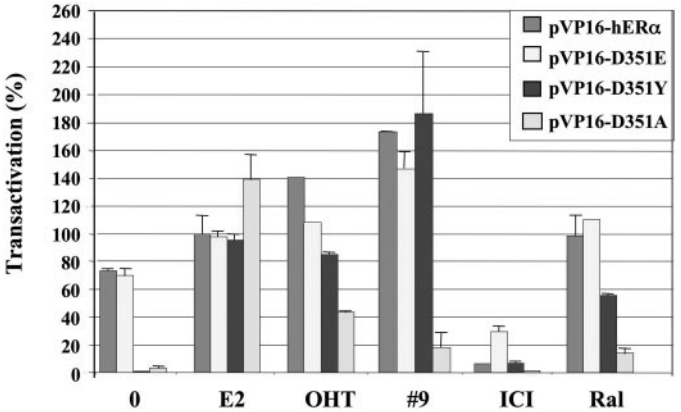

Fig. 9. Fusion with the VP16 transcriptional activation function releases transcriptional activity in the presence of raloxifene and compound **9**. Transient transfection analysis in HepG2 cells of the transcriptional activity of the wild-type ERα, the D351E, -Y, and -A mutants, or derivatives thereof fused to the VP16 activation domain was performed as in Fig. 5. Relative luciferase activity is shown.

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