Binding Thermodynamics as a Tool To Investigate the Mechanisms of Drug−Receptor Interactions: Thermodynamics of Cytoplasmic Steroid/Nuclear Receptors in Comparison with Membrane Receptors

Paola Gilli,[†] Gastone Gilli,*[,†,‡] Pier Andrea Borea,[‡,§] Katia Varani,[§] Angelo Scatturin,[∥] and Alessandro Dalpiaz*[,∥]

Centro di Strutturistica Diffrattometrica and Dipartimento di Chimica, University of Ferrara, via Borsari 46, 44100, Ferrara, Italy, Dipartimento di Medicina Clinica e Sperimentale, University of Ferrara, via Fossato di Mortara 19, 44100, Ferrara, Italy, Dipartimento di Scienze Farmaceutiche, University of Ferrara, via Fossato di Mortara 19, 44100, Ferrara, Italy, and Interdisciplinary Center for the Study of Inflammation (ICSI), University of Ferrara, via Fossato di Mortara 19, 44100, Ferrara, Italy

Received June 2, 2004

Drug−receptor binding thermodynamics has proved to be a valid tool for pharmacological and pharmaceutical characterization of molecular mechanisms of receptor-recognition phenomena. The large number of membrane receptors so far studied has led to the discovery of enthalpy−entropy compensation effects in drug−receptor binding and discrimination between agonists and antagonists by thermodynamic methods. Since a single thermodynamic study on cytoplasmic receptors was known, this paper reports on binding thermodynamics of estradiol, ORG2058, and R1881 bound to estrogen, progesterone, and androgen steroid/nuclear receptors, respectively, as determined by variable-temperature binding constant measurements. The binding at 25 °C appears enthalpy/entropy-driven ($-53.0 \le \Delta G° \le -48.6$, $-34.5 \le \Delta H° \le -19.9$ kJ/mol, $0.057 \le \Delta S° \le 0.111$, and $-2.4 \le \Delta C_p° \le -1.7$ kJ mol^{-1} K^{-1}) and is interpreted in terms of hydrophobic and hydrogen-bonded specific interactions. Results obtained for cytoplasmic receptors are extensively compared with those known for typical membrane receptors, in particular the adenosine A_1 receptor, to investigate the thermodynamic bases of drug−receptor binding from the most general point of view.

Introduction

Thermodynamic data for a great number of drug−receptor equilibria are currently available.[1,2] The significance of the thermodynamic approach is related to the more complete information on drug−receptor interaction mechanisms obtainable by full thermodynamic methods with respect to the simple affinity constants measurements.[3−5] In fact, the determination of drug−receptor binding constants (association, K_A, or dissociation, $K_D = 1/K_A$) allows us to calculate the standard free energy $\Delta G° = -RT \ln K_A$ ($T = 298.15$ K) of the binding equilibrium, but not its two components, as defined by the Gibbs equation $\Delta G° = \Delta H° - T\Delta S°$, where $\Delta H°$ and $\Delta S°$ are the equilibrium standard enthalpy and entropy, respectively. The determination of K_A values is indispensable for the screening of active drugs toward receptor subtypes and for the pharmacological characterization of these proteins, including the identification of signal transduction pathways. On the other hand, the knowledge of the molecular mechanisms underlying the drug−receptor interactions appears poor when based only on $\Delta G°$ and not on the $\Delta H°$ and $\Delta S°$ related values. It can be indeed assumed, in a simplified form, that these two last thermodynamic terms are directly related to the two main classes of molecular events responsible for the drug receptor recognition and interaction phenomena: intermolecular interactions, such as hydrogen bonding and multipolar or dispersive forces (mostly related to $\Delta H°$), on one side, and solvent reorganization (mostly related to $\Delta S°$), on the other.[6,7]

Nearly a dozen receptorial systems have been so far studied in greater detail from a thermodynamic point of view, most of which concern membrane receptors, including six G-protein coupled receptors (GPCRs: adenosine A_1,[8−18] A_{2A},[17,19,20] A_3;[21] β-adrenergic;[22−24] dopamine D_2;[24−27] and 5-HT_{1A} [28,29]), four ligand-gated ion channel receptors (LGICRs: glycine;[30,31] $GABA_A$;[31−33] 5-HT_3;[31,34−37] and nicotinic[37−41]), and one modulator receptor for benzodiazepines,[42−44] while only one concerns cytoplasmatic receptors, i.e., the receptor for glucocorticoid hormones.[45]

$\Delta G°$, $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ (standard heat capacity) values have been collected for a remarkable number of ligands, including agonists, partial agonists, inverse agonists, or antagonists, both in the absence and in the presence of suitable modulators. Our database includes, at present, thermodynamic parameters for more than 430 ligand−receptor interactions concerning some 300 different ligands. The information provided by these data could be very useful from a pharmacological and pharmaceutical point of view, allowing us to discover new thermodynamic relationships related to drug−receptor interactions and their molecular mechanisms.

As an example, $\Delta H°$ and $\Delta S°$ values can be used, in some membrane receptors, as indicators of the agonist or antagonist behavior of the ligands, the agonist and antagonist binding being respectively entropy-driven

* To whom correspondence should be addressed. A.D.: tel, +39-0532-291273; fax, +39-0532-291296; e-mail, dla@dns.unife.it. G.G.: tel, +39-0532-291141; fax, +39-0532-240709; e-mail: vg3@dns.unife.it.

† Centro di Strutturistica Diffrattometrica and Dipartimento di Chimica.

‡ Interdisciplinary Center for the Study of Inflammation.

§ Dipartimento di Medicina Clinica e Sperimentale.

∥ Dipartimento di Scienze Farmaceutiche.

10.1021/jm040842z CCC: $30.25
Published on Web 09/21/2004

($\Delta S° \gg 0$; $\Delta H° \geq 0$) or enthalpy-driven ($\Delta H° \ll 0$; $\Delta S° \leq 0$ or >0), or vice versa. This phenomenon, called thermodynamic discrimination,[10] has been monitored for β-adrenergic, adenosine (A_1, A_{2A}, A_3), glycine, $GABA_A$, serotonin 5-HT_3, and nicotinic membrane receptors,[2] a conclusion which would hold even if the antagonists are to be classified in a different way, in agreement with the fact that, recently, a large number of antagonists of several membrane receptors have been recognized as inverse agonists,[46−49] in touch with theoretical predictions indicating neutral antagonists as minority species in pharmacological space.[50]

Another thermodynamic aspect, which characterizes all membrane receptors, is the $\Delta C_p°$ value nearly zero,[51] a phenomenon which is not completely understood and is not usual in reactions involving biomacromolecules in solution.[52]

Finally, the great number of thermodynamic data available for membrane drug−receptor interactions has made it possible to observe that the enthalpic ($\Delta H°$) and entropic ($-T\Delta S°$) terms of the Gibbs equation are strongly correlated by the linear regression according to the equation[51]

$$-T\Delta S° \text{ (kJ mol}^{-1}) = (-1.01 \pm 0.01)\Delta H \text{ (kJ mol}^{-1}) - 42.4 \pm 0.5 \text{ kJ mol}^{-1} \quad (1)$$

$$(n = 436, R = 0.979, P < 0.0001)$$

This behavior, which has been called enthalpy−entropy compensation, is normally imputed to solvent reorganization phenomena accompanying the receptor binding processes.[51,53,54]

