Molecular Basis of the Interaction Specificity between the Human Glucocorticoid Receptor and Its Endogenous Steroid Ligand Cortisol

Johannes von Langen,[c] Karl-Heinrich Fritzemeier,[d] Stephan Diekmann,[c] and Alexander Hillisch*[a, b]

We analyzed the binding of five steroids to the human glucocorticoid receptor (hGR) experimentally as well as theoretically. In vitro, we measured the binding affinity of aldosterone, cortisol, estradiol, progesterone, and testosterone to hGR in competition with the ligand dexamethasone. The binding affinity relative to the endogenous ligand cortisol (100%) is reduced for progesterone (22%) and aldosterone (20%) and is very weak for testosterone (1.5%) and estradiol (0.2%). In parallel, we constructed a homology model of the hGR ligand-binding domain (LBD) based on the crystal structure of the human progesterone receptor (hPR). After docking the five steroids into the hGR model ligand-binding pocket, we performed five separate 4-ns molecular dynamics (MD) simulations with these complexes in order to study the complex structures. We calculated the binding affinities with two different approaches (MM/PBSA, FlexX) and compared them with the values of the experimentally determined relative binding affinities. Both theoretical methods allowed discrimination between strongly and weakly binding ligands and recognition of cortisol as the endogenous ligand of the hGR in silico. Cortisol binds most strongly due to a nearly perfect steric and electrostatic complementarity with the hGR binding pocket. Chemically similar ligands such as estradiol, testosterone, and progesterone also fit into the hGR binding pocket, but they are unable to form all those contacts with the amino acids of the protein that are necessary to yield a stable, transcriptionally active receptor conformation. Our analysis thus explains the selectivity of the human glucocorticoid receptor for its endogenous ligand cortisol at a molecular level.

Introduction

The glucocorticoid receptor (GR) is a hormone-activated transcription factor known to regulate, either directly or indirectly, target genes involved in glucose homeostasis, bone turnover, cell differentiation, lung maturation, and inflammation.[1] It is therefore widely recognized as a therapeutically important target. The transcriptional regulation of the GR is mediated by hormone binding, which leads to receptor dimerization and coactivator recruitment. GR ligands are commonly used to treat diverse medical conditions such as asthma, allergic rhinitis, rheumatoid arthritis, and leukemia.[2] However, clinical use is limited due to a number of side effects ranging from increased bone loss and growth retardation to suppression of the hypothalamic–pituitary–adrenal axis. Discovery of GR agonists without the undesired side effects is the subject of intense research efforts.[3]

The glucocorticoid receptor is a member of a family of steroid/nuclear receptors (NR). They are eukaryotic transcription factors and regulate gene expression in response to binding of small hydrophobic ligands.[4–6] In humans, the NR superfamily includes 48 proteins,[7] with the estrogen receptors α and β and the glucocorticoid (GR), mineralocorticoid (MR), progesterone (PR), and androgen (AR) receptors forming a single family. Phylogenetic analysis and sequence alignments show that, within the steroid receptors, the GR, MR, PR, and AR form a subfamily of oxosteroid receptors that is distinct from the estrogen receptor (ER) subfamily.[8]

The GR is a modular protein that is organized into three major domains: an N-terminal activation function-1 domain (AF-1), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The ability of a nuclear receptor to activate specific gene transcription requires the binding of cognate ligands to their LBDs[9] and binding of the steroid actively modulates the structure of the receptor into a DNA-binding transcriptionally active complex.[10–12] The steroid is thus an integral part of the transcriptionally active receptor/ligand complex and is completely buried within the fold of the LBD. In the absence of ligand, the GR is retained in the cytoplasm by association with chaperone proteins such as hsp90 and p23, which bind to the LBD.[13] In the nucleus, the GR binds to DNA promoter elements and can either activate or repress

[a] *Dr. A. Hillisch*
EnTec GmbH
Adolf-Reichwein-Strasse 20, 07745 Jena (Germany)
E-mail: alexander.hillisch@bayerhealthcare.com

[b] *Dr. A. Hillisch*
Current address: Bayer HealthCare AG
Apratherweg 18a, 42096 Wuppertal (Germany)
Fax: (+49) 202-368149

[c] *Dr. J. von Langen, Prof. Dr. S. Diekmann*
Institute for Molecular Biotechnology
Beutenbergstrasse 11, 07745 Jena (Germany)

[d] *Dr. K.-H. Fritzemeier*
Schering AG
Müllerstrasse 178, 13342 Berlin (Germany)

Supporting Information for this article is available on the WWW under http://www.chembiochem.org or from the author.

  DOI: 10.1002/cbic.200400361 ChemBioChem **2005**, 6, 1110 – 1118

transcription depending on the context of the target promoters.

For almost all members of the steroid-receptor superfamily, the DBDs and LBDs are relatively well conserved.[14–16] The receptors of the GR subfamily (AR, GR, MR, PR) have overlapping steroid-binding specificity and they bind to the same steroid-responsive elements in target genes.[15,17,18] The mineralocorticoid receptor, for example, recognizes mineralocorticoids and most glucocorticoids with high affinity, while the glucocorticoid receptor only binds glucocorticoids with high affinity.[15,18–20]

NR LBD crystal structures[10,12,21,22] reveal that the LBDs fold into a canonical three-layer helical sandwich that embeds a hydrophobic pocket for ligand binding. They also highlight the importance of the C-terminal helix 12 in ligand-dependent regulation. Agonist binding induces a conformational change of the C-terminal activation function AF-2 (helix 12),[12,23,24] thereby stabilizing the receptor in an active conformation in order to facilitate its association with coactivator proteins, such as steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor 2 (TIF2).[20,25,26] Given its biological and pharmaceutical importance, the GR LBD structure is of particular interest. It was determined in complex with dexamethasone and a coactivator motif derived from TIF[27] and in complex with Mifepristone (RU486).[20] The structure shows a distinct steroid-binding pocket with features that explain dexamethasone binding in detail.

However, the molecular basis of how GR distinguishes between cortisol and other important steroid hormones has not previously been understood in detail. Aldosterone, progesterone, and testosterone in particular are based on the same steroid skeleton as cortisol, while the estradiol steroid skeleton is similar.

In this work, we measured and theoretically studied the relative binding affinity of aldosterone, cortisol, estradiol, progesterone, and testosterone for the GR. In order to explain the experimental binding data, we developed a model of the human glucocorticoid receptor (hGR). This receptor-protein model was then used to study the interactions between the hGR and its endogenous ligand cortisol, as well as the interactions with the other four steroids, by using various theoretical methods.

