Cysteine 530 of the Human Estrogen Receptor α Is the Main Covalent Attachment Site of 11*β*-(Aziridinylalkoxyphenyl)estradiols[†]

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ABSTRACT: The efficiency of 11*β*-[*p*(aziridinylethoxy)phenyl]estradiol **1** and 11*β*-[*p*(aziridinylpentoxy)-phenyl]estradiol **2** affinity labeling of the estrogen receptor α (ERα) was evaluated on the basis of their capacity to inhibit [^{3}H]estradiol binding to lamb and human ERαs. Relative to RU 39 411 (11*β*-[*p*(dimethylaminoethoxy)phenyl]estradiol), the most closely related and chemically inert analogue of **1**, the two electrophiles irreversibly inhibited [^{3}H]estradiol binding to the lamb ERα. The fact that the compound effects were prevented (i) when the ERα hormone-binding site was occupied by estradiol and (ii) when the ERα-containing extracts were pretreated with methyl methanethiosulfonate (an SH-specific reagent) suggested that the compounds specifically alkylated ERα at cysteine residues. Wild-type human ERα was alkylated as efficiently as lamb ER, whereas the quadruple cysteine → alanine mutant, in which all cysteines of the hormone-binding domain (residues 381, 417, 447, and 530) were changed to alanines, showed no significant electrophile labeling. The single C530A mutant was much less sensitive to the action of the electrophiles than the three other single mutants (C381A, C417A, and C447A). Moreover, analysis of the three double mutants (C381A/C530A, C417A/C530A, and C447A/C530A) showed that only the C381A/C530A mutant was less susceptible to electrophile labeling than the single C530A mutant. We concluded that in the hormone-binding pocket C530 was the main covalent attachment site of aziridines **1** and **2**, whereas C381 could be a secondary site. These results agreed with the crystal structure of the hormone-binding domain of the human ERα bound to estrogen or antiestrogen, since C381 and C530 appeared to be (i) located in structural elements involved in delineating the hormone-binding pocket and (ii) in spatial proximity to each other, which was closer in the crystal structure of the ER:antiestrogen complex than in that of the ER:estrogen complex. Since C530 and C381 were also the main and secondary covalent attachment sites of tamoxifen aziridine (a nonsteroidal affinity-labeling agent), we propose a selective mode of superimposition of tamoxifen-class antiestrogens with RU 39 411-class antiestrogens, which could account for the relative positioning of the two types of ligands in the ERα hormone-binding pocket.

Two estrogen receptor subtypes (ERs)[1] belonging to the nuclear receptor superfamily (*1*) are presently known. One ER subtype, now termed ERα, was cloned in 1985 (*2*, *3*); another subtype, termed ER*β*, was recently characterized from various mammal tissues (*4*, *5*). In the uterus, (i) the relative abundance of ERα and ER*β* mRNAs (*6*), and (ii) the consequences of the ERα gene disruption (*7*) indicated that in this organ ERα was much more abundant than ER*β*.

The crystal structures of the hormone-binding domain of the human ERα bound to estrogen or antiestrogen were recently established. Tanenbaum et al. (*8*) determined the structure of covalently dimerized domain bound to estradiol, whereas structures of the S-carboxymethylated domain bound (i) to estradiol or raloxifene (a benzothiophene antiestrogen), (ii) to diethylstilbestrol (a diphenylethylene estrogen), or to 4-hydroxytamoxifen (a triphenylethylene antiestrogen) were determined by Brzozowski et al. (*9*) and Shiau et al. (*10*), respectively. The overall architecture of the ERα ligand-binding domain was similar to those highlighted in the crystal structure of various nuclear receptor hormone-binding domains (*11*−*15*). The ERα hormone-binding domain, which includes 12 α-helices and 2 *β*-strands, is folded into a three-layered antiparallel α-helical sandwich. According to the authors (*8*−*10*), the ligand-binding pocket is predominantly formed by hydrophobic residues located in six segments of the secondary structure (parts of five α-helices and of the *β*-sheet), whereas polar amino acids E353 and R394 are directly involved in hydrogen bonds with the phenolic hydroxyl of estradiol (or above-mentioned nonsteroidal estrogens and antiestrogens), and H524 is involved in a single hydrogen bond with the 17*β*-hydroxyl of the estradiol D-ring (or the second phenolic hydroxyl of diethylstilbestrol or raloxifene). Agonists and antagonists bind at the same site within the core of the ligand-binding domain but demonstrate different binding modes. Moreover, each of the four above-mentioned estrogens or antiestrogens showed specific interactions with different amino acid residues in the hormone-binding pocket (*9*, *10*).

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[1] Abbreviations: DME medium, Dulbecco's modified Eagle's tissue culture medium; ER, estrogen receptor; MMTS, methyl methanethiosulfonate; PBS, phosphate-buffered saline; RAC, relative affinity constant; T_{20}, 20 mM Tris-HCl buffer.

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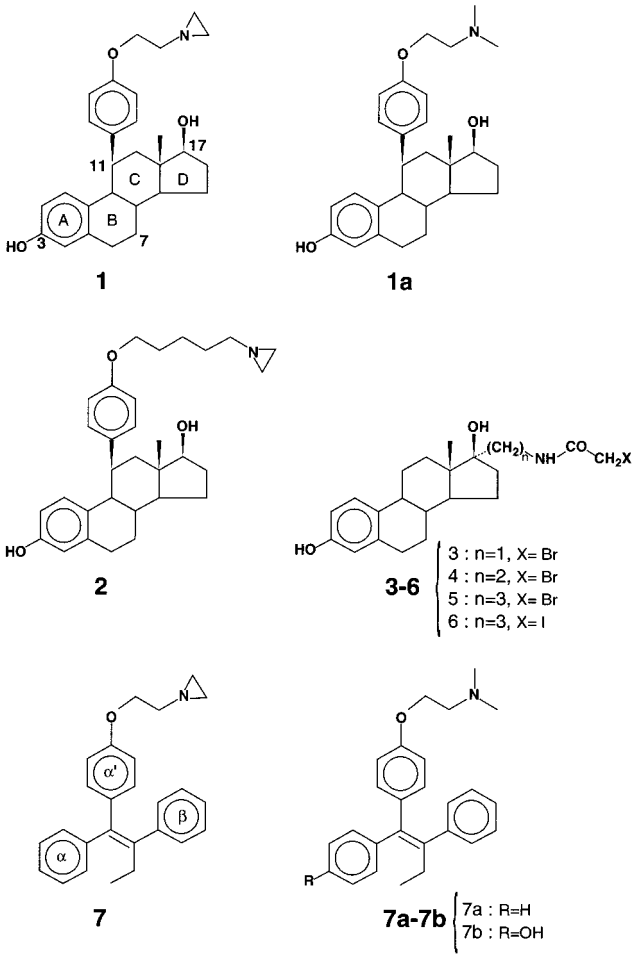

FIGURE 1: Structures of electrophilic ligands of estrogen receptors and related chemically inert antiestrogens. The two electrophilic ligands of ERs evaluated in the study are 11β-[*p*(aziridinylethoxy)-phenyl]estradiol **1** and 11β-[*p*(aziridinylpentoxy)phenyl]estradiol **2**. RU 39 411 (compound **1a**), the most closely related and chemically inert analogue of **1**, was used as a blank in ER affinity-labeling experiments. Other electrophilic molecules which proved to be efficient ER affinity-labeling agents in previous studies (*18*, *20*) and which are described in the paper are 17α-(haloacetamidoalkyl)-estradiols **3**−**6** and tamoxifen aziridine **7**, an electrophile closely related to triphenylethylene antiestrogens, tamoxifen **7a** and 4-hydroxytamoxifen **7b**.