The main difficulties arise from the impossibility of comparing membrane with cytoplasmic receptors for lack of data on the latter. Indeed, thermodynamic data are reported in the literature only for the binding of two steroids (cortisol and dexamethasone, DEX) to the glucocorticoid receptor (GR).[45] In this case, strongly negative values of $\Delta C_p°$ have been detected (-1.6 kJ $mol^{-1}K^{-1}$ for cortisol and -4.6 kJ mol^{-1} K^{-1} for DEX), a thermodynamic behavior which is totally different from that displayed by membrane receptor systems, where $\Delta C_p°$ is always nearly zero. We have, therefore, undertaken a systematic analysis on the drug interactions toward cytoplasmic receptors, with the aim to evaluate if the thermodynamic behavior associated with negative $\Delta C_p°$ values is specific for GR, or a general feature of all cytoplasmic receptors. The analysis has been focused on the superfamily of human steroid/nuclear receptors[55] by evaluating the binding at different temperatures of (i) [^{3}H]17β-estradiol ([^{3}H]estradiol or [^{3}H]E_2) to the estrogen receptor (hER), (ii) [^{3}H]16α-ethyl-21-hydroxy-19-nor-pregn-4-ene-3,20-dione ([^{3}H]-ORG2058) to the progesterone receptor (hPR), and (iii) [^{3}H]17α-methyltrienolone ([^{3}H]R1881) to the androgen receptor (hAR). The structures of the ligands are reported in Figure 1.

The experimental results for cytoplasmic receptors will be described and finally discussed in comparison with the behavior of the adenosine A_1 receptor, i.e., the membrane receptor most widely known from a thermodynamic point of view, with the aim to investigate on a thermodynamic basis the molecular mechanisms determining drug−receptor interactions from the most general point of view.

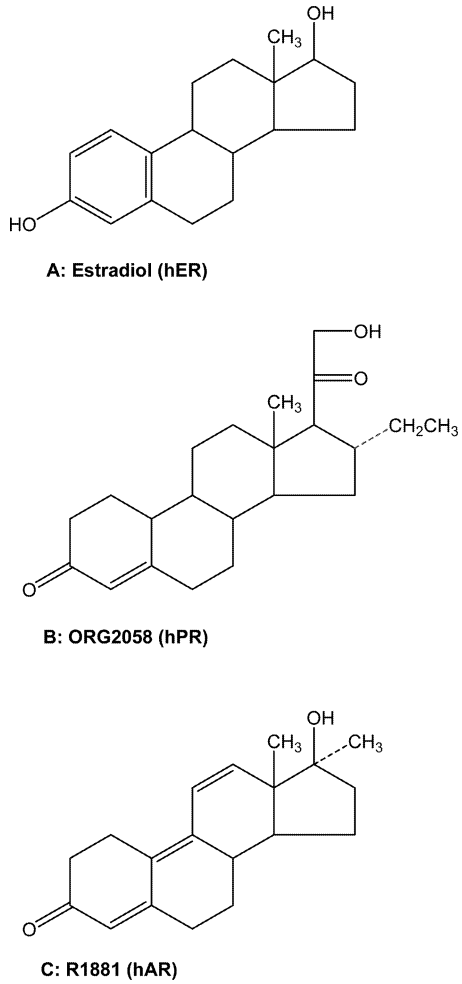

Figure 1. Structures of estradiol, ORG2058, and R1881, analyzed for interaction toward estrogen (hER), progesterone (hPR), and androgen receptors (hAR), respectively.

Results

The thermodynamics of cytoplasmic receptor binding has been characterized by measuring the dissociation constants (K_D) at nine different temperatures (0, 5, 10, 15, 20, 25, 30, 35, 37 °C) as reported in Table 1. The K_D values were obtained from saturation experiments followed by Scatchard analysis. Experimental data obtained at 15 and 37 °C are reported, as representative, in Figure 2. All Scatchard plots appear linear in the concentration range investigated, in agreement with the computer analysis of the saturation experiments which indicates a one-site rather than a two-site binding model. Whereas B_{max} values appear to be independent of temperature for all the drug−receptor interactions investigated (Table 1), the affinity constants $K_A = 1/K_D$ show an appreciable temperature dependence. This dependence is represented in Figure 3 in the form of ln K_A versus T plots which fit significantly better the equation of a parabola rather than being described by linear regressions. These results suggest $\Delta C_p°$ values different from zero, in agreement with the thermodynamic parameters at 298.15 K reported in Table 2. At this temperature the binding appears to be enthalpy/entropy-driven ($-35 \leq \Delta H° \leq -20$ kJ mol^{-1}; $0.057 \leq$

Table 1. Equilibrium Binding Parameters Obtained from Saturation Experiments at Nine Different Temperatures[a]

receptor		0 °C (273 K)	5 °C 278 (K)	10 °C (283 K)	15 °C (288 K)	20 °C (293 K)	25 °C (298 K)	30 °C (303 K)	35 °C (308 K)	37 °C (310 K)
hER	K_D	0.72 ± 0.03	0.62 ± 0.02	0.53 ± 0.01	0.43 ± 0.01	0.45 ± 0.01	0.53 ± 0.01	0.59 ± 0.02	0.79 ± 0.01	0.87 ± 0.03
	B_{max}	32.3 ± 0.9	34.4 ± 0.3	35.9 ± 0.3	33.9 ± 0.9	32.3 ± 0.3	34.1 ± 0.9	36.2 ± 0.9	35.3 ± 0.5	37.8 ± 0.9
hPR	K_D	3.28 ± 0.09	2.70 ± 0.13	2.49 ± 0.03	2.32 ± 0.12	2.64 ± 0.13	3.16 ± 0.03	4.13 ± 0.09	4.56 ± 0.08	5.42 ± 0.03
	B_{max}	331 ± 20	325 ± 33	329 ± 11	311 ± 18	315 ± 15	312 ± 14	326 ± 14	333 ± 15	329 ± 34
hAR	K_D	0.61 ± 0.01	0.52 ± 0.02	0.56 ± 0.02	0.68 ± 0.04	0.74 ± 0.02	0.99 ± 0.04	1.21 ± 0.06	1.51 ± 0.04	1.95 ± 0.04
	B_{max}	22.6 ± 1.2	23.7 ± 2.7	21.9 ± 1.2	22.5 ± 1.1	22.2 ± 0.4	21.2 ± 0.5	21.6 ± 0.3	22.3.2 ± 1.1	21.8 ± 0.2

[a] Dissociation constants K_D (nM) and B_{max} values (fmol/mg protein) are referred to the binding of (i) [^{3}H]estradiol to estrogen receptor (hER), (ii) [^{3}H]ORG2058 to progesterone receptor (hPR), and (iii) [^{3}H]R1881 to androgen receptor (hAR). Values and SEM obtained from at least four independent experiments performed in duplicate.