Results and Discussion

Relative-binding-affinity measurements

The relative binding affinities (RBA) of estradiol, progesterone, testosterone, aldosterone, and cortisol for the GR were measured in competition experiments. [^{3}H]-dexamethasone was used as a radioligand for the competition measurements. The results are normalized with respect to cortisol, which is set to 100% binding. As expected, cortisol shows the highest binding affinity followed by progesterone (22.0%), aldosterone (20.2%), testosterone (1.5%), and estradiol (0.2%; see Table 1). RBA values for these ligands had previously been determined by others for the GR from rat thymus, liver, and hepatoma tissue cells (HTC) in culture[28] and from transiently transfected CV1 cells.[29] The RBA values depend on the experimental conditions, as well as the tissues/cells used in the studies.[28] However, the relative rankings agree well in most studies with our data shown here (Table 1).

Table 1. Experimental and calculated receptor-binding data.

Ligand	Experimental RBA[a] [%]	MM/PBSA[b] ΔG_b [kcal mol^{-1}]	ΔG_{MM} [kcal mol^{-1}]	ΔG_{so} [kcal mol^{-1}]	FlexX initial[c]	FlexX mean[c]
aldosterone	20.2	−31.1 ± 3.7	−82.0	50.9	−21.0	−23.9
cortisol	100.0	−35.0 ± 4.2	−96.2	61.2	−11.8	−26.2
estradiol	0.2	−30.0 ± 3.6	−66.1	36.0	−13.4	−19.3
progesterone	22.0	−28.2 ± 4.0	−65.3	37.1	−5.0	−4.8
testosterone	1.5	−27.6 ± 3.7	−62.6	35.1	−9.0	−4.7

[a] Experimental RBA: experimentally determined relative binding affinity (given as a percentage) in relation to the binding of cortisol to the hGR (100%). [b] MM/PBSA: $\Delta G_b = \Delta G_{MM} + \Delta G_{sol} - T\Delta S$; ΔG_b = calculated binding free energy. ΔG_{MM} and ΔG_{sol} are the force-field interaction and solvation terms for ΔG_b. $T\Delta S$ is not included (see text). [c] FlexX scores obtained by docking the five ligands into the inital homology model (FlexX initial) and the mean structure (FlexX mean) of the hGR LBD simulated by molecular dynamics over 4 ns.

In order to interpret the experimentally determined binding affinities on a molecular basis, molecular dynamics simulations were performed, by using a homology model of the hGR-LBD/ligand complexes.

Initial homology model of the hGR LBD in complex with cortisol

Since there is a 54% sequence identity between the hPR and hGR, a homology model of the hGR LBD was built based on the X-ray crystal structure of the hPR LBD.[16] After energy minimization including an explicit solvent environment (see the Experimental Section), the protein structure was analyzed with regard to conformational parameters. More than 97% of the residues in the Ramachandran plot are in the most favored or allowed regions and 100% show stereochemical correctness when checked with the PROCHECK program.[30] These results suggest that the obtained initial hGR model is of good quality and is suitable for further studies regarding details of receptor/ligand interactions. Indeed, the comparison of this model with the later-solved X-ray crystal structure of hGR in complex with dexamethasone[27] reveals an RMSD value for the backbone atoms of 2.1 Å. A comparison between the initial homology model and the X-ray crystal structure is displayed in Figure 1a. Our hGR model and the hGR X-ray crystal structure are very similar, especially in the regions of helices 1, 3, 5, 6, 8, and 9. Differences occur mainly at the loop regions in the outer parts,

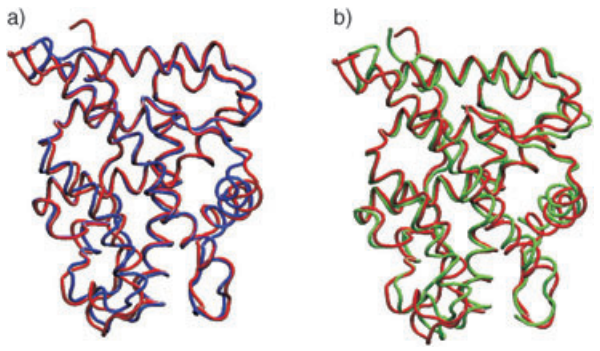

Figure 1. Comparison between the experimental and the simulated models of the hGR. Superposition of the X-ray crystal structure (in red) with a) the initial homology model (in blue) and b) the final model (in green), which was calculated by averaging the MD simulation.

which are responsible for dimerization, but there are also differences in helix 12. The higher degree of variation within the loop regions is probably due to simulating a monomer instead of a dimer. This leads to higher flexibility in these areas due to the lack of stabilization by the second monomer.

hGR-LBD/cortisol interactions in the initial homology model

In the initial hGR structure model (only energy minimization, no MD), most of the functional groups of cortisol are involved in hydrogen bonds. Only the hydroxy group at the 17α position remains unbound and Gln642 is in a rotameric state that differs from the X-ray crystal structure. The energy-minimized complex shows a pronounced hydrogen-bonding pattern around the A ring, involving Arg611 and Gln570 in hydrogen bonds with O3 of cortisol. A central water molecule is thereby bound to Gln570 and the backbone carbonyl oxygen atom of Met605 and it also accepts a hydrogen bond from Arg611. Thus, this water molecule positions Arg611 and Gln570 ideally in order to form further hydrogen bonds with the carbonyl oxygen atom O3 of the steroid. Direct hydrogen-bond contacts of this water molecule with O3 are not observed. This observation is in agreement with the hydrogen-bonding pattern found in X-ray crystal structures for both the hPR[16] and hGR.[27] Further hydrogen-bond contacts between the ligand and receptor are observed for the hydroxy functional groups at the 11β and 21 positions of cortisol with Asn564.

Docking of steroids to the initial homology model

We then asked whether the initial homology hGR model is accurate enough to reflect the specific binding affinity of cortisol in comparison to other steroid hormones. A selection of five different steroids (aldosterone, cortisol, estradiol, progesterone, and testosterone) were docked into the binding pocket of the model by using the FlexX program.[31] The results of the docking studies are summarized in Table 1 (FlexX initial), with the FlexX scores listed.