To understand the agonistic and antagonist properties of ER ligands and further to design more selective ER modulators (*16*), it is important to specify the different types of interactions of steroidal and nonsteroidal estrogen agonists or antagonists with ERα and ERβ at the molecular level. Nucleophilic amino acid residues of a receptor hormone-binding pocket in close proximity to electrophilic ligands could be identified by affinity labeling of the receptor. This was carried out, using both steroidal (*17*−*19*) and nonsteroidal (*20*−*24*) reactive ligands (Figure 1, compounds **3**−**7**), to study the ERα hormone-binding pocket. The use of methyl methanethiosulfonate revealed that, for most of these reactive ligands, cysteine residues of the hormone-binding domain were the major if not the exclusive covalent attachment sites of the compounds (*17*, *18*, *23*). This domain includes only four cysteine residues (C381, C417, C447, and C530 for the human ERα hormone-binding domain), which are perfectly conserved from birds to mammals (*25*, *26*). In some instances, the cysteine residues involved in the affinity-labeling processes were identified through Edman degradation of alkylated ER (*22*) or the concomitant use of the wild-type and single, double, and the quadruple cysteine → alanine ERα mutants (*19*, *23*, *24*). Three out of the four cysteine residues were found to be covalent attachment sites of different electrophilic ligands. C530 was the covalent attachment site of an aziridine derivative of tamoxifen (*22*); C381 was involved in the alkylation of the C530A ER mutant by the compound (*24*), whereas C417 and C530 were the covalent attachment sites of 17α-alkyl derivatives of estradiol bearing a terminal halomethylketo group (*19*). Interestingly, all of these three cysteines (and not C447) appeared to be accessible to small molecules since they reacted with the thiol reagent, iodoacetic acid (*27*). These results agreed with the crystal structure of the ERα ligand-binding domain, since the three cysteines are located at the extreme border or in structural elements involved in delineating the hormone-binding pocket (*9*).

In two recent studies, we described ERα alkylation by electrophilic estradiol 11β-derivatives, in which a terminal electrophilic functionality was linked to carbon 11 of the steroid by an aliphatic (*28*) or an aromatic arm (*29*). We found that a "7-bond" distance between the electrophilic center and carbon 11 did not allow the electrophilic carbon to react with a nucleophilic residue in the hormone-binding pocket (*28*), whereas compounds with ≥10 bonds between carbon 11 and the electrophilic carbon were efficient affinity labels (*28*, *29*). Moreover, discrete changes (i) in the structure of haloacetamido compounds (e.g., substitution of Cl by Br or NH by NCH_3) or (ii) in the ERα structure (e.g., SH → S−SCH_3) profoundly affected the ability of the compounds to specifically alkylate ERα (*29*). We concluded that ERα cysteine residues located on the β side of the bound steroid, but relatively remote from carbon 11, were covalent attachment sites for most of these affinity labels.

In the present study, we first use lamb ERα to demonstrate affinity labeling of ER by 11β-[*p*(aziridinylethoxy)phenyl]-estradiol and 11β-[*p*(aziridinylpentoxy)phenyl]estradiol (Figure 1, compounds **1** and **2**), two electrophilic compounds closely related to the 11β-aryl estradiol derivatives that we recently evaluated as ERα affinity labels (*29*), and whose distance between the electrophilic center and the steroid nucleus included 9 and 12 bonds, respectively. We then use expression vectors coding for the wild-type and single, double, and the quadruple cysteine → alanine mutants of the human ERα to identify potential ERα cysteines which would be alkylated by these two electrophiles. Finally, taking into account the target cysteines, we examine the potential relative positioning of 17α- and 11β-substituted steroidal compounds and of triphenylethylene derivatives in the ERα hormone-binding pocket.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. The electrophilic ligands of ER used in this study, i.e., 11β-[*p*(aziridinylethoxy)phenyl] estradiol **1** and 11β-[*p*(aziridinylpentoxy)phenyl]estradiol **2**, were synthesized from 11β-[*p*(iodoethoxy)phenyl]estradiol and 11β-[*p*(iodopentoxy)phenyl]estradiol, respectively (*29*). The synthesis and physicochemical characteristics of the compounds will be described elsewhere. RU 39 411 was from Hoechst Marion Roussel (Romainville, France) and 4-hydroxytamoxifen was obtained from Zeneca (Maccles-

field, England). [6,7-^{3}H]Estradiol (specific activity 1.89 PBq/mol, radiochemical purity of 98%) was purchased from Amersham International (Amersham, England). Estradiol, 4-hydroxytamoxifen, and estradiol 11β-derivatives used for the binding studies were solubilized in absolute ethanol. Solutions were stored at −20 °C in the dark. The purity of solubilized compounds was checked before use by thin-layer chromatography.

Estrogen Receptor Expression Vectors. The plasmids coding for the wild-type, the four single cysteine → alanine mutants (C381A, C417A, C447A, and C530A), the three double cysteine → alanine mutants (C381A/C530A, C417A/C530A, and C447A/C530A), and the quadruple cysteine → alanine mutant (C381A/C417A/C447A/C530A) of human ERα were given by B. S. Katzenellenbogen; preparations of these plasmids have been described previously (*23*, *24*).

Cell Culture Conditions and Tranfections. COS cells were passaged in phenol red-free Dulbecco's modified Eagle's tissue culture medium (DME medium) supplemented with 5% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). COS cells, transfected for use in binding affinity constant determination and in ER affinity-labeling studies, were plated in T150 tissue culture flasks in DME medium plus 10% fetal calf serum at a density of $\sim 10^6$ cells in a humidified 5% CO_2 atmosphere. The cells were transfected by the calcium phosphate coprecipitation method, with 30 μg of expression plasmid (pRER) coding for wild-type, or one of the various (single, double, or quadruple) cysteine → alanine mutants of human ERα. Fifteen hours later, the medium was changed, and the cells were then grown for 48 h in DME medium containing 5% charcoal/dextran-treated fetal calf serum, penicillin, and streptomycin.

Cytosolic Estrogen Receptors. (1) Lamb Receptor. Lamb uteri were homogenized in 5 vol of chilled 20 mM Tris-HCl buffer (T_{20}) pH 7 or 8.5 using an ultraturrax. The homogenate was centrifuged at $10^5 \times$ g for 30 min at 4 °C to yield the cytosol.

(2) Human Receptor. COS cells, transfected with ERα expression vectors, were harvested in phosphate-buffered saline (PBS) containing 1 mM EDTA by scraping with a rubber policeman. Cells, collected by centrifugation, were washed in ice-cold PBS and then resuspended in 0.25 mL/plate chilled T_{20} (pH 7 or 8.5) containing 1.5 mM EDTA. Cells were disrupted by sonication. The homogenate was then centrifuged at $10^5 \times$ g for 30 min at 4 °C to yield the cytosol.

Competitive Binding Assay. Apparent Relative Affinity Constants. Cytosols from lamb uteri (3 mg of protein/mL) and COS cells transfected with human ERα expression vectors (1−2 mg of protein/mL) prepared in T_{20} (either at pH 7 or 8.5) were incubated for 24 h at 20 °C with 10 nM [^{3}H]estradiol and increasing concentrations of nonradioactive estradiol, 4-hydroxytamoxifen, RU 39 411, or estradiol 11β-derivative **1** or **2**.

Binding of [^{3}H]estradiol in samples was determined by charcoal assay: 0.3 mL aliquots of samples were treated with an equal volume of charcoal suspension (1% charcoal and 0.1% dextran T70 in T_{20} at pH 7.4) for 30 min at 0 °C; charcoal was pelleted by 5-min centrifugation at $10^3 \times g$, and then radioactivity in supernatants was measured. Apparent relative affinity constants (RACs) of competitors, relative to that of estradiol, were calculated from the concentration of unlabeled estradiol (E) and competitor (C), which inhibited 50% of the specific binding of [^{3}H]estradiol, according to Korenman (*30*):

$$\mathrm{RAC} = \frac{\dfrac{E_t^* - E_b^*}{E^*_{sb}} \times \dfrac{E}{C}}{\dfrac{E_t^* - E_b^*}{E^*_{sb}} + 1 - \dfrac{E}{C}}$$

where E_t* is the total concentration of [^{3}H]estradiol, E_b* and E_{sb}* are the respective concentrations of bound and specifically bound [^{3}H]estradiol at 50% inhibition, E_t* − E_b* was assumed to be the unbound [^{3}H]estradiol concentration at 50% inhibition.