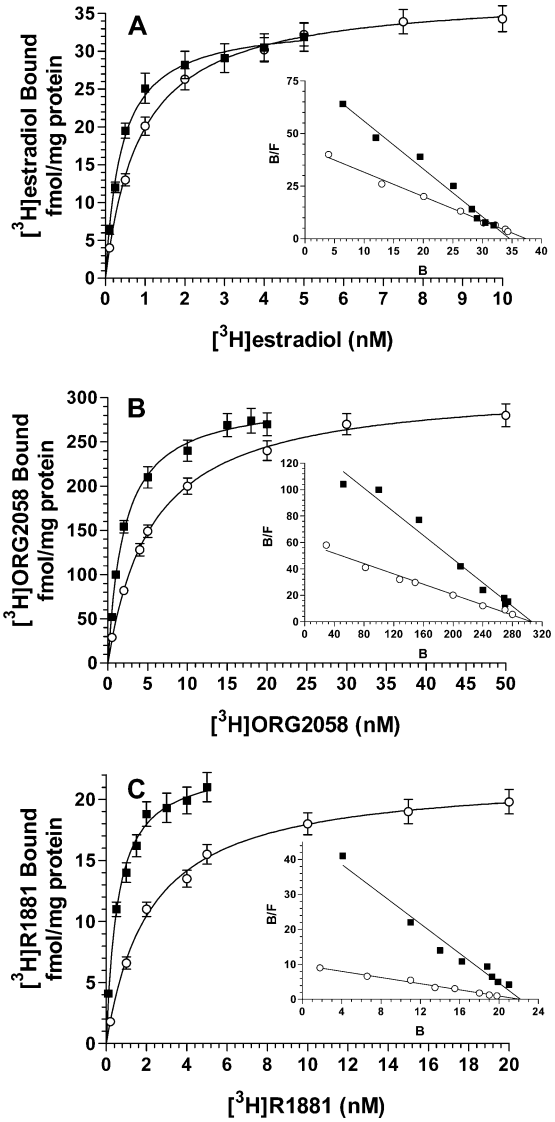

Figure 2. Saturation curves and their Scatchard plots obtained at 15 °C (■) and 37 °C (○) for the binding equilibrium of (A) [^{3}H]estradiol to estrogen receptor, (B) [^{3}H]ORG2058 to progesterone receptor, and (C) [^{3}H]R1881 to androgen receptor. The linearity of the Scatchard plots ($R > 0.98$, $P < 0.0001$) is indicative of the presence of a single class of binding sites in each experimental system. Data and SEM were obtained from at least four independent experiments performed in duplicate.

$\Delta S° \leq 0.111$ kJ mol^{-1} K^{-1}) for all systems examined, and accompanied by great negative standard heat capacities ($-2.4 \leq \Delta C_p° \leq -1.7$ kJ mol^{-1} K^{-1}). ΔC_p variations in the range of temperature investigated appear negligible in relation with their standard deviations, and, therefore, they can be considered essentially independent of temperature.

The dependence of ΔG, ΔH, and $T\Delta S$ values on temperature is depicted in Figure 3. The changes of ΔG values are small with respect to the modifications of the ΔH and $T\Delta S$ values which decrease linearly with the temperature from 0 to 37 °C, gradually converting the drug−receptor binding from entropy-driven to enthalpy-driven through entropy/enthalpy-driven, while only relatively small modifications of the affinities occur. A strictly similar behavior was observed for the binding of cortisol and dexamethasone to the glucorticoid receptor.[45] Figure 4 reports, the $-T\Delta S$ versus ΔH plot for all cytoplasmic steroid/nuclear receptors so far known at all the temperatures investigated. All points of the plot are arranged on a same regression line of the equation

$$-T\Delta S\ (\text{kJ mol}^{-1}) = (-1.00 \pm 0.02)\Delta H°\ (\text{kJ mol}^{-1}) - 48.2 \pm 0.7\ \text{kJ mol}^{-1} \quad (2)$$

$$(n = 45, R = 0.995, P < 0.0001)$$

which evidences a typical enthalpy−entropy compensation phenomenon.

As previously discussed by other authors,[45,52] the observed behavior (negative $\Delta C_p°$, entropy-driven binding at low temperatures which becomes enthalpic at higher ones) is a clear indication that hydrophobic interactions make a significant contribution to the molecular mechanisms of drug interaction to steroid/nuclear cytoplasmic receptors.

To better quantify these hydrophobic interactions, we have taken advantage of three different semiempirical treatments, the first of which, originally proposed by Sturtevant,[47] makes use of the unitary entropies, $\Delta S_u°$, calculated at 298.15 K[56] to partition the experimental heat capacities ($\Delta C_p°$) and entropies ($\Delta S_u°$) in their components resulting from hydrophobic (H) and vibrational (V) effects, which are considered the main causes of heat capacity changes during the binding.[45,52] Results are reported in Table 3. The larger and positive $\Delta S_{u(H)}°$ values evidence the prevailing role of hydrophobic forces with respect to vibrational effects, whose $\Delta S_{u(V)}°$ values are negative. In a similar way, the changes of standard heat capacity appear to be mainly imputable to hydrophobic interactions rather than to changes of the vibrational state.

The second method[57] makes it possible to calculate the contribution of hydrophobic forces to the binding constants ($K_{D(hydro)}$) according to the equation

$$\Delta G_{(hydro)} = RT \ln K_{D(hydro)} = \gamma\ \Delta\text{SAHSA}_{D-R} \quad (3)$$