All the steroids are correctly placed in the binding pocket in an orientation similar to cortisol, having the gonane skeleton properly superimposed. However, the FlexX scores do not reflect the measured relative binding affinities (see Table 1). In this conformation of the protein, a hydrogen-bonding partner for the 17α-hydroxy group of cortisol is missing, since Gln642 is placed outside the binding pocket. Thus, the polar 17α-hydroxy group of cortisol is placed in a mainly lipophilic environment, thereby reducing the lipophilic-contact surface area. In contrast to the experiments, aldosterone was identified as the ligand with the highest binding affinity (−21.0; see Table 1). The reason is that this steroid lacks the 17α-hydroxy group. Even estradiol (−13.4) showed a more favorable score than cortisol (−11.8). This indicates that the initial model of the hGR LBD is not accurate enough to explain the experimental binding-affinity data correctly.

Molecular dynamics simulations of the hGR/cortisol complex

In order to obtain a better understanding of the energetic contributions to the binding, the initial model of the hGR/cortisol complex was subjected to a molecular dynamics simulation including 7925 water molecules and periodic boundary conditions. The trajectory was subsequently analyzed by using the MM/PBSA method.[32] This approach considers the contribution of the electrostatic and deformation energy terms as well as the desolvation energy.

The simulation can be regarded as a stable trajectory. When the initial model and the model obtained by energy minimizing are compared, the averaged structure of the MD simulation shows important differences in hormone binding (Figure 2). In comparison to the initial model, the MD simulation model allowed the formation of additional tight hydrogen bonds between cortisol and the hGR. In particular, Gln642 changed its conformation and built a stable hydrogen bond with the 17α-hydroxy group of cortisol. This interaction was observed in 99% of the snapshots taken during the MD simulation (as shown in Figure 3a). Figure 3 displays the frequency of hydrogen bonds formed during the MD simulation by stating the percentage of connections measured by analyzing the trajectory. Further particularly strong interactions were observed for the 11β-hydroxy function binding to the carbonyl oxygen atom of Asn564. Less frequent hydrogen-bond contacts were observed for the cortisol 21-hydroxy group with Asn564 (64% of snapshots) and the carbonyl O3 atom with Arg611 (47%). Weak hydrogen bonds between the steroid and the protein were formed between O20/21 and Thr739. These data are consistent with interactions observed in the X-ray crystal structure of dexamethasone and the hGR (Figure 4),[20,27] simulations of cortisol with other homology models,[33,34] and site-directed mutagenesis experiments.[35] Neither the X-ray crystal structures nor our model show direct hydrogen-bond contacts of Cys736 to the ligand. This observation is supported by mutagenesis experiments.[36]

The MD simulation also allowed an assignment of the hydrogen-bonding pattern around the A ring of cortisol, including a central water molecule. A conserved single structural water molecule was also found in the hPR, human-androgen-receptor (hAR), and hGR structures. Since X-ray crystallography fails to detect hydrogen atoms, the pattern of these atoms had to be

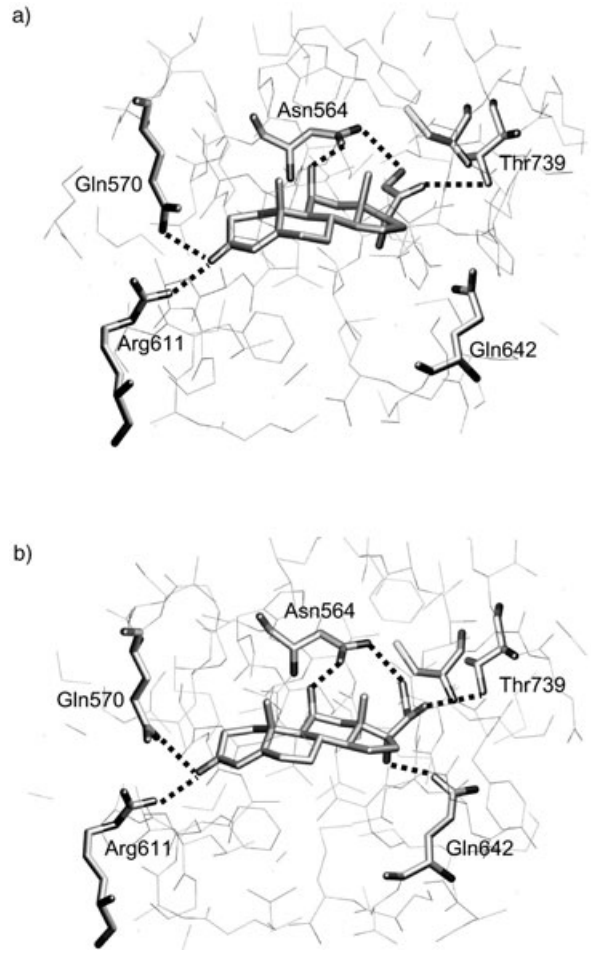

Figure 2. Comparison of binding modes of cortisol in a) the initial hGR model and b) the averaged structure of the MD simulation. Dotted lines have been inserted afterwards to indicate potential hydrogen bonds with regard to general hydrogen-bonding parameters. Hydrogens have been removed and further structural elements—covering a distance of 8 Å around the ligand—are shown as thin lines for the sake of clarity.