Standard Irreversible Binding Assay. Since the electrophiles used are nonradiolabeled ER ligands, alkylation of lamb and human ER hormone-binding sites by electrophiles was determined indirectly on the basis of the fact that the difference between the concentration of specific binding sites for [^{3}H]estradiol in control cytosol and electrophile-exposed cytosol reflects the extent of ER affinity labeling (*17*). Except where otherwise mentioned, ERs were labeled with electrophiles **1** and **2** by incubation of lamb uterine cytosol (6 mg of protein/mL; pH 8.5) or COS cytosol (2−6 mg of protein/mL; pH 8.5) with 50 nM compound for 2 h at 25 °C. As controls, other cytosol aliquots were incubated without steroid or with RU 39 411 (11β-[*p*(dimethylaminoethoxy)-phenyl]estradiol), the most closely related and chemically inert analogue of **1**. To remove unbound steroids, samples were treated with an equal volume of charcoal suspension (1% charcoal and 0.1% dextran T70 in T_{20} at pH 7.4) for 30 min at 0 °C and then centrifuged. The concentration of specific estradiol-binding sites in supernatants was determined by incubation of aliquots with 20 nM [^{3}H]estradiol, under exchange conditions (20 h at 20 °C), in the absence and presence of 5 μM radioinert estradiol, respectively. Total and nonspecific estradiol binding were measured by charcoal assay.

Protein Concentration. The protein concentration in lamb uterine and COS cytosols was determined according to the method of Bradford (*31*).

Molecular Mechanics Studies. Molecular structures were built and optimized using the Builder Module of Insight II software package (MSI) from Biosym Technologies. Graphical visualization and molecular superimposition were monitored using the Insight software package.

RESULTS

Relative Affinity Constants for Estrogen Receptors α. Affinity labeling of ERs by a nonradioactive electrophilic ligand is usually shown by a decrease in the concentration of specific binding sites for [^{3}H]estradiol in estrogen-target cell extracts initially exposed to the electrophile. This method was efficient for evaluating the affinity-labeling ability of electrophiles displaying low or moderate affinity for ERs (*17*−*19*, *21*). With high-affinity electrophiles, problems could arise as a result of incomplete [^{3}H]estradiol labeling of ER noncovalently occupied by the electrophile (incomplete [^{3}H]-estradiol labeling of ER at the exchange step due to competition between [^{3}H]estradiol and reversibly bound and

Table 1: Apparent Relative Affinity Constants for Lamb and Human Estrogen Receptors α[a]

	lamb ER		human ER	
	pH 7	pH 8.5	pH 7	pH 8.5
estradiol	1	1	1	1
aziridine **1**	0.22 ± 0.06 (3)	0.52 ± 0.05 (3)	ND	ND
aziridine **2**	0.26 ± 0.03 (3)	0.46 ± 0.08 (3)	ND	ND
RU 39 411 **1a**	0.28 ± 0.04 (3)	2.91 ± 1.09 (5)	15.2 ± 2.9 (2)	12.3 ± 2.8 (4)
4-hydroxytamoxifen **7b**	0.39 ± 0.04 (3)	0.29 ± 0.05 (5)	8.86 ± 1.71 (2)	3.77 ± 1.29 (4)
tamoxifen **7a**	0.0015[b]		0.0015[c]	

[a] Apparent binding affinity constants of aziridines **1** and **2**, RU 39 411 and 4-hydroxytamoxifen for lamb and human ERαs, were determined by competitive binding radiometric assay using [^{3}H]estradiol as tracer as described in the Experimental Procedures. Data given are means ± SD with the number of independent determinations given in parentheses. [b] Value determined from a 20 h competition at 20 °C [Borgna, J. L., and Scali, J. (1988) *J. Steroid Biochem. 31*, 427−436]. [c] Value determined from a 24 h competition at 0 °C, pH 7.4 [Coezy, E., et al. (1982) *Cancer Res. 42*, 317−323].

unbound electrophile); this would result in false positive affinity-labeling assessment. When studying affinity labeling of ERs by electrophilic ligands, it would therefore be necessary to assess the classical (reversible) binding affinity of these electrophiles. In competitive binding experiments using [^{3}H]estradiol as tracer and the electrophile as competitor, the potential irreversible binding of the latter to both ER and other cell extract components (which would tend to overestimate and underestimate, respectively, the classical binding affinity of the compound) could markedly alter the compound affinity value. Especially in the case of a high-affinity electrophile, it is then particularly important to use a chemically inert compound that is a very close analogue of the electrophile. This analogue could be used to obtain a reliable estimate of (i) the classical binding affinity constant of the electrophile for ER and (ii) the level of [^{3}H]estradiol binding inhibition due to classical binding of the electrophile to ER in affinity-labeling experiments.

Numerous 11β-derivatives of estradiol were synthesized. The structure/affinity relationships indicate that large substituents at the 11β-position are tolerated for binding ERα, provided that polar functions (if present) are remote from the ligand core (*32*). In fact, for various estradiol 11β-derivatives, the binding affinity for ERα is similar and in some cases much higher than that of estradiol. This is the case for RU 39 411 (Figure 1, compound **1a**) (*33*) which can be considered as the steroidal homologue of 4-hydroxytamoxifen (compound **7b**), a high affinity triphenylethylene antiestrogen (*34*). Since the aziridine **1** evaluated in this study is very closely related to RU 39 411, the reversible binding affinity of aziridine **1** to ERα should be very similar to the binding affinity of RU 39 411. Moreover, the latter could constitute an appropriate control in the ERα affinity-labeling experiments performed with aziridines **1** and **2**.

The apparent relative affinity constants of compounds for binding lamb and human ERαs were determined on the basis of competitive binding experiments between [^{3}H]estradiol and unlabeled estradiol, the two aziridines and RU 39 411, using lamb uterine cytosol and cytosol from COS cells transfected with a human ERα expression vector, as sources of ERαs. Since the reactivity of nucleophilic amino acid residues with electrophiles normally increases with pH, a first series of experiments was carried out at pH 7 to minimize alkylation processes. To determine the binding affinity constants under covalent labeling trial conditions, another series of competitive binding experiments was performed at pH 8.5. Apparent binding affinity constants of the two aziridines and of RU 39 411 for lamb ER at pH 7 were roughly similar (Table 1), i.e., they accounted for 22, 26, and 28%, respectively, of the estradiol binding affinity constant. At pH 8.5, the apparent affinity of RU 39 411 (291% that of estradiol) was ∼6-fold higher than values obtained for the aziridines (52 and 46% that of estradiol, respectively). The marked increase in the apparent affinity constant of RU 39 411 for lamb ER observed when the pH was raised from 7 to 8.5 was not a general phenomenon since the affinity of 4-hydroxytamoxifen slightly decreased concomitantly. The relative affinity constants of RU 39 411 and 4-hydroxytamoxifen for the human ERα at pH 7 were ∼15- and ∼9-fold higher than that of estradiol, respectively. In sharp contrast with the results obtained with lamb ER, the 7−8.5 rise in pH did not increase the apparent relative affinity of RU 39 411 for human ERα. In fact, upon the pH transition, the relative affinities of RU 39 411 and 4-hydroxytamoxifen for human ERα decreased slightly but were still much higher than that of estradiol. The striking increase in the relative affinity of RU 39 411 and of 4-hydroxytamoxifen observed when human ERα instead lamb ER was used for compound affinity determination, especially at pH 7, was unexpected since in their hormone-binding domains the two ERs show an homology >95% (*25*) with only one nonconservative change (R503 in lamb ERα is changed to Q502 in human ERα).