where γ is a free/surface area coefficient frequently cited

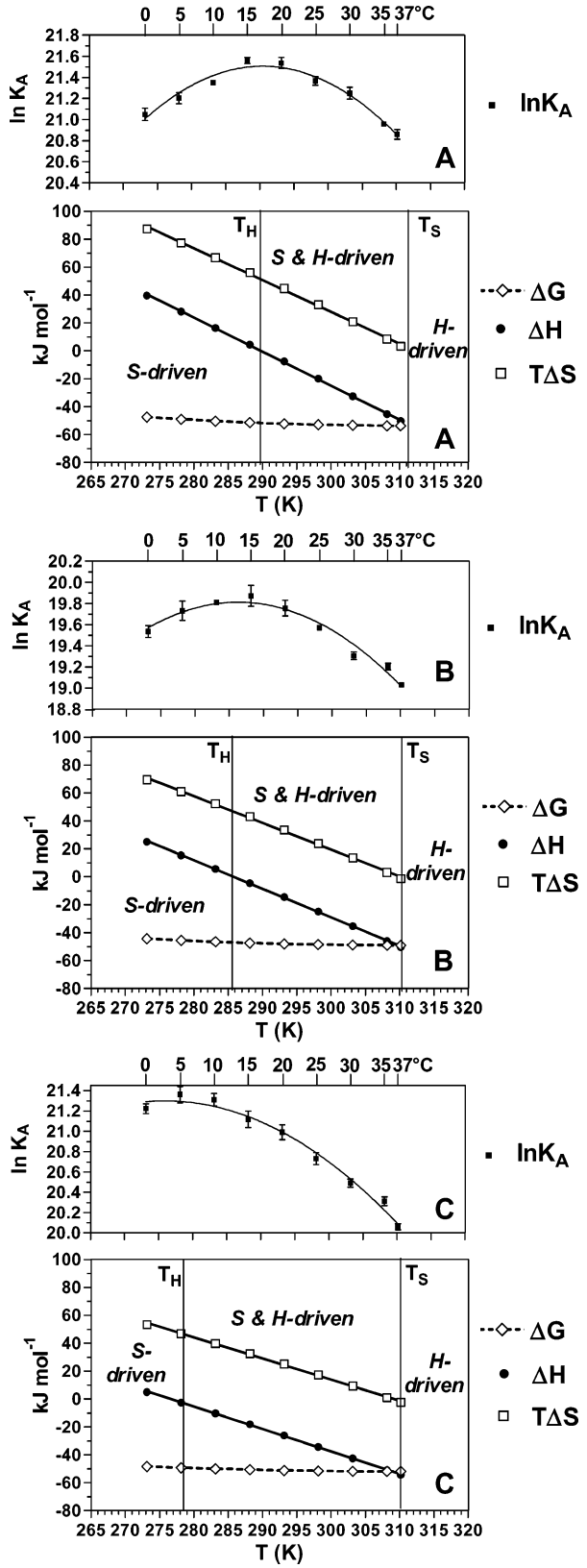

Figure 3. ln K_A versus T plots showing the effect of temperature on the association constants, K_A, and temperature dependence of ΔG, ΔH, and $T\Delta S$ values referred to the binding of (A) estradiol to estrogen receptor, (B) ORG 2058 to progesterone receptor, and (C) R1881 to androgen receptor. Data referred to $\Delta H°$ and $-T\Delta S°$ can be fitted according to a linear regression for each receptor system ($R > 0.999$, $P < 0.0001$). $\Delta C_p°$ values appear to be essentially independent of temperature. T_H and T_S are the temperatures where the ΔH and $T\Delta S$ values, respectively, are zero.

as 1.046 kJ mol^{-1}Å^{-2},[58–60] while ΔSAHSA$_{D-R}$ is the difference between the solvent accessible hydrophobic surface area (SAHSA) of the drug–receptor complex and that of separate drug and receptor molecules. Related quantities are the total (SASA) and polar (SAPSA) solvent accessible surface areas. Table 4 reports SASA, SAHSA, and SAPSA values for the three hormones studied with the corresponding $K_{D(hydro)}$ values obtained from eq 3. Steroid hormones show an average SAHSA which is nearly 80% of SASA and can bind to their receptors with hydrophobic contributions ($K_{D(hydro)}$) ranging from 9.5 to 84 nM, which are an order of magnitude higher than the experimental K_D values, which are found to range from 0.53 to 3.16 nM. Accordingly, the experimental $\Delta G°$ is some 8 kJ mol^{-1} lower than the $\Delta G_{(hydro)}$ ones.

Finally, SAHSA and SAPSA values are employed to calculate $\Delta C_{p(hydro)}$ values according to the method proposed by Spolar and Record [61] (see Experimental Section). The values, reported in Table 4, are nearly -0.5 kJ mol^{-1} K^{-1} for all the receptors studied.

Discussion

Thermodynamics of Cytoplasmic Steroid/Nuclear Receptors. The quantities directly derived from the equilibrium binding measurements to steroid receptors are the B_{max} and K_D values reported in Table 1. The essential invariance of the B_{max} values with the changes of temperature shows that the experimental conditions chosen are reasonably correct, avoiding the main problem of receptor thermolability. Conversely, the affinity values of the drug–receptor interaction show a definite temperature dependence, as represented by the ln K_A versus T plots of Figure 3, where the data are fitted according to a polynomial quadratic equation, at variance with membrane receptors whose corresponding plots are linear.[51] Similar parabolic patterns have been already observed for GR[45] and are indicative of molecular mechanisms inducing large and negative standard heat capacity values ($-2.4 \le \Delta C_p° \le -1.7$ kJ mol^{-1} K^{-1}, Table 2) essentially independent of temperature. The facts that $\Delta C_p = (\delta\Delta H/\delta T)_p = (\delta T\Delta S/\delta T)_p$ and $\Delta C_p < 0$ completely explain the ΔH and $T\Delta S$ temperature dependence shown in Figure 3. Indeed, the binding of steroid receptors is always entropy-driven at low temperatures, but the decrease of both ΔH and $T\Delta S$ terms gradually converts it to enthalpy/entropy-driven until totally entropy-driven while the temperature increases. The plots of Figure 3 also evidence that ln K_A has a maximum for $\Delta H = 0$ at T_H. The T_H value is not the same for the three receptors (289.6 K for hER, 285.6 K for hPR, and 278.2 for hAR), while the temperature T_S for which $T\Delta S$ became zero is confined in a strict interval with an average value of 310 ± 2 K. It may be of interest to remark that T_S is much larger (383–393 K) for other hydrophobic processes such as protein denaturation and dissolution of apolar substances in water.[62]

The observed thermodynamic behavior can be straightforwardly interpreted in terms of the so-called hydrophobic effect. This is due to the fact that apolar molecules (or parts of molecules) cannot dissolve in water but become surrounded by cages of water molecules which are characterized from a thermodynamic

Table 2. Thermodynamic Parameters for the Binding Equilibrium of (i) [^{3}H]Estradiol to Estrogen Receptor (hER), (ii) [^{3}H]ORG2058 to Progesterone Receptor (hPR), and (iii) [^{3}H]R1881 to Androgen Receptor (hAR)[a]

receptor	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (kJ mol^{-1} K^{-1})	$\Delta C_p°$ (kJ mol^{-1} K^{-1})	$\Delta S°/\Delta C_p°$
hER	-53.04 ± 0.04	-19.9 ± 0.9	0.111 ± 0.003	-2.4 ± 0.1	-0.046
hPR	-48.62 ± 0.04	-25.3 ± 0.9	0.078 ± 0.003	-2.2 ± 0.2	-0.035
hAR	-51.56 ± 0.05	-34.5 ± 1.5	0.057 ± 0.005	-1.7 ± 0.2	-0.034

[a] $\Delta G°$, $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ values are given at 298.15 K.