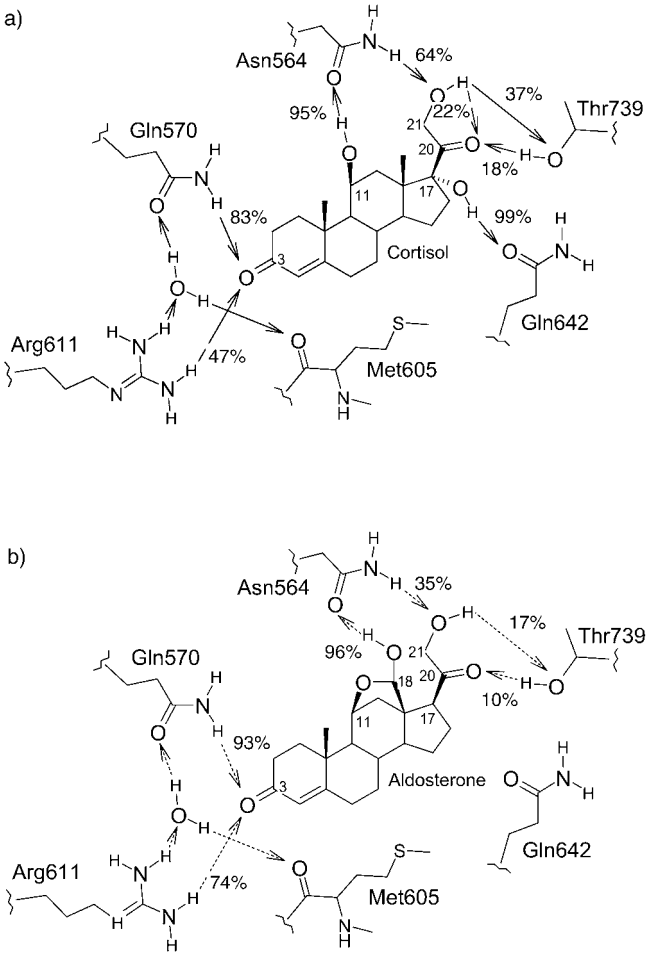

Figure 3. Schematic representation of the interaction of a) cortisol and b) aldosterone with selected amino acids in the hGR ligand-binding pocket. Analysis of the MD simulation gives an estimate of the frequency of the hydrogen bonds formed (as a percentage) during the entire simulation. Arrows demonstrate the direction of binding force from donor to acceptor. The protein backbone atoms have been removed for the sake of clarity.

modeled.[37] The central water molecule, binding to Gln570 and the backbone of Met605, seems to be crucial for stabilizing the interaction of Gln570 and Arg611 with the carbonyl O3 group of cortisol. Interestingly, throughout the MD simulation we observed that this water molecule exchanged several times with other water molecules residing in a cavity near Arg611 and Gln570.

Molecular dynamics simulations of the hGR/steroid complexes

The MD simulations of hGR with aldosterone, estradiol, progesterone and testosterone were performed identically to that for the hGR/cortisol complex. All simulations resulted in stable trajectories. The hGR/aldosterone complex is shown in Figure 3b as an example of a medium-strength-binding hGR ligand. The aldosterone A ring forms the same hydrogen bonds as cortisol. The 18β-hydroxy function of aldosterone forms a hydrogen bond to Asn564 similar to that of the 11β-hydroxy group of cortisol. This influences the hydrogen-bond frequency of the

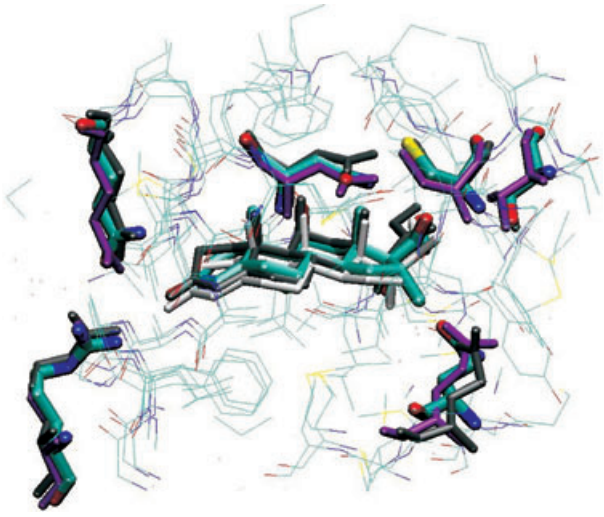

Figure 4. Comparison of experimentally determined and predicted binding modes of hGR ligands. The X-ray crystal structure of the hGR in complex with dexamethasone is shown in light blue, the initial homology model is dark grey, and the averaged conformation of the MD simulation is shown in pink, with the ligand cortisol in white.

21-hydroxy group negatively. The missing 17α-hydroxy group contributes to the weaker interactions of aldosterone with the hGR. Hydrogen bonds to the 11-ether oxygen atom of aldosterone were not observed throughout the MD simulation.

MM/PBSA calculations for all five hGR complexes

Calculations of the binding free energy, ΔG_b, between hGR and the five different steroids were carried out by using the MM/PBSA method.[32] The results of these calculations are displayed in Table 1 together with the relative binding affinities measured in vitro. These calculations qualitatively allow us to discriminate between the strongly and the weakly binding compounds. Cortisol is correctly predicted by the calculation to be the ligand with the highest binding free energy for the hGR (-35.0 kcal mol^{-1}; see Table 1). However, the weakest binder estradiol is predicted by the MM/PBSA approach to have a higher binding free energy (-30.0 kcal mol^{-1}) than the second strongest binding ligand progesterone (-28.2 kcal mol^{-1}), which is not in agreement with the RBA data. The method is thus not able to correctly discriminate between several low-affinity ligands.

The MM/PBSA analysis allows the binding free energy to be split into a protein–ligand interaction and a solvation free energy part. The cortisol/hGR association is calculated to have the highest solvation free energy of all the reactions. However, due to seven hydrogen bonds between the ligand and protein, the interaction contributions overwhelm the desolvation penalty. In contrast, the much more lipophilic estradiol, testosterone, and progesterone complexes have a decreased solvation free energy but are unable to form the necessary interactions with the hGR (forming only four, three, and two stable hydrogen bonds, respectively); this results in a lower binding affinity.

Conformation changes with dependence on the ligand

We than compared the behavior of the entire hGR-LBD conformation in complex with the five steroids throughout the MD simulations. Each of the five ligands displayed a different influence on the global conformation of the protein. Figure 5 shows a representation of the root mean square fluctuation (RMSF) of the Cα atoms of the hGR LBD for each complexed steroid. This analysis allows rigid and flexible regions to be detected.

The calculations demonstrate a noticeable conformational flexibility of the protein when it is simulated with estradiol, progesterone, and testosterone. Although the overall structures of these hGR complexes remained close to the starting conformation, changes occurred in the loop between helices 11 and 12 and within helix 12. Helix 12 is known to play an important role in ligand binding to nuclear receptors.[12] The most stable structures were obtained from the simulations with cortisol and aldosterone (Figure 5). Obviously, the hydrogen-bonding network inferred by these two ligands in the binding pocket stabilizes the conformation of the entire protein. Cortisol, for example, stabilizes the interaction of helices 3 and 8 by forming strong hydrogen bonds with Asn564 and