Covalent Labeling of Lamb Estrogen Receptor α by 11β-[(Aziridinylalkoxy)phenyl]estradiols. The potential reversible high-affinity binding of the two aziridines to ERαs fully justified the use of RU 39 411 as a control in experiments to determine the potential of the compounds to specifically alkylate ERαs. In fact, exposure of ERα-containing lamb uterine extracts to RU 39 411 decreased their capacity to bind [^{3}H]estradiol. This decrease, which ranged from ∼20 to ∼60% according to experiments, was observed at an RU 39 411 concentration as low as 10 nM and did not markedly increase until 100 nM RU 39 411. The decrease was less pronounced at pH 7.5 than at pH 8.5 (not shown) and in methyl methanethiosulfonate-treated extracts than in untreated extracts (cf. next paragraph). The influence of time, temperature, pH, and concentration on the ability of aziridines **1** and **2** to decrease the [^{3}H]estradiol-binding capacity in lamb uterine extracts was then determined. According to the incubation conditions we used, the effects of aziridinyl derivatives markedly varied relative to the level of [^{3}H]-estradiol binding measured in cytosol exposed to an equivalent RU 39 411 concentration. Aziridines **1** and **2** were at

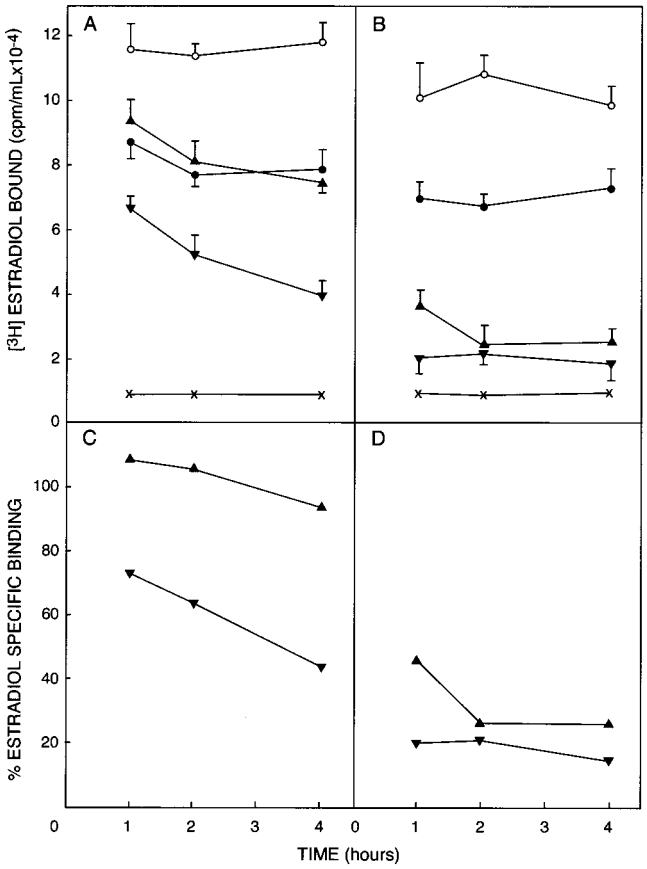

FIGURE 2: Time course of inactivation of specific estradiol-binding sites in lamb uterine cytosol after exposure to 11β-[(aziridinylalkoxy)phenyl]estradiols. Uterine cytosol (6 mg of protein/mL, pH 8.5) was incubated at 0 or 25 °C without steroid, with 50 nM RU 39 411, aziridine **1,** or aziridine **2**. After various periods of time, aliquots were removed and treated for 30 min with an equal volume of charcoal suspension. Charcoal was pelleted by centrifugation, and then the total and nonspecific binding of [^{3}H]estradiol occurring in the supernatants under exchange conditions were determined by the standard irreversible binding assay, as described in Experimental Procedures. (A, B) The total binding of [^{3}H]estradiol in supernatants corresponding to cytosol incubated at 0 °C (A) or 25 °C (B) without steroid (○), with RU 39 411 (●), aziridine **1** (▲), or aziridine **2** (▼), and the nonspecific binding of [^{3}H]estradiol (×), which did not significantly vary according to the compound incubated with cytosol, are represented as functions of the incubation time. Values are means of duplicate determinations; errors bars indicate standard deviations. (C, D) The specific [^{3}H]estradiol binding in cytosol exposed to aziridine **1** (▲) or aziridine **2** (▼) is expressed as a percentage of specific binding measured in cytosol exposed to RU 39 411; (C) 0 °C-incubation; (D) 25 °C-incubation.

least as efficient as RU 39 411 for decreasing [^{3}H]estradiol-binding capacity at 0 °C, pH 7.5 (not shown) or pH 8.5 (Figure 2A). At 25 °C, pH 7.5 (not shown) or pH 8.5 (Figure 2B), the two compounds were much more efficient than RU 39 411, with aziridine **2** being more potent than aziridine **1**, especially at pH 7.5. The effect of compounds, which did not significantly increase at concentrations above 50 nM, was more pronounced at pH 8.5 than at pH 7.5. At 25 °C, pH 8.5 the effect of compounds was completed within 2 h.

Therefore, further ERα affinity-labeling studies were performed via 2 h exposure of cell extracts at 25 °C, pH 8.5, to 50 nM aziridine **1** or **2**, RU 39 411, or vehicle. Using the [^{3}H]estradiol-binding capacity in RU 39 411-exposed cytosol as the reference value, high-binding inhibition levels were obtained in aziridine-exposed cytosol (Figure 2D). In the different experiments, the inhibition level was between 45 and 85% and higher for aziridine **2** than for aziridine **1**. Finally, as previously observed with other electrophilic estradiol derivatives (*17*), preincubation of cytosol with unlabeled estradiol totally blocked the effect of the two aziridines (not shown), suggesting that the compounds specifically alkylated ERα.

Cysteine Residues Are the Main Covalent Attachment Sites of 11β-[(Aziridinylalkoxy)phenyl]estradiols to Estrogen Receptors α. Most ERα affinity-labeling studies involving both steroidal (*17*−*19*) and nonsteroidal electrophilic ligands (*20*−*24*) indicated that cysteine residues were the main or exclusive covalent attachment sites of these electrophiles. Methyl methanethiosulfonate, a cysteine-specific reagent which transforms SH into S-SCH$_3$ groups, was therefore used to highlight the potential involvement of cysteine residues in ERα alkylation by aziridines **1** and **2**. In lamb uterine cytosol subsequently incubated at 25 °C without steroid, increasing concentrations of methyl methanethiosulfonate progressively decreased the estradiol-binding capacity (Figure 3A). This effect was much less marked in samples subsequently exposed to RU 39 411. Conversely, increasing concentrations of methyl methanethiosulfonate progressively and substantially increased the estradiol-binding capacity of cytosol when the latter was subsequently exposed to aziridine **1** or **2**; for concentrations of ≥1 mM of the reagent, the binding capacity measured in aziridine-exposed samples was higher than that in RU 39 411-exposed samples and very similar to that found in samples not exposed to steroid. Hence, using either cytosol not exposed to steroid or cytosol exposed to RU 39 411 as control, the decrease in the estradiol-binding site concentration elicited by the two aziridines was totally prevented by methyl methanethiosulfonate (Figure 3B), suggesting that aziridines **1** and **2** alkylated the lamb ERα at cysteine residues.

To obtain further evidence that cysteine residues are actually the covalent attachment sites of 11β-[(aziridinylalkoxy)phenyl]estradiols to ERα, we used expression vectors coding for the wild-type and a mutant of human ERα in which all four cysteines of the hormone-binding domain were replaced with alanines (*24*). The wild-type and mutant ERαs were expressed in COS cells, and their ability to be affinity labeled by the two aziridines was evaluated. As observed with lamb uterine cytosol, aziridines **1** and **2** were more efficient than RU 39 411 for decreasing the estradiol-binding capacity of wild-type ERα-containing cell extracts (Figure 4A). The amplitude of the decrease (Figure 4C) was similar to that previously determined using lamb ERα. With COS extracts containing the quadruple cysteine → alanine ERα mutant, there was no significant effect of RU 39 411 or aziridines **1** and **2** relative to the control (Figure 4, panels B and D), indicating that the effect of aziridines was dependent on the presence of cysteines in the hormone-binding domain of human ERα.