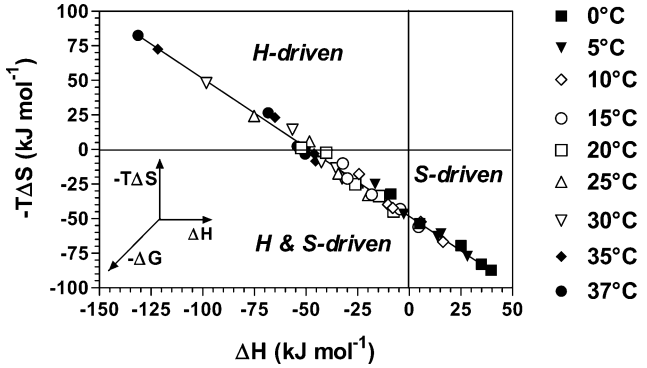

Figure 4. $-T\Delta S$ versus ΔH (kJ mol^{-1}) scatter plot for the binding at different temperatures of estradiol to estrogen receptor, ORG2058 to progesterone receptor, R1881 to androgen receptor, and cortisol and dexamethasone to glucocorticoid receptor. The linear regression of the points ($R = 0.995$, $P < 0.0001$) evidences a typical enthalpy−entropy compensation phenomenon with respect to the temperature.

Table 3. Changes of Unitary Entropy ($\Delta S_u°$) and Standard Heat Capacity ($\Delta C_p°$) Resulting from Hydrophobic (H) and Vibrational (V) Effects at 298.15 K for the Binding of (i) [^{3}H]Estradiol to Estrogen Receptor (hER), (ii) [^{3}H]ORG2058 to Progesterone Receptor (hPR), and (iii) [^{3}H]R1881 to Androgen Receptor (hAR)[a]

receptor	$\Delta S°$	$\Delta S_u°$	$\Delta S_{u(H)}°$	$\Delta S_{u(V)}°$	$\Delta C_p°$	$\Delta C_{p(H)}°$	$\Delta C_{p(V)}°$
hER	0.111	0.144	0.54	−0.40	−2.45	−2.07	−0.38
hPR	0.078	0.111	0.47	−0.36	−2.15	−1.81	−0.34
hAR	0.057	0.090	0.38	−0.28	−1.73	−1.46	−0.27

[a] Units of kJ mol^{-1} K^{-1}.

point of view for having large heat capacities and low entropies, respectively imputed to the lack of hydrogen bonding between the enclosed molecule and the surrounding cage and to the tightening of the water structure at the cage borderline. Cages are unstable structures which tend to reduce their surface by associating two or more hydrophobic molecules in a unique cage, a process causing a large number of important effects such as separation of oil from water, binding of nonpolar ligands to hydrophobic receptor binding sites (as in our case), or protein folding, and thermodynamically characterized by a decrease of free energy ($\Delta G < 0$) and heat capacity ($\Delta C_p < 0$) and an increase of entropy ($\Delta S > 0$).[52,63]

As observed by Sturtevant,[52] the heat capacity and entropy changes related to the binding of ligands to proteins may arise from both hydrophobic effects (water-cage disruption) and decrease of the number of internal vibrational modes of the protein. Such a partitioning of observed $\Delta C_p°$ and $\Delta S_u°$ is reported in Table 3 and suggests that the hydrophobic effects have a prevalent role, with respect to vibrational forces, in influencing the molecular mechanisms related to the binding of steroids to their receptors.

According to this point of view, the entropy-driven binding observed at low temperatures for the three steroid receptors is an indication that, during the binding process, the partial withdrawal of nonpolar groups from water induces an entropically favorable dispersion of previously ordered water molecules. With the increase of temperature, the water molecules may assume more conformations (higher entropy) but at the cost of breaking a part of the hydrogen bonds among them (higher enthalpy). Accordingly, the association of the ligand with the receptor in hot water becames increasingly controlled by the decrease of enthalpy ($\Delta H°$) at the expense of the increase of entropy ($T\Delta S°$).[64] It is interesting to observe that the T_S values near 37 °C for the three receptor systems imply a minimum of water organization at this physiological temperature, which could be interpreted also as a minimum of the activation energy necessary for water-cage disruption.

The contribution of the hydrophobic forces alone to hormone−cytoplasmic receptor interactions ($K_{D(hydro)}$) can be evaluated as a function of the solvent accessible hydrophobic surface area of the hormones involved (SAHSA), according to eq 3.[57−60] Data of Table 4 show that hydrophobic interactions contribute, on average, 84% of the total free energy of binding ($\Delta G°_{(hydro)} = 43$ against $\Delta G° = 51$ kJ mol^{-1}), the remaining 16% having to be imputed to specific interactions, such as hydrogen bonding. This value of $\Delta G°_{(hydro)}$ would give an average dissociation $K_{D(hydro)} = 46$ nM against the experimental K_D of 1.6 nM.

Similar semiempirical considerations can be made for the evaluation of the $\Delta C_{p(hydro)}$ of the binding equilibrium, which can be evaluated from SAHSA and SAPSA values using the expression, proposed by Spolar and Record,[61] $\Delta C_{p(hydro)} = 0.00058$ SAPSA $- 0.00134$ SAHSA kJ mol^{-1} K^{-1}. The $\Delta C_{p(hydro)}$ values shown in the last column of Table 4 amount to some -0.5 kJ mol^{-1} K^{-1} which, thought remarkably large, are only one-third of the experimental values. Reasons may be that the equation has been parametrized on the basis of rather different protein−DNA interactions, or, more probably, that the binding to steroid receptors includes large and negative vibrational contributions due to the tightening of the ligand-binding domain (LBD) and to the closing up of helix 12 (see below).

These thermodynamic results are much better understood in the light of the most recent X-ray crystallographic studies. In fact, many crystal structures of the steroid/nuclear receptor superfamily LBDs have been determined in the last years, including ER, PR, AR, GR, and the retinoid RAR and RXR receptors,[65−72] some of which correspond exactly to the steroid−LBD systems studied here from a thermodynamic point of view, namely E_2−hER, [65] R1881−hAR [69] and DEX−hGR, [70] the former being schematically shown in Figure 5. A comparison among the determinants of hormone bind-

Table 4. Solvent Accessible Surface Area (SASA), Solvent Accessible Hydrophobic Surface Area (SAHSA), and Solvent Accessible Polar Surface Area (SAPSA) Calculated for the Three Steroid Hormones Examined, Calculated $\Delta G_{(hydro)}$, $K_{D(hydro)}$, and $\Delta C_{p(hydro)}$ Values, Experimental $\Delta G°$ and K_D Values, and Averages[a]

	SASA (Å²)	SAHSA (Å²)	SAPSA (Å²)	$\Delta G_{(hydro)}$ (kJ mol^{-1})	$K_{D(hydro)}$ (nM)	$\Delta G°$ (kJ mol^{-1})	K_D (nM)	$\Delta C_{p(hydro)}$ (kJ mol^{-1} K^{-1})
estradiol	473.9	386.1	87.9	−40.4	84	−53.04	0.53	−0.465
R1881	488.1	401.1	87.1	−42.0	45	−48.62	3.16	−0.486
ORG2058	571.1	437.6	133.5	−45.8	9.5	−51.56	0.99	−0.506
av	511	408	103	−43	46	−51	1.6	−0.486

[a] The $\Delta G_{(hydro)}$, $K_{D(hydro)}$, and $\Delta C_{p(hydro)}$ values were calculated according to the equations $\Delta G_{(hydro)} = RT \ln K_{D(hydro)} = 0.1046\ \Delta$SAHSA kJ mol^{-1} and $\Delta C_{p(hydro)} = 0.58$ SAPSA − 1.34 SAHSA J mol^{-1} K^{-1}. Experimental $\Delta G°$ and K_D values and averages are reported as terms of comparison.