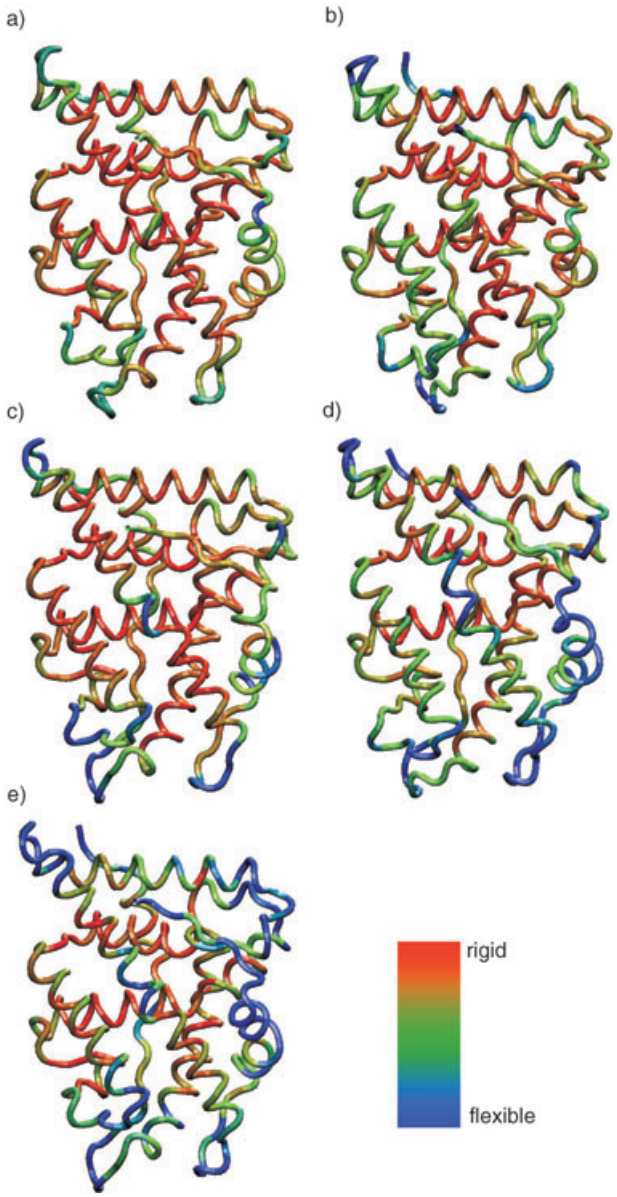

Figure 5. Flexibility of the hGR Cα-backbone atoms during the MD simulations with dependence on the ligand. hGR complexes with, a) aldosterone, b) cortisol, c) estradiol, d) progesterone, and e) testosterone. The models are colored according to the RMS deviations of the backbone atoms throughout the simulations. Red corresponds to RMSD values of 0–2 Å and shows regions of the structure that remained rigid during the simulations. Green indicates regions with RMS deviations of 2–3 Å, and blue areas indicate larger fluctuations with RMSD values of >3 Å.

Gln642. Asn564 contacts cortisol with two hydrogen bonds and forms an additional hydrogen bond with Glu748 in the loop between helix 11 and 12. This interaction pattern is only observed for cortisol and aldosterone and may be responsible for the higher stability of these complexes. Simulation of the hGR complexes with the other steroids (estradiol, progesterone, and testosterone) shows much higher fluctuations in these loop regions. We speculate that the stability of the conformation is correlated with the transcriptional potency of the steroids. This speculation is supported by comparison of other

glucocorticoid-receptor binding and transactivation data.[29] Steroids that lack the 11β-hydroxy functionality (that is, 11-deoxycorticosterone, 11-deoxycortisol, progesterone, and testosterone) show larger discrepancies between receptor binding and transactivation. (They show strong receptor binding but weak transactivation.) The conformational stability of the resulting complexes may be one reason for this discrepancy.

Observation of the plasticity of the binding pocket

Calculations of the volume of the binding pocket were carried out in order to determine the plasticity of the binding pocket with dependence on the different ligands. We find that the behavior of the binding pocket varies strongly among the different ligands (see Figure 6). When simulated with its endogenous ligand cortisol, the volume of the binding pocket stays within its starting dimensions, with a volume of 607 Å^3 and a variable plasticity of $\pm$44 Å^3. When compared with the remaining steroids in complex with the hGR, cortisol shows the best fit to the binding pocket by occupying 61% of its volume. When the complex is simulated with estradiol (the smallest ligand), the pocket collapses to a volume of (499$\pm$47) Å^3. When simulated with progesterone, the binding pocket adopts an unusually large space. Although progesterone (with 315 Å^3 of volume) is smaller than cortisol (with 338 Å^3), it forces the pocket to expand to a mean volume of (664$\pm$51) Å^3. This might be due to a lack of potential hydrogen-bonding partners, which would be required for contracting the binding pocket to form an optimal space around the bound steroid. The volumes of the pockets for aldosterone and testosterone are (598$\pm$48) Å^3 and (608$\pm$44) Å^3. In our studies the volume of the binding pocket is thus not correlated with the volume of the ligands. Ligands that are unable to form important contacts with the receptor lead to a collapse or an expansion of the binding pocket. This destabilizes the entire conformation and leads to larger fluctuations during the MD simulations, as discussed above.

Only cortisol and aldosterone resulted in stable MD trajectories with a low flexibility of the protein backbone and a constant volume of the binding pocket. This is in agreement with published transactivation data.[29]

Docking to the refined homology model

The averaged structure of the MD simulation was used for second docking experiments with the program FlexX. This was done in order to check if the conformational changes from the MD simulation lead to a structure that explains ligand recognition better than the initial homology model. Performing docking on the initial homology model resulted in FlexX scores that suggest aldosterone to be the strongest binding ligand of hGR (see Table 1, FlexX initial). Cortisol was not identified as the endogenous ligand of the hGR. By contrast, when the averaged structure from the MD simulation was used, the FlexX program was able to identify cortisol as the ligand showing the highest binding affinity($-$26.2; see Table 1, FlexX mean). The different abilities of the two hGR homology models to recognize cortisol is grounded in the fact that Gln642 points out of the binding pocket in the initial model (Figure 2) and the docking program FlexX is unable to identify a hydrogen bond between the 17α-hydroxy functional group of cortisol and Gln642. During the MD simulation, Gln642 flips into the binding pocket, thereby forming a hydrogen bond to the cortisol 17α-hydroxy group. When the FlexX program is run on the averaged structure of the MD simulation, a correct binding pose of cortisol, including a hydrogen bond to Gln642, is identified. Cortisol is now recognized as the ligand with the best score. In all five MD simulations, Gln642 was inside the pocket. Thus, even the complexes with the "wrong" ligands were better starting points than the initial homology model.