Cysteine 530 of the Human Estrogen Receptor Is the Main Covalent Attachment Site of 11β-[(Aziridinylalkoxy)phenyl]-estradiols. When only one cysteine is the covalent attachment site of 11β-[(aziridinylalkoxy)phenyl]estradiols, the four single cysteine → alanine mutants could be used for identification of the involved cysteine. Such ERα mutants (C381A, C417A, C447A, and C530A) (*23*) were therefore exposed to the aziridine derivatives. As observed with the

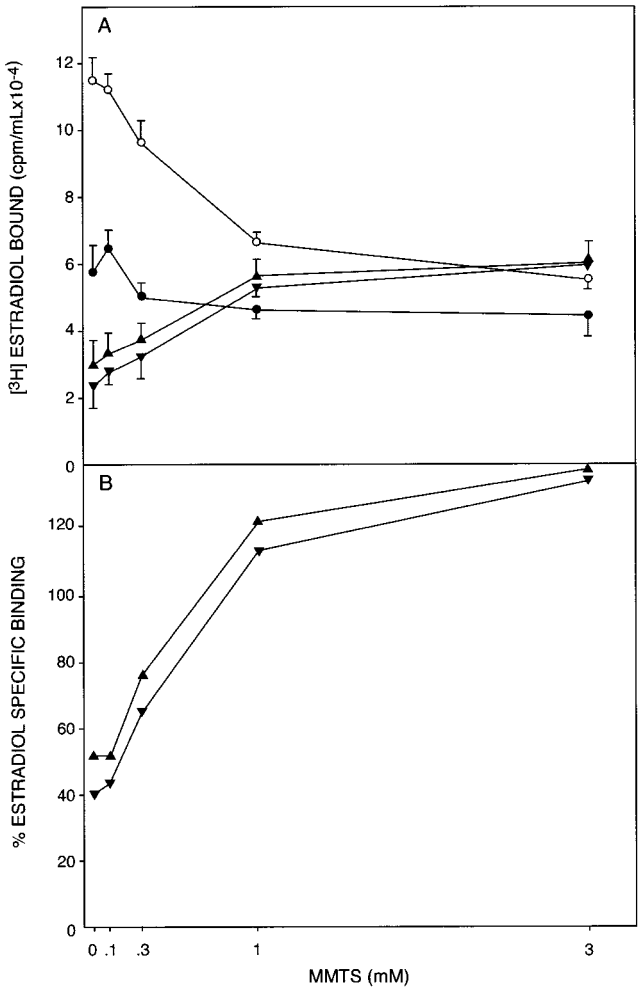

FIGURE 3: Effect of methyl methanethiosulfonate on the inactivation of specific estradiol-binding sites in lamb uterine cytosol by 11β-[(aziridinylalkoxy)phenyl]estradiols. Lamb uterine cytosol (6 mg of protein/mL, pH 8.5) was incubated for 4 h at 0 °C with increasing concentrations (0−3 mM) of methyl methanethiosulfonate (MMTS). Aliquots of the various samples were then incubated for 2 h at 25 °C without steroid, with 50 nM RU 39 411, aziridine **1** or aziridine **2**. Aliquots were treated with an equal volume of charcoal suspension. Charcoal was pelleted by centrifugation, then the total and nonspecific binding of [^{3}H]estradiol occurring in the supernatants under exchange conditions were determined by the standard irreversible binding assay, as described in Experimental Procedures. (A) The specific [^{3}H]estradiol binding in cytosol not exposed to steroid (○) or exposed to RU 39 411 (●), aziridine **1** (▲), or aziridine **2** (▼) is represented as a function of the MMTS concentration. Values are means of duplicate determinations; errors bars indicate standard deviations. (B) The specific [^{3}H]estradiol binding in cytosol exposed to aziridine **1** (▲) or aziridine **2** (▼) is expressed as a percentage of the specific binding in cytosol exposed to RU 39 411.

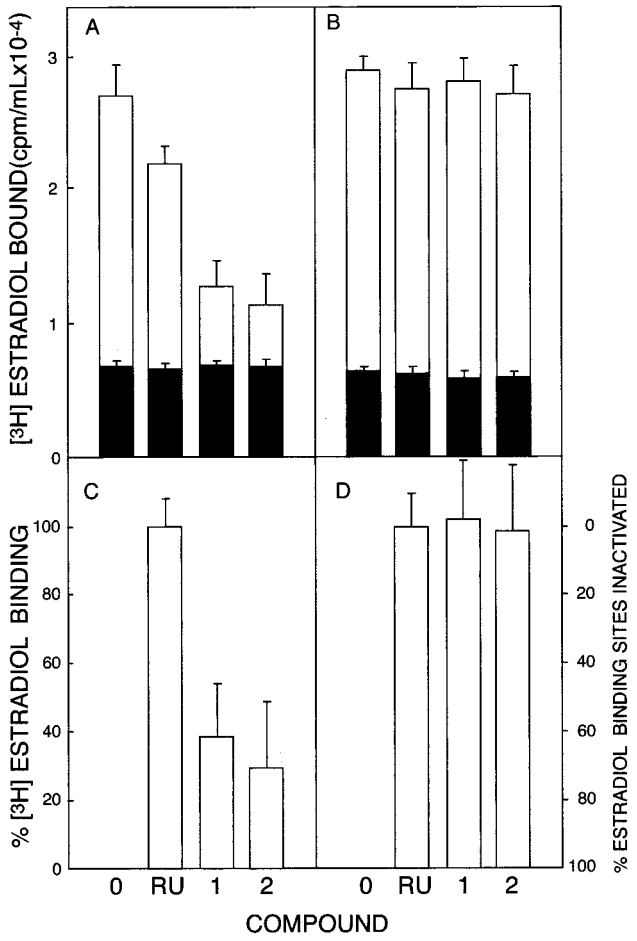

FIGURE 4: Estradiol binding to wild-type and quadruple cysteine → alanine mutant human estrogen receptors α after exposure to 11β-[(aziridinylalkoxy)phenyl]estradiols. Cytosols (3 mg of protein/mL, pH 8.5) from COS cells transfected with pRER containing wild-type or quadruple cysteine → alanine mutant human ERα cDNA were incubated for 2 h at 25 °C without steroid, with 50 nM RU 39 411, aziridine **1**, or aziridine **2**. Unbound steroids in aliquots were removed by charcoal treatment and the total and nonspecific binding of [^{3}H]estradiol occurring in samples under exchange conditions were determined by the standard irreversible binding assay as described in Experimental Procedures. (A, B) Concentrations of total (solid bars + open bars) and nonspecific (solid bars) bound [^{3}H]estradiol in cytosol containing wild-type ERα (A) or quadruple cysteine → alanine ERα mutant (B) are represented according to the compound incubated with the cytosolic extracts: without steroid (0), with RU 39 411 (RU), aziridine **1** (**1**), or aziridine **2** (**2**). Values are means of duplicate determinations; error bars indicate standard deviations. (C, D) The specific [^{3}H]-estradiol binding in cytosols exposed to aziridine **1** or aziridine **2** is expressed as a percentage of the specific binding measured in cytosols exposed to RU 39 411; (C) wild-type ERα-containing cytosol; (D) quadruple cysteine → alanine mutant ERα-containing cytosol.

wild-type ERα, exposure to aziridine **1** or **2** decreased the estradiol-binding capacity of extracts containing the C381A, the C417A, or the C447A ERα mutant (Figure 5, panels A−C). The decrease (∼65−80%, relative to the binding site concentration measured in extracts exposed to RU 39 411) was similar to that obtained with the wild-type ERα. Relative to the effect of RU 39 411, there was no significant effect of aziridine **1** or **2** on the C530A mutant-containing extracts (Figure 5D). These results strongly suggested that C530 of the human ERα would be the main covalent attachment site of aziridines **1** and **2**.