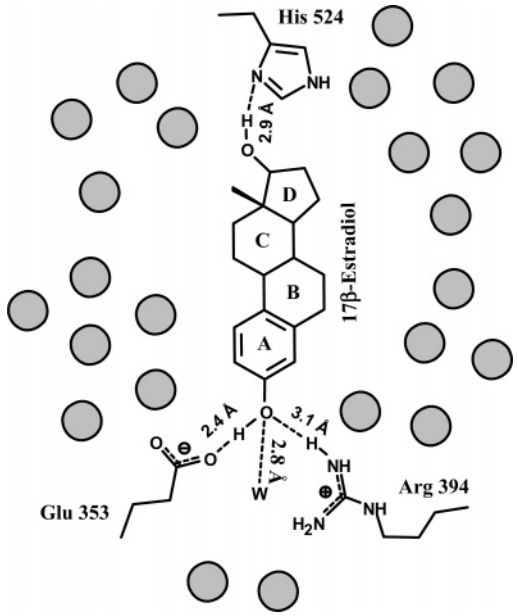

Figure 5. Schematic representation of the interactions made by 17β-estradiol (E_2) within the hERα ligand binding site. The approximate positions of the receptor hydrophobic residues are shown by small gray circles. The ligand makes also direct hydrogen bonds with residues Glu 353, Arg 394, and His 524 of the receptor and a water molecule (W). Adapted from ref 60.

ing to the different receptors reveals a common structural theme of mutually supported hydrophobic and hydrogen-bonded interactions involving highly conserved residues[67] which ensures shape complementarity of the binding cavity for and specific recognition of the polar regions of the ligand, respectively. The pattern (Figure 5) of three hydrogen bonds linking the hydroxyl of ring A through Glu 353, Arg 394, and a water molecule (W) is conserved in all the receptors studied with the only exchange of Gln for Glu when the OH is substituted by a ketonic function as, for instance, in progesterone or dihydrotestosterone. The binding of agonists induces a relevant tightening of the receptor and the closing up of the binding cavity through a dramatic repositioning of helix 12, the last of the 12 α-helices building up the receptor structure.[71,72] This repositioning cannot occur with antagonists because of their larger dimensions, so hindering the binding of the coactivator peptide and any further effect.[65,70] It is worthwhile mentioning that the pattern of Figure 5 has been recently validated by a number of molecular dynamics simulations of the E_2−ERα complex in solution[73,74] which confirmed the essential role played by the triad Glu 353-water-Arg 394 in the binding of estradiol to the ERα−LBD.

The particular shape of the LBD of nuclear receptors allows us to conclude that their binding process can be most probably partitioned in two independent thermodynamic contributions: (i) the aspecific hydrophobic binding of the central body of the steroid to the nonpolar LBD cavity; and (ii) the specific binding of mostly enthalpic nature due to the hydrogen bonds formed. The latter is hard to evaluate because of the insufficient accuracy of the hydrogen-bond geometries achievable by protein-crystallography methods, while the former can be evaluated semiempirically by the methods applied in this paper.[52,57,58,61]

Comparison between Cytoplasmic and Membrane Receptors. The thermodynamic behavior of all known membrane receptors[1,2] is quite different from that just described for the cytoplasmic ones. The $\Delta C_p°$ of the binding equilibrium is normally zero, or at least smaller than its experimental standard deviation. For this reason both ΔH and ΔS values are independent of temperature and their standard values, $\Delta H°$ and $\Delta S°$, can be obtained by linear van't Hoff plots. In spite of these substantial differences the two types of binding conserve an important similarity: the phenomenon of extrathermodynamic enthalpy−entropy (E/E) compensation.[51,53,54] Membrane receptors are well-known to be in E/E compensation, as shown by the correlation eq 1, which is practically based on all data available, a total of 436 binding experiments performed on 17 membrane receptorial systems with more than 300 different ligands. In the frame of this general E/E compensation, some membrane receptors display the phenomenon of thermodynamic discrimination, for which agonists and antagonists (or rather inverse agonists, as recently suggested[50,75]) share different regions of the correlation line, being agonist binding enthalpy-driven and antagonist binding entropy-driven, or vice versa, as illustrated in Figure 6 for the A_1 adenosine receptor.

Steroid receptors display the same compensation effect (Figure 4) but, in this case, for two different reasons: (i) one of purely thermodynamic nature, due to the fact that both ΔH and $T\Delta S$ depend on temperature according to the $\Delta C_p = (\delta \Delta H/\delta T)_p = (\delta T \Delta S/\delta T)_p$ equation; and (*ii*) the other, truly extrathermodynamic, for which data of different receptors do not split in a set of parallel lines, but cluster on a same regression line of eq 2. The reasons for this general behavior are difficult to understand, although, from a mechanistic point of view, there is the possibility that the thermodynamic discrimination of the entropy- and enthalpy-driven binding to membrane receptors can be in some way assimilated to low- and high-temperature binding to cytoplasmic receptors, respectively.

Table 5. Solvent Accessible Surface Area (SASA), Solvent Accessible Hydrophobic Surface Area (SAHSA), and Solvent Accessible Polar Surface Area (SAPSA) Calculated for Five A_1 Membrane Receptor Ligands, Calculated $\Delta G_{(hydro)}$, $K_{D(hydro)}$, and $\Delta C_{p(hydro)}$ Values, Experimental $\Delta G°$ and K_D Values, and Averages[a]

	SASA (Å²)	SAHSA (Å²)	SAPSA (Å²)	$\Delta G_{(hydro)}$ (kJ mol^{-1})	$K_{D(hydro)}$ (mM)	$\Delta G°$ (kJ mol^{-1})	K_D (μM)	$\Delta C_{p(hydro)}$ (kJ mol^{-1} K^{-1})
caffeine	347.9	44.9	302.9	−4.7	150	−24.7[b]	45[b]	0.116
theophylline	341.3	142.4	198.9	−15.0	2.5	−28.2[b]	12[b]	−0.074
IBMX	412.7	256.9	155.8	−26.9	19	−30.1[b]	5.9[b]	−0.253
adenosine	433.1	116.9	316.1	−12.2	7			−0.023
NECA	485.5	196.4	289.1	−20.5	0.25	−46.0[b]	0.0086[b]	−0.095
av	404	151	253	15.3	36	−32	16	−0.066

[a] The $\Delta G_{(hydro)}$, $K_{D(hydro)}$, and $\Delta C_{p(hydro)}$ values were calculated according to the equations $\Delta G_{(hydro)} = RT \ln K_{D(hydro)} = 0.1046\ \Delta$SAHSA kJ mol^{-1} and $\Delta C_{p(hydro)} = 0.58$ SAPSA − 1.34 SAHSA J mol^{-1} K^{-1}. Experimental $\Delta G°$ and K_D values and averages are reported as terms of comparison. [b] Data obtained from ref 10.