This example has some general implications for docking ligands to homology models. In our case, the MD simulations provided conformations of homology models that were better suited for predicting the correct ligands by using docking methods than the simple energy-minimized homology models. It might thus be advantageous to use homology models that have been simulated in complex with known ligands for, for example, (high-throughput) docking studies. Protein conformations that have been simulated with natural ligands or substrates might thus give biologically more meaningful results.

Conclusion

We determined the relative binding affinities of five chemically similar steroid ligands to the human glucocorticoid receptor (hGR) and obtained results in agreement with previous data.[35,36] In order to understand the stronger binding of the endogenous ligand cortisol as compared to the other steroid ligands on a molecular basis, we constructed a homology model of the hGR ligand-binding domain (LBD). The model was derived from an X-ray crystal structure of the human progesterone receptor (hPR) since hPR and hGR show a 54% sequence identity. The resulting model and the ligand-binding mode (obtained by manual docking of glucocorticoids to the model) are virtually identical to those observed in the complexed hGR X-ray crystal structure.[26] Our molecular dynamics simulations show that the hGR/cortisol and hGR/aldosterone complexes maintain their integrity during the entire simulation. This is in contrast with simulations of complexes with other steroids, which lead either to a collapse or an expansion of the ligand-binding pocket and an increased flexibility in certain regions of the protein structure. A detailed MM/PBSA analysis of the energetics of the binding process enabled us to identify cortisol as the strongest binding steroid and to discriminate between the strongest and the weaker binding ligands in silico. Although the other steroids, aldosterone, estradiol, testosterone, and progesterone, are characterized by lower desolvation penalties, cortisol binds most strongly due to a nearly perfect steric and electrostatic complementarity with the hGR binding pocket. Our analysis thus explains the selectivity of the hGR for cortisol on a molecular level. Chemically similar ligands such as estradiol, testosterone, and progesterone also fit into the hGR binding pocket, but they are unable to form all those contacts with the amino acids of the protein

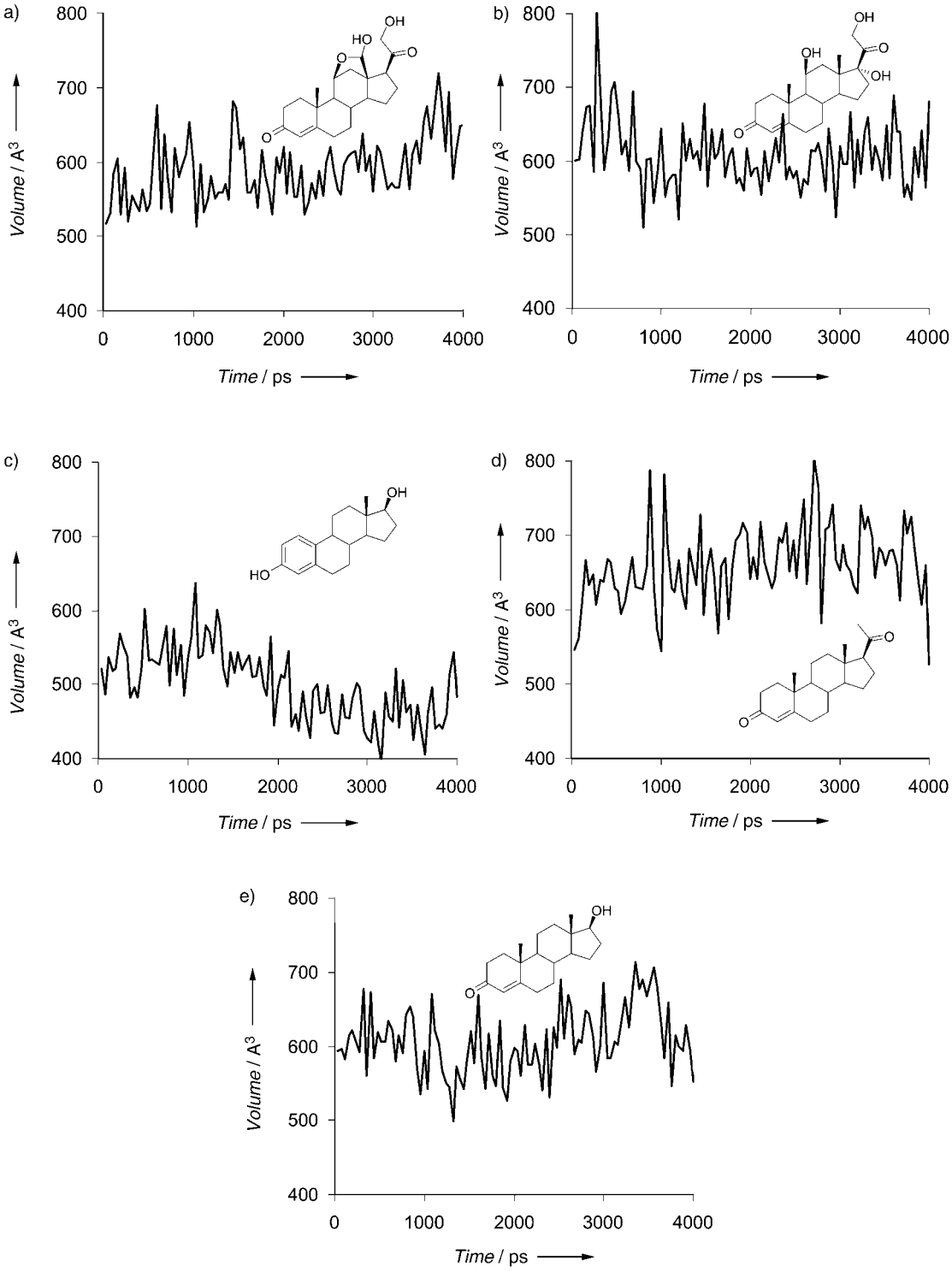

Figure 6. Plasticity of the hGR ligand-binding pocket recorded during MD simulations with five steroids: a) aldosterone, b) cortisol, c) estradiol, d) progesterone, and e) testosterone. The *x* axis shows the simulation time (up to 4 ns) and the *y* axis shows the volume of the binding pocket, measured in Å^3.

that are necessary to yield a stable, transcriptionally active receptor conformation. This approach provides an opportunity to estimate the binding affinity of even theoretical compounds to the glucocorticoid receptor, which should be helpful for the discovery of future, highly selective ligands.