As for nonsteroidal and steroidal affinity labels of ERα (*19*, *23*, *24*), in addition to the C530 residue, at least one other cysteine residue could be involved in the covalent attachment of aziridines **1** and **2** to the human ERα. To determine which of the three other residues would be involved, the three double cysteine → alanine mutants, C381A/C530A, C417A/C530A, and C447A/C530A (*24*) were submitted to the action of the aziridines. With the C381A/C530A double mutant, there was no effect of aziridines **1** and **2**, since the binding site concentration in extracts exposed to aziridines **1** or **2** was 15−25% higher

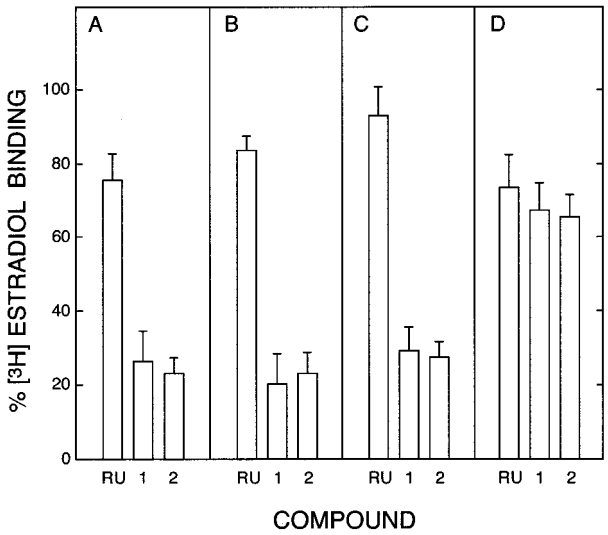

FIGURE 5: Estradiol binding to single cysteine → alanine mutants of the human estrogen receptor α after exposure to 11*β*-[(aziridinylalkoxy)phenyl]estradiols. Cytosols from COS cells transfected with the expression vector coding for (A) C381A, (B) C417A, (C) C447A, or (D) C530A mutant ERα (4.5 mg of protein/mL, pH 8.5) were incubated for 2 h at 25 °C without steroid or with 50 nM RU 39 411, aziridine **1** or aziridine **2**. Total and nonspecific binding of [^{3}H]estradiol in cytosol samples were then determined as described in Experimental Procedures. The specific [^{3}H]estradiol binding in cytosol samples incubated with RU 39 411 (RU), aziridine **1** (**1**) or aziridine **2** (**2**) is expressed as a percentage of the specific binding measured in samples incubated without steroid. Data were obtained from three independent determinations; error bars indicate standard deviations.

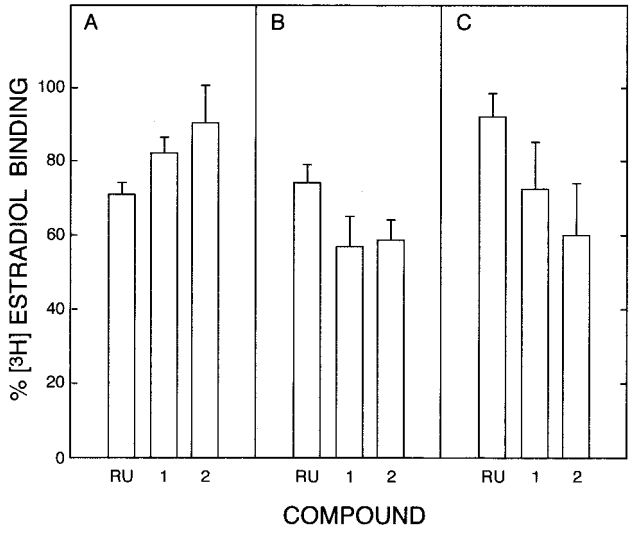

FIGURE 6: Estradiol binding to double cysteine → alanine mutants of the human estrogen receptor α after exposure to 11*β*-[(aziridinylalkoxy)phenyl]estradiols. Cytosols from COS cells transfected with the expression vectors coding for (A) C381A/C530A, (B) C417A/C530A, or (C) C447A/C530A mutant ERα (3 mg of protein/mL, pH 8.5) were incubated for 2 h at 25 °C without steroid or with 50 nM RU 39 411, aziridine **1** or aziridine **2**. Total and nonspecific binding of [^{3}H]estradiol in cytosol samples were then determined as described in the Experimental Procedures. The specific [^{3}H]estradiol binding in cytosol samples incubated with RU 39 411 (RU), aziridine **1** (**1**), or aziridine **2** (**2**) is expressed as a percentage of the specific binding measured in samples incubated without steroid. Data were obtained from three independent determinations; error bars indicate standard deviations.

than in extracts exposed to RU 39 411 ($p < 0.025$) and only 10−20% lower than that found in extracts not exposed to steroids (Figure 6A). Conversely with C417A/C530A and C447A/C530A, ERα double mutants (Figure 6, panels B and C), the estradiol-binding site concentration in COS extracts exposed to aziririne **1** or **2** was 20−30% lower than measured in extracts exposed to RU 39 411 ($p < 0.025$ for the C417A/C530A mutant and $p < 0.05$ for the C447A/C530 mutant). This effect was lower than obtained with C381A, C417A, and C447A ERα single mutants but slightly higher than that obtained with the C530A mutant. These results suggested that C381 of the human ERα could be a secondary covalent attachment site of aziridines **1** and **2**.

DISCUSSION

In this study, we first evaluated two aziridine derivatives of 11*β*-(alkoxyphenyl)estradiols as ER affinity-labeling agents, using both lamb and human ERαs. The evaluation was based on the ability of compounds to irreversibly inhibit the binding of [^{3}H]estradiol to ERs under exchange conditions. Since the compounds appeared to be high-affinity ligands of ERαs, RU 39 411, the most closely related and chemically inert analogue of aziridine **1**, was used as a blank in the affinity-labeling experiments (note that RU 39 411 lowered the [^{3}H]estradiol binding capacity from 20 to 60% in samples). The fact that in various situations, for instance when cysteine residues of ERα were either modified by methyl methanethiosulfonate or changed to alanine residues, the [^{3}H]estradiol binding in samples incubated with aziridines **1** or **2** was higher than that in samples incubated with RU 39 411 suggested that the latter constituted a hard control, which could have lead to underestimation of the labeling of ERα by aziridines **1** and **2**. Nonetheless, aziridines **1** and **2** appeared to be affinity labels of ERαs since, relative to RU 39 411, (i) they elicited a marked decrease in the [^{3}H]-estradiol-binding site concentration of lamb and human ERα-containing extracts and (ii) these effects did not occur when the ERα hormone-binding site was occupied by estradiol.

Our second goal was to characterize and possibly identify the ERα covalent attachment sites of the compounds. The absence of effect of aziridines on ERαs in which the cysteine residues were either chemically modified or mutated indicated that the labeling of these ERαs depended on the presence of intact cysteine residues. The sharp decrease in the efficiency of aziridines **1** and **2** observed with the C530A human ERα mutant and not with the three other single cysteine → alanine mutants indicated that C530 of the human ERα was probably a major covalent attachment site of the compounds. Moreover, the results obtained with the three double cysteine → alanine mutants, in which C530 was mutated along with one of the other three cysteines in the hormone-binding domain, suggested that C381 could be a covalent aziridine attachment site in addition to C530. The assumption that C381 and C530 would be the covalent attachment sites of aziridines **1** and **2** in the hormone-binding pocket of the human ERα is valid only if we assume that the cysteine(s) → alanine(s) mutations and especially the C530A and C381A/C530A mutations did not change, except locally at the mutated sites, the tridimensional structure of the ERα hormone-binding pocket. This seems to be reasonable since these ERα mutations did not markedly modify the apparent estradiol affinity for human ERα or the estradiol ability to transactivate it (*24*).

The indirect approach used to demonstrate the covalent attachment sites of aziridines **1** and **2** do not exclude the possibility that in wild-type ER, as observed with tamoxifen aziridine (*24*), C530 would be the major covalent attachment site and that C381 would be a covalent attachment site only in ER forms including the C530A mutation. Regardless of the actual situation, the results indicated that C381 would be in a more favorable position than C417 or C447 to react with the aziridine function of **1** and **2**. Different conclusions were drawn in another study involving both C417 and C530 as covalent attachment sites with respect to the affinity labeling of ERα by 17α-(haloacetamidoalkyl) estradiols (*19*). This suggests that the aziridinyl carbons of **1** and **2** and those of tamoxifen aziridine, compounds which strongly differ in their structural type and ERα binding affinity, are in a similar environment when compounds are in the ligand-binding pocket. Conversely, the terminal halogen-bearing carbon in the estradiol 17α-derivatives and aziridinyl carbons in the estradiol 11*β*-derivatives **1** and **2**, although located in proximity of C530, are probably not in the same environment when the compounds are in the ligand-binding pocket.

These different patterns of residue labeling with the aziridine-based and haloacetamide-based affinity labels could reflect (i) inherent differences in the reactivity of haloacetamides versus aziridines, (ii) different orientations that these ligands may have when bound within ERα, and (iii) different conformations that ERα may adopt around agonists versus antagonists. The estrogenic/antiestrogenic profile of an ERα ligand is thought to largely result from the structure of the ER:ligand complex, especially at the transactivation domains (*35*, *36*) involved in the interaction of ER with its cognate coactivators or corepressors (*37*) that modulate ER-mediated transcription of estrogen-target genes. The fact that RU 39 411 and tamoxifen (or 4-hydroxytamoxifen) display the same estrogenic/antiestrogenic profile (*33*, *38*, *39*) suggests that, irrespective to their structural type, the two compounds induce very close ERα conformations and the binding mode of the two compounds within ER is similar. Conversely, the fact that RU 39 411 and 17α-(haloacetamidoalkyl)estradiols display very different estrogenic/antiestrogenic profiles (*18*) suggests that although they are steroidal derivatives, when bound within the hormone-binding pocket the two types of compounds induce different ERα conformations. This effect probably results from dissimilar binding modes of the two types of compounds.

The fact that in tamoxifen (or tamoxifen aziridine), the trans-stilbene element formed by the ethylenic bond and the α- and *β*-rings mimics, as for the potent estrogen diethylstilbestrol, the whole structure of steroidal estrogens, limits the number of possible superimpositions of the molecule with RU 39 411 (or aziridine **1**). When the α- or *β*-ring is overlayed on the A-ring of RU 39 411, with the carbon 4 of the α- or *β*-ring on the carbon 3 of the A-ring, there are four different types of superimpositions of the two molecules. With the α-ring of tamoxifen playing the role of the RU 39 411 A-ring, there are two alternative possibilities related to the orientation of the ethylenic bond: (i) either close to that of the carbon 9-carbon 8 bond of the steroid (type 1 superimposition, Figure 7A) or (ii) close to that of the carbon 9-carbon 11 bond (type 2 superimposition, Figure 7B). Similar considerations with the *β*-ring (instead of the α-ring) playing the role of the RU 39 411 A-ring will give type 3 (Figure 7C) and type 4 (Figure 7D) superimpositions, respectively. In type 1 and type 4 superimpositions, the substituted phenyl ring (α′) of tamoxifen is above the median plane of the steroid, whereas in type 2 and type 3 superimpositions, the α′-ring is under the median plane. This last positioning of the α′-ring, which considerably differs from that of its 11*β*-homologue in RU 39 411, is not compatible with the results obtained in affinity-labeling experiments, since the latter indicate that the positions of the aziridine functions of tamoxifen aziridine and of compound **1** in the hormone-binding pocket are very similar. Moreover, the fact that (i) in the crystal structures of ERα complexed with estradiol (*9*) or with 4-hydroxytamoxifen (*10*), the α-ring of 4-hydroxytamoxifen plays the role the estradiol A-ring, and (ii) in triphenylethylene series, 4-hydroxylated derivatives displayed a much higher affinity for ER when the hydroxyl was on the α-ring than when it was on the *β*-ring (*40*) strongly suggests that the α-ring (not the *β*-ring) of tamoxifen plays the role of the steroid A-ring. Therefore, from the four initial types selected, type 1 superimposition of tamoxifen with RU 39 411 probably best reflects the binding mode of the two compounds within ERαs.

Superimposition of compound **1** (or RU 39 411) with 17α-(bromoacetamidomethyl)estradiol **3** could be used to account for the different patterns of residue labeling by the two electrophiles. Superimposition could be obtained either by direct overlay of the two quasi invariant steroid nuclei of the compounds or after a 180° rotation of one of the two molecules along the carbon 3−carbon 10 axis. In the latter case, although the two steroid nuclei do not coincide, they could demonstrate a good overlap (*41*, *42*). In the first case, the two electrophilic centers located on the *β* side and the α side, respectively, of superimposed molecules would be relatively distant. In the hormone-binding pocket, the relative location of the compound common attachment site C530 should thus be intermediate between those of the two electrophilic centers, whereas C381 would be closer to the first electrophilic center than to the second and conversely for C417. In the second case, the two electrophilic centers would be located on the same side of superimposed molecules, which would better account the common labeling of C530 by the two electrophiles. However, it is not certain that optimized superimposition of two ligands which display opposite hormonal effects could account for their relative interactions with ERα. Since, as discussed above, the two compounds probably interact with ER according to dissimilar binding modes, i.e., the ER hormone-binding pocket adopts different conformations around compound **1** and compound **3**; the common labeling of C530 by the two electrophiles may not reflect close proximity of the two electrophilic centers but rather a different positioning of C530 when the molecules are in the hormone-binding pocket. This difference between the binding modes of RU 39 411 and estradiol (or 17α-derivatives) around the 17*β*-hydroxy function was previously revealed by the very contrasting decreases in the relative affinity of estradiol (240-fold) and RU 39 411 (2-fold) upon ER modification by diethylpyrocarbonate, a specific histidine reagent (*43*).

Previous results on ERα affinity labeling by tamoxifen aziridine (*24*) and 17α-(haloacetamidoalkyl)estradiols (*19*) and the present results and hypotheses are in agreement with the crystal models of the human ERα ligand-binding domain

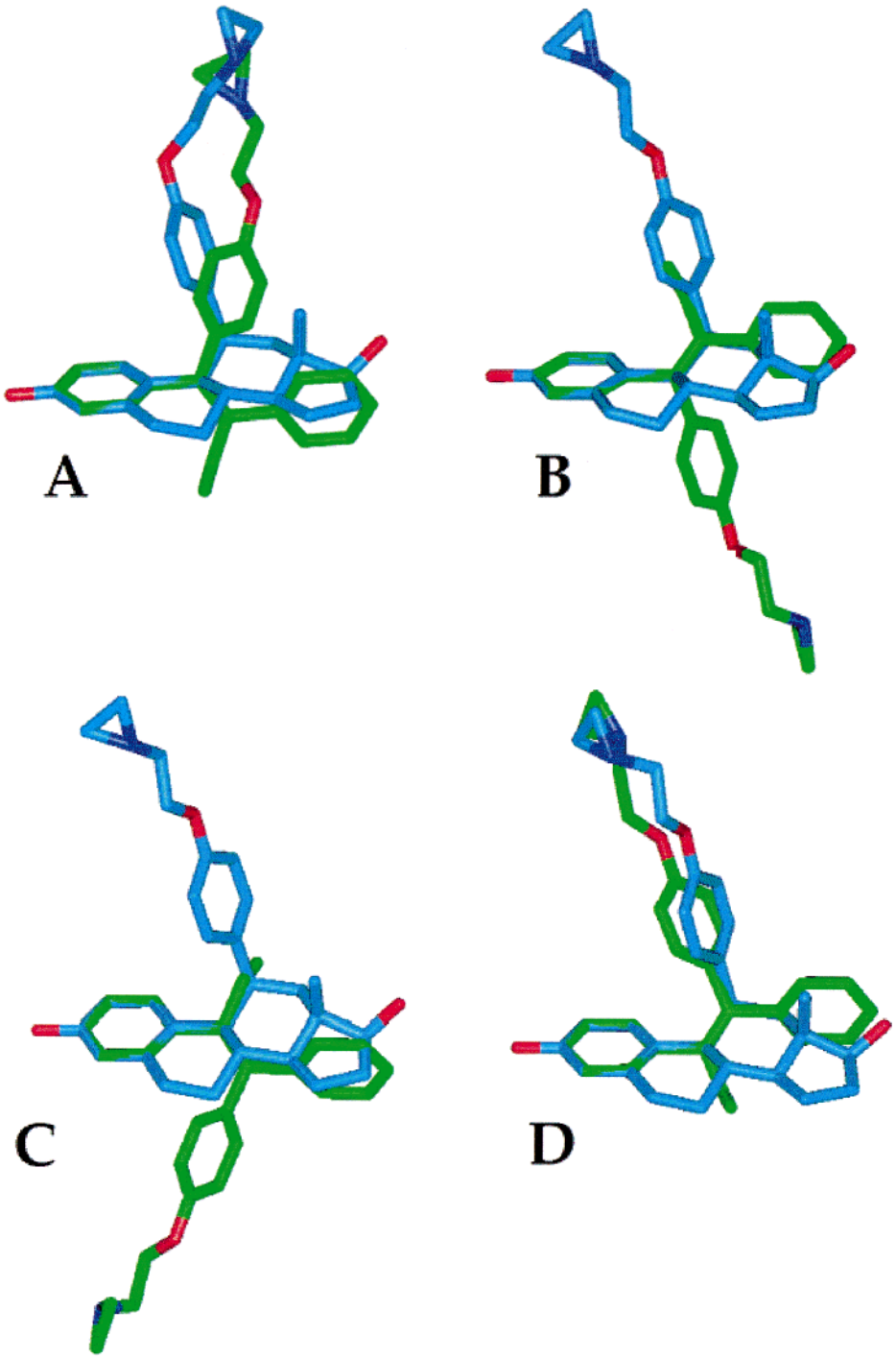

FIGURE 7: Spatial proximity of aziridinyl carbons in superimposed 11β-[(aziridinylethoxy)phenyl]estradiol and tamoxifen aziridine. Structure of 11β-[(aziridinylethoxy)phenyl]estradiol **1** was determined by molecular mechanics. The structure of tamoxifen aziridine **7** is derived from the tamoxifen conformation established by X-ray cristallographic studies [Précigoux et al. (1979) *Acta Crystallogr., Sect. B 38,* 312−315]; values for dihedral angles between the plane of the ethylenic bond and the α-, α′-, or β-ring are 51.1, 51.1, and 49.8°, respectively [Pons et al. (1984) in *Progress in Cancer Research and Therapy* (Bresciani et al., Eds.) Vol. 31, pp 27−36, Raven Press, New York]. The α-ring (A, B) or the β-ring (C, D) of tamoxifen aziridine (green lines) is superimposed on the A-ring of compound **1** (blue lines) so that carbon 4 of tamoxifen aziridine coincides with carbon 3 of the A-ring of compound **1**. The α′-ring of tamoxifen aziridine is then oriented either (i) above the median plane of the steroid nucleus, in close proximity to the 11β-phenyl of compound **1** (A, D) or (ii) under the median plane of the steroid nucleus, remote from the 11β-phenyl of compound **1** (B, C). (A, D) Mobilities of the aziridinylethoxy groups included in compound **1** and tamoxifen aziridine were used to bring the aziridinyl carbons of the two molecules closer, without considerably increasing the energies of the molecules; in the conformations shown, the distances between the aziridinyl carbons of **1** and those of tamoxifen aziridine, respectively, are from 0.9 to 2.2 Å (A) and from 0.3 to 1.7 Å (D), whereas from minimal levels, the increase in the molecule energies were 6.1 kcal (A) and 4.1 kcal (D) for compound **1** and 4.8 kcal (A) and 3.3 kcal (D) for tamoxifen aziridine.

filled with either estrogens or antiestrogens (*9*, *10*). In estradiol-filled and diethylstilbestrol-filled ligand-binding domain models, C417 (located at the C-terminal end of helix 7) but not C381 (located in helix 6) appears to lie in close proximity to C530 (located at the C-terminal end of helix 11). In raloxifene-filled and 4-hydroxytamoxifen-filled ligand-binding domain models, C417 appears to be more remote from C530 (located in the helix 11−helix 12 loop) and C381

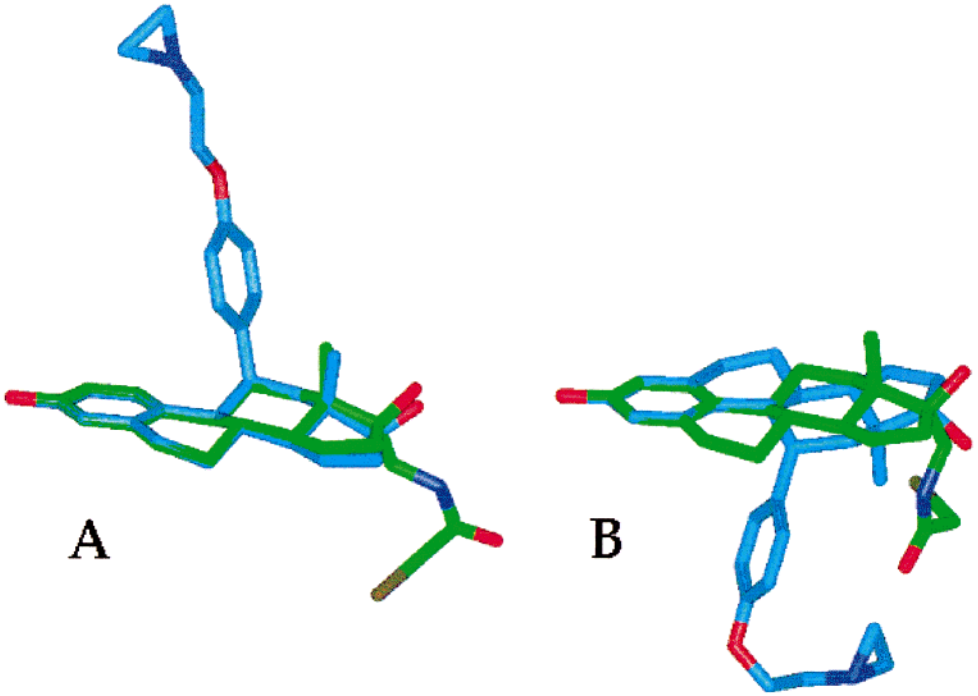

FIGURE 8: Spatial proximity of electrophilic carbons in superimposed 11β-[(aziridinylethoxy)phenyl]estradiol and 17α-(bromoacetamidomethyl)estradiol. Structures of 11β-[(aziridinylethoxy)phenyl]estradiol **1** (blue lines) and of 17α-(bromoacetamidomethyl)estradiol **3** (green lines) were determined by molecular mechanics. The two molecules could be superimposed by overlaying the A-rings of the compounds either (i) directly so that the two steroid nuclei practically coincide (A) or (ii) after a 180° rotation of compound **1** along the carbon 3−carbon 10 axis (B). In the first type of superimposition, the aziridinyl carbons of **1** and the bromine-bearing carbon of **3**, each located on a different side of the median plane of the steroid nuclei, are remote from each other (distance > 16 Å). In the second type of superimposition, relative to the median plane, the electrophilic carbons of **1** and **3** are located on the same side. (B) The mobility of the aziridinylethoxy group was used to bring the aziridinyl carbons of **1** closer to the bromine-bearing carbon of **3**, without considerably increasing the energy of molecule **1**; in the conformations shown, the distances between the aziridinyl carbons and the bromine-bearing carbon are 3.2 and 4.0 Å, whereas from the minimal level, the increase in the energy of **1** was 11.4 kcal.

closer to C530 than they are in estrogen-filled ligand-binding domain models. This indicates that the interaction of RU 39 411-class antiestrogens with ERα would be roughly similar to that of raloxifene or 4-hydroxytamoxifen.

In recent studies (*28*, *29*), we observed that electrophilic estradiol 11β-derivatives were effective ERα affinity-labeling agents only when the electrophilic center was relatively remote from the steroid nucleus (more than seven bonds between carbon 11 and the electrophilic center). In aziridine **1**, the electrophilic center is nine bonds away from carbon 11 and, although the presence of the 11β phenyl ring in the linking group restricts the aziridinyl carbons to the β side, there are still four degrees of freedom for the phenyl-anchored chain bearing the electrophilic center. Theoretically, on the β side of the molecule and centered on the oxygen atom, the aziridinyl carbons could scan a large and quasi-hemispheric volume, relatively remote from the steroid nucleus. This suggests that when aziridine **1** or RU 39 411 is in the ligand-binding pocket (i) both C381 and C530 would be on the β side, but remote from the steroid, and (ii) the two cysteines could be relatively far from each other.

Further studies are still necessary to obtain details at the molecular level that would differentiate the ER binding of the various classes of estrogens and antiestrogens. Work is under way to (i) identify in wild-type human ERα, by mass spectrometry, the cysteinyl and noncysteinyl covalent attachment sites of a large series of electrophilic estradiol 11β-derivatives (*29*) and (ii) to determine the effects of specific amino acid modifications on the ability of various steroidal and nonsteroidal estrogens or antiestrogens to interact with ERα. The results of the studies will be used for docking the compounds into models of the ERα ligand-binding pocket. These studies should improve our understanding of ERα/ligand interactions and possibly help in the design of new selective modulators of ERα.

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