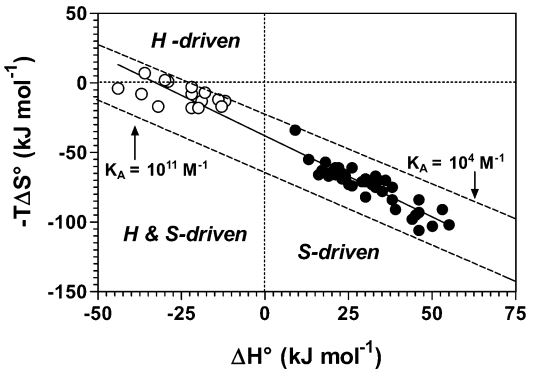

Figure 6. $-T\Delta S°$ versus $\Delta H°$ (kJ mol^{-1}; $T = 298.15$ K) scatter plot for adenosine A_1 receptor agonists (full circles) and antagonists (open circles). All points lie on the same regression line of the equation $-T\Delta S$ (kJ mol^{-1}) $= -1.16 \pm 0.02\ \Delta H°$ (kJ mol^{-1}) $- 38.0 \pm 1.1$ kJ mol^{-1} ($n = 54$, $R = 0.975$, $P < 0.0001$). The two dashed lines indicate the loci of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values which give rise to two different association constants ($K_A = 10^4$ M^{-1} and 10^{11} M^{-1}.

The main problem remains, however, to explain why membrane receptor binding occurs without significant changes of ΔC_p, while it could be imagined that disruption of the water cage of the ligand should be always been associated with some hydrophobic decrease of heat capacity. This indicates that some ΔC_p compensation mechanism must be acting, for which several valid reasons can be suggested. To simplify the discussion, it is reasonable to focus the attention on only two receptorial systems, one of cytoplasmic and the other of membrane type, whose molecular structures are reasonably known. This choice has been already done above with the steroid/nuclear superfamily of receptors (Figure 5), while for membrane receptors we have decided to focus on the adenosine A_1 subtype. This is a G-protein coupled receptor (GPCR) characterized by a structure with seven α-helical transmembrane domains connected by intra- and extracellular loops,[76] for which several simulation computer models, based on the bacteriorhodopsine structure,[77] are known.[78] A further advantage of this receptor is that its binding site is known to be located in the inter-α-helix region and not in the peripheral loops.[79,80]

The possible reasons for which $\Delta C_p°$ is so small for membrane receptors can be summarized as follows: (a) the ligand is more hydrophilic than hydrophobic; (b) the ligand binding site includes several water molecules;[10] (c) the ligand has access to a large vibrational space in its interhelix binding site; and finally, (d) while in cytoplasmic receptors the binding is associated with an often relevant tightening of the protein structure (inducing a large decrease of its $\Delta C_{p\ (vib)}$), no conformational changes of this type can occur in this multichain type of membrane receptor to avoid perturbation of the hydrophobic interactions between the membrane and the receptor itself. Discrimination among these different hypotheses is not a simple matter. A check of vibrational hypotheses, c and d, could probably be achieved by accurate and complex molecular dynamics simulations. What can be easily done here is to check hypothesis a by computing solvent accessible surface areas for a number of representative agonists and antagonists at the adenosine A_1 receptor (Table 5). It is evident that the hydrophobic part of these molecules is rather small with respect to the polar (or hydrophilic) part. This leads to $\Delta G_{(hydro)}$ values which are remarkably smaller (or $K_{D(hydro)}$ values remarkably higher) than the experimental ones. In a similar way, the $\Delta C_{p(hydro)}$ contributions turn out to be remarkably decreased with respect to those computed for the steroid receptors in Table 4. This seems to indicate that hydrophobic forces may be far less relevant for membrane receptors, at least for the adenosine A_1 case, than for cytoplasmic ones.

This final statement can be considered with some caution, however, because there is a very large number of membrane receptor ligands, which have relevant hydrophobic surface areas and will, accordingly, display relevant hydrophobic contributions to the binding. What is certainly true, however, is that hydrophilic molecules can equally bind to the membrane adenosine A_1 receptor, at variance with what is known to occur for all the steroid /nuclear ones.

Experimental Section

[^{3}H]17β-Estradiol (specific activity 96 Ci/mmol), [^{3}H]ORG 2058 (specific activity 52 Ci/mmol), and [^{3}H]R1881 (specific activity 84.5 Ci/mmol) were obtained from NEN Research Products (Boston, MA). Diethylstilbestrol, ORG 2058, R1881, and dithiothreitol were obtained from Sigma Chemicals (St. Louis, MO). All other chemicals were obtained from standard sources.

Preparation of Tissues. Healthy human tissues of uterus and prostate were provided by the Obstetric-Gynaecologic Clinic, University of Ferrara, and by the Urology Department of the S. Anna Hospital, Ferrara (Italy). Connective tissue was removed by dissection, and the freshly collected uterus and prostate were washed in cold isotonic saline solution, promptly cut into small pieces, and quickly frozen in liquid nitrogen. These tissues were transported on solid CO_2 to the laboratory and stored at −70 °C until analysis.

Preparation of Cytosol. The frozen pieces of uterine and prostate tissues were pulverized in a dismembrator (Braun, Melsungen, Germany) precooled in liquid nitrogen. The final

powder was suspended in five volumes of ice cold buffer 10 mM K_2HPO_4/KH_2PO_4, 1.5 mM EDTA, 5 mM dithiothreitol, 3 mM NaN_3, glycerol 10% W/W, 10 mM Na_2MoO_4; pH 7.4. The sample was centrifuged (Beckman L8-50 M/E) at 100000*g* for 20 min. The temperature was maintained at 2 °C throughout the centrifugation. The protein concentration in each cytosol was determined according to a Bio-Rad method[81] with bovine albumine as reference standard.

Dextran-Coated Charcoal (DCC) Assays: Saturation Studies. Aliquots of cytosol containing 200 μg of proteins were incubated in 200 μL of the phosphate buffer described above with 8 to 10 different concentrations of radiolabeled estradiol and ORG 2058 (for uterine cytosol) or R1881 (for prostate cytosol) ranging from 0.1 to 50 nM. Nine different incubation temperatures were chosen: 0, 5, 10,15, 20, 25, 30, 35, and 37. The experiments were carried out in a thermostatic bath ensuring a temperature of ±0.1 °C, and incubation times were variable temperature-depending (from 20 min at 37 °C to 5 h at 0 °C) according to the results of previous time-course experiments. All buffer solutions were adjusted to maintain a constant pH of 7.4 at the desired temperature. Nonspecific binding (always lower than 15% of total binding) was defined in the presence of 10 μM diethylstilbestrol (for radiolabeled estradiol), or 10 μM unlabeled ORG 2058 and R1881. Separation of bound from free radiolabeled ligand was obtained by DCC adsorption. DCC suspensions were prepared according to the procedure of Paganetto et al.[82] with minor modifications. Dry, washed charcoal (0.5%) was suspended in a solution containing 0.05% dextran and phosphate buffer pH 7.4. Samples were treated for 15 min with 100 μL of dextran coated charcoal and centrifuged at 1000*g* for 20 min at 4 °C in a Beckman JS centrifuge. Supernatant fluid was removed (100 μL) and mixed with 4 mL of Atomlight scintillation fluid (NEN Research Products, Boston, MA) and counted for radioactivity determination in a LS 1800 Beckman scintillation counter.

Determination of Water Accessible Surface Area. Solvent accessible surface areas (SASA), hydrophobic solvent accessible surface areas (SAHSA), and polar solvent accessible surface area (SAPSA) have been obtained using the MOE suite of programs (Chemical Computing Group Inc. MOE 2003.2. 1997−2003).

Calculations: Affinity Constant Determination. For a generic binding equilibrium

$$L + R \rightleftarrows LR \quad (L = \text{ligand}, R = \text{receptor})$$

affinity constants are calculated as $K_A = [LR]/([L][R]) = [LR]/([L_{max} - LR][B_{max} - LR]) = 1/K_D$, where $[L_{max}]$ = total concentration of the ligand added, $[B_{max}]$ = total concentration of the binding sites, and K_D = dissociation constant. Since $[LR]/[L_{max} - LR] = [\text{bound/free}] = [B_{max}]K_A - K_A[\text{bound}]$, the K_A and the B_{max} values can be obtained from the slope and the intercept of the plot [bound/free] versus [bound] (Scatchard plot).[83]

Calculations: Thermodynamic Parameters Determination. Equilibrium thermodynamic parameters were calculated by two different methods, methods A and B.

Method A. The observed ΔG values ($\Delta G = -RT \ln K_A$) are fitted by the quadratic expression[45,84]

$$\Delta G = A + BT + CT^2$$

It can be shown that

$$\Delta H = \left(\frac{\delta \frac{\Delta G}{T}}{\delta \frac{1}{T}}\right)_p = A - CT^2 \qquad \Delta S = -\left(\frac{\delta \Delta G}{\delta T}\right)_p = -B - 2CT$$

$$\Delta C_p = \left(\frac{\delta \Delta H}{\delta T}\right)_p = -2CT$$

(ΔC_p = equilibrium heat capacity difference)

Because experimental measurements are performed in a narrow range around $T° = 298.15$ K, equilibrium standard parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ are better obtained by interpolation in this range,[79] i.e.,

$$\Delta G° = A' + B'(T - T°) + C'(T - T°)^2$$

for which

$$\Delta G° = A' \qquad \Delta H° = A' - B'T° \qquad \Delta S° = -B'$$

$$\Delta C_p{}^o = -2C'T°$$

The condition $C = C' = 0$ (i.e., $\Delta C_p{}^o = 0$) corresponds to the case of a linear van't Hoff plot for which $\Delta H = \Delta H°$ and $\Delta S = \Delta S°$ at all temperatures. This condition is considered to be verified whenever the second-order C' coefficient is statistically not significant.

The ΔC_p and ΔS values resulting from hydrophobic ($\Delta C_{p(H)}$; $\Delta S_{(H)}$) or vibrational effects ($\Delta C_{p(V)}$; $\Delta S_{(V)}$) can be obtained as suggested by Sturtevant[52] according to the equations

$$\Delta C_p{}^o = \Delta C_{p(H)}{}^o + \Delta C_{p(V)}{}^o \qquad \Delta C_{p(H)}{}^o = \frac{1.05\Delta C_p{}^o - \Delta S_u{}^o}{1.31}$$

$$\Delta S_u{}^o = \Delta S^o - \Delta S_{CR}{}^o$$

$$\Delta S_{CR}{}^o = R \ln \frac{1}{55.6} = -0.0334 \text{ kJ K}^{-1} \text{ mol}^{-1}$$

$\Delta S_u{}^o$ is the change of unitary entropy, i.e., the standard entropy corrected for the cratic entropy $\Delta S_{CR}{}^o$. This correction is necessary for the very diluted concentrations of the ligands employed in the experimental conditions.[56]

$$\Delta S_u{}^o = \Delta S_{u(H)}{}^o + \Delta S_{u(V)}{}^o \qquad \Delta S_{u(H)}{}^o = -0.26\Delta C_{p(H)}{}^o$$

$$\Delta S_{u(V)}{}^o = 1.05\Delta C_{p(V)}{}^o$$

The contribution of the hydrophobic effect to affinity (K_D) was obtained as $K_{D(hydro)}$ from the corresponding ΔG, according to $\Delta G_{(hydro)} = RT \ln K_{D(hydro)}$, where $\Delta G_{(hydro)} = \gamma\,\Delta$SAHSA, with $\gamma = 0.1046$ kJ mol^{-1} Å^{-2} [57,58] and ΔSAHSA is taken as the SAHSA values calculated for each ligand as described above.

SAHSA and SAPSA values have also been employed to calculate the $\Delta C_{p(hydro)}$ values, as reported by Spolar and Record,[61] according to the expression

$$\Delta C_{p(hydro)} = 0.00058 \text{ SAPSA} - 0.00134 \text{ SAHSA}$$

(kJ mol^{-1} K^{-1})

Method B. $\Delta C_p{}^o$ is assumed a priori to be essentially zero. This situation is not common in reactions involving biomacromolecules in solution but has been frequently recognized for the binding of drugs to membrane receptors.[51] In this case the van't Hoff equation $\ln K_A = -\Delta H°/RT + \Delta S°/R$ gives a linear plot $\ln K_A$ versus $1/T$. The standard free energy can be calculated from the van't Hoff plot $\ln K_A$ versus $1/T$ as $\Delta G° = -RT \ln K_A$ at 298.15 K, the standard enthalpy from the slope, $-\Delta H°/R$, and the standard entropy from the intercept, $\Delta S°/R$, or as $(\Delta H° - \Delta G°)/T$, with $T = 298.15$ K and $R = 8.314$ J K^{-1} mol^{-1}.

Role of Dithiothreitol and Sodium Molybdate. The binding of hormones to steroid/nuclear receptors induces a conformational change resulting in a dissociation of the heat-shock protein complex which allows dimerization of the receptor and DNA binding to a hormone response element to produce a transcriptionally productive complex.[55] Since these processes are consequent to the steroid receptor activation, it is important to hinder them during the thermodynamic measurements. The presence of dithiothreitol and Na_2MoO_4 in the reaction mixture makes it possible to perform measurements strictly related to the drug−receptor interactions, excluding the processes of dissociation of heat shock proteins, dimerization, and binding to DNA. Indeed, dithiothreitol and

Na_2MoO_4 allow one to prevent the thermolability of steroid receptors.[45,86,87]

Acknowledgment. The authors thank Prof. Adriano Piffanelli (Dipartimento di Medicina Clinica e Sperimentale, Sezione di Medicina Nucleare, Corso Giovecca 203, Ferrara, Italy) for providing the uterine and prostate tissues and the methodology for their treatment.

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JM040842Z