Experimental Section

Glucocorticoid receptor binding: The binding affinity of the compounds to the hGR was measured by in vitro competition experiments with [^{3}H]-dexamethasone as the ligand and unlabeled dexamethasone as the reference. The study was performed essentially

as described in ref. [38]. In short, the hGR was isolated from SF-9 insect cells infected with a recombinant baculovirus coding for the human GR. Cells were harvested 2 days postinfection by centrifugation for 15 min at 600 *g*. Pellets were resuspended in 1/10th of the culture volume in homogenization buffer (20 mM tris(hydroxymethyl)aminomethane (Tris)/HCl at pH 7.5, 0.5 mM ethylenediaminetetraacetate (EDTA), 2 mM 1,4-dithiothreitol (DTT), 20% glycerol, 400 mM KCl, 20 mM sodium molybdate, 0.3 μM Aprotinin, 1 μM Pepstatin, and 10 μM Leupeptin (protease inhibitors were freshly added from a 100-fold ethanol stock solution just before the preparation)) and were shock frozen in liquid nitrogen. After 3 cycles of freezing and thawing, the homogenate was centrifuged for 1 h at 100,000 *g*. The protein concentration of the resulting supernatant was typically 5–7 mg mL^{-1} (determined by the Pierce bicinchoninic acid (BCA) method).

The binding affinity of the test compounds is given as relative binding affinity (RBA), which is defined as (IC_{50}(reference)/IC_{50}(test compound))×100 from the competition experiment. The IC_{50} value of dexamethasone is 10 nM. The results were normalized with respect to cortisol, arbitrarily set to 100% relative binding affinity. The mean deviation of the test method is 15%.

Dexamethasone[1,2,4,6,7-^{3}H] with a specific activity of 89 Ci mmol^{-1} (2.85 TBq mmol^{-1}) was obtained from Amersham/England, Charcoal Norit A was from Serva, and Dextran T70 was from Pharmacia. All other substances were commercially available in analytical grade.

Estradiol, testosterone, aldosterone, cortisol, and progesterone were provided by the Medicinal Chemistry Section of Schering AG, Berlin, Germany.

Comparative modeling: A homology model of the GR LBD was built based on the X-ray crystal structure of the PR in complex with progesterone.[16] The sequence alignment was taken from ref. [16] and shows a 54% sequence identity between the PR template structure and the GR target sequence. The model-building process was carried out with the program MODELLER (Version 4).[39] For evaluation of this initial homology model, the structure was checked by using the programs PROCHECK[30] and WhatCheck,[40] to spot faults in backbone folding and clashes between side chains. The resulting model was energy minimized by using the AMBER program (see below).

The positioning of the five different ligands within the binding pocket of the hGR starting structure was taken from the position of progesterone in the human progesterone receptor (hPR)[16] by fitting the model of the hGR onto the X-ray crystal structure of the hPR and copying progesterone into the hGR structure. The remaining hGR-receptor/ligand combinations were built by replacing the progesterone with the corresponding new ligand after superpositioning of the steroid scaffold.

Force field simulations:*Molecular dynamics simulations*: Molecular dynamics (MD) simulations were performed by using the AMBER software package.[41] The energy-minimum conformations of the five steroids were obtained by using ab initio calculations and manually docked into the binding cavities. The complexes were protonated, neutralized, solvated (with approximately 8000 water molecules), and simulated in a box of water. Long-range electrostatic interactions were dealt with by using the Particle Mesh Ewald method (PME). A detailed description of the method used is provided in the Supporting Information.

Analysis of protein flexibility during the MD simulations: The analyses of the fluctuations of atoms about their mean position were performed by using the CARNAL module in the AMBER software package. Each conformation of the protein recorded as a snapshot was superpositioned on to the averaged structure of the MD simulation. Root mean square deviation (RMSD) values were calculated from the distance change between each Cα atom of the mean structure and the actual conformation of the protein. RMSD values were averaged over all snapshots.

Molecular mechanics (MM)/Poisson–Boltzmann solvation area (PBSA) method: The overall binding free energy consists of three major parts and is calculated as the sum of the interaction energies and the solvation free energies of the components as well as the conformational entropy contribution to the binding. A detailed description of the single terms contributing to the overall binding free energy is provided in the Supporting Information.

Docking: The FlexX program (version 1.8)[31] implemented with the SYBYL 6.7 software[42] was used to perform the docking experiments with the hGR. Docking of steroidal compounds was performed with two receptor conformations. The "initial structure" refers to the conformation of the GR/cortisol complex obtained from the MODELLER program after solvation with TIP3P water molecules and full energy minimization with the AMBER force field. The "averaged structure" refers to the conformation of the GR/cortisol complex averaged over the last two nanoseconds of the MD simulation. All water molecules in the averaged structure were removed, and a short, steepest descent energy minimization was performed to relax any existing clashes. This was necessary due to the fluctuations of the water molecules, which made it impossible to include them during the averaging process. Subsequently this structure was solvated in a box of TIP3P water molecules and minimized by using the conjugated gradient method until the energy difference was below 0.01 kcal mol^{-1}.

For the docking procedure, all water molecules, ions, and protons were removed and a 6-Å sphere around the binding site was used as the template structure. Removal of the water molecules and ions prevented steric and volumetric irritation of the docking process, thereby allowing a broader variety of conformations to be considered. All default parameters, as implemented in the SYBYL 6.7 software, were used. The FlexX scoring function was used to estimated binding free energies with the FlexX program.[43]

Acknowledgements

We thank the Bundesministerium für Bildung und Forschung (BMBF) for support (EXIST HighTEPP), J. Sühnel and M. Zacharias for helpful discussions, and F. Haubensak and G. Flossmann for technical assistance.

Keywords: binding affinity · cortisol · molecular dynamics · receptors · steroids

[1] H. M. Reichardt, F. Tronche, S. Berger, C. Kellendonk, G. Schutz, *Adv. Pharmacol.* **2000**, *47*, 1–21.
[2] P. J. Barnes, *Clin. Sci.* **1998**, *94*, 557–572.
[3] H. Schacke, A. Schottelius, W. D. Docke, P. Strehlke, S. Jaroch, N. Schmees, H. Rehwinkel, H. Hennekes, K. Asadullah, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 227–232.
[4] R. M. Evans, *Science* **1988**, *240*, 889–895.
[5] M. J. Tsai, B. W. O'Malley, *Annu. Rev. Biochem.* **1994**, *63*, 451–486.
[6] R. C. Ribeiro, P. J. Kushner, J. D. Baxter, *Ann. Pharm. Belg. Ann. Rev. Med.* **1995**, *46*, 443–453.
[7] T. M. Willson, S. A. Jones, J. T. Moore, S. A. Kliewer, *Med. Res. Rev.* **2001**, *21*, 513–522.

[8] V. Laudet, C. Hanni, J. Coll, F. Catzeflis, D. Stehelin, *EMBO J.* **1992**, *11*, 1003 – 1013.
[9] J. M. Wurtz, W. Bourguet, J. P. Renaud, V. Vivat, P. Chambon, D. Moras, H. Gronemeyer, *Nat. Struct. Biol.* **1996**, *3*, 87 – 94.
[10] R. L. Wagner, J. W. Apriletti, M. E. McGrath, B. L. West, J. D. Baxter, R. J. Fletterick, *Nature* **1995**, *378*, 690 – 697.
[11] M. G. Parker, R. White, *Nat. Struct. Biol.* **1996**, *3*, 113 – 115.
[12] A. M. Brzozowski, A. C. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engström, L. Öhman, G. L. Greene, J. A. Gustafsson, M. Carlquist, *Nature* **1997**, *389*, 753 – 758.
[13] W. B. Pratt, D. O. Toft, *Endocr. Rev.* **1997**, *18*, 306 – 360.
[14] S. M. Hollenberg, C. Weinberger, E. S. Ong, G. Cerelli, A. Oro, R. Lebo, E. B. Thompson, M. G. Rosenfeld, R. M. Evans, *Nature* **1985**, *318*, 635 – 641.
[15] J. L. Arriza, C. Weinberger, G. Cerelli, T. M. Glaser, B. L. Handelin, D. E. Housman, R. M. Evans, *Science* **1987**, *237*, 268 – 275.
[16] S. P. Williams, P. B. Sigler, *Nature* **1998**, *393*, 392 – 396.
[17] D. Pearce, K. R. Yamamoto, *Science* **1993**, *259*, 1161 – 1165.
[18] R. Rupprecht, J. M. Reul, B. van Steensel, D. Spengler, M. Soder, B. Berning, F. Holsboer, K. Damm, *Eur. J. Pharmacol.* **1993**, *247*, 145 – 154.
[19] R. Rupprecht, J. L. Arriza, D. Spengler, J. M. Reul, R. M. Evans, F. Holsboer, K. Damm, *Mol. Endocrinol.* **1993**, *7*, 597 – 603.
[20] B. Kauppi, C. Jakob, M. Farnegardh, J. Yang, H. Ahola, M. Alarcon, K. Calles, O. Engström, J. Harlan, S. Muchmore, A. K. Ramqvist, S. Thorell, L. Öhman, J. Greer, J. A. Gustafsson, J. Carlstedt-Duke, M. Carlquist, *J. Biol. Chem.* **2003**, *278*, 22748 – 22754.
[21] W. Bourguet, M. Ruff, P. Chambon, H. Gronemeyer, D. Moras, *Nature* **1995**, *375*, 377 – 382.
[22] J. P. Renaud, N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer, D. Moras, *Nature* **1995**, *378*, 681 – 689.
[23] A. C. Pike, A. M. Brzozowski, J. Walton, R. E. Hubbard, T. Bonn, J. A. Gustafsson, M. Carlquist, *Biochem. Soc. Trans.* **2000**, *28*, 396 – 400.
[24] A. C. Pike, A. M. Brzozowski, R. E. Hubbard, T. Bonn, A. G. Thorsell, O. Engström, J. Ljunggren, J. A. Gustafsson, M. Carlquist, *EMBO J.* **1999**, *18*, 4608 – 4618.
[25] J. J. Voegel, M. J. Heine, C. Zechel, P. Chambon, H. Gronemeyer, *EMBO J.* **1996**, *15*, 3667 – 3675.
[26] S. A. Onate, S. Y. Tsai, M. J. Tsai, B. W. O'Malley, *Science* **1995**, *270*, 1354 – 1357.
[27] R. K. Bledsoe, V. G. Montana, T. B. Stanley, C. J. Delves, C. J. Apolito, D. D. McKee, T. G. Consler, D. J. Parks, E. L. Stewart, T. M. Willson, M. H. Lambert, J. T. Moore, K. H. Pearce, H. E. Xu, *Cell* **2002**, *110*, 93 – 105.
[28] T. Ojasoo, J. C. Dore, J. Gilbert, J. P. Raynaud, *J. Med. Chem.* **1988**, *31*, 1160 – 1169.
[29] T. S. Berger, Z. Parandoosh, B. W. Perry, R. B. Stein, *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 733 – 738.
[30] R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, *J. Appl. Crystallogr.* **1993**, *26*, 283 – 291.
[31] M. Rarey, B. Kramer, T. Lengauer, G. Klebe, *J. Mol. Biol.* **1996**, *261*, 470 – 489.
[32] I. Massova, P. A. Kollman, *Perspect. Drug Discovery Des.* **2000**, *18*, 113 – 135.
[33] R. Dey, P. Roychowdhury, C. Mukherjee, *Protein Eng.* **2001**, *14*, 565 – 571.
[34] S. Hammer, I. Spika, W. Sippl, G. Jessen, B. Kleuser, H. D. Holtje, M. Schafer-Korting, *Steroids* **2003**, *68*, 329 – 339.
[35] U. Lind, P. Greenidge, M. Gillner, K. F. Koehler, A. Wright, J. Carlstedt-Duke, *J. Biol. Chem.* **2000**, *275*, 19041 – 19049.
[36] U. Lind, J. Carlstedt-Duke, J. A. Gustafsson, A. P. Wright, *Mol. Endocrinol.* **1996**, *10*, 1358 – 1370.
[37] A. Hillisch, J. von Langen, B. Menzenbach, P. Droescher, G. Kaufmann, B. Schneider, W. Elger, *Steroids* **2003**, *68*, 869 – 878.
[38] U. Fuhrmann, E. P. Slater, K. H. Fritzemeier, *Contraception* **1995**, *51*, 45 – 52.
[39] A. Sali, T. L. Blundell, *J. Mol. Biol.* **1993**, *234*, 779 – 815.
[40] R. W. Hooft, G. Vriend, C. Sander, E. E. Abola, *Nature* **1996**, *381*, 272.
[41] D. A. Pearlman, D. A. Case, J. W. Caldwell, W. S. Ross, T. E. Cheatham III, AMBER 4.1, University of San Francisco, **1995**.
[42] SYBYL 6.7, Tripos Inc., 1699 S. Hanley Road, Suite 303, St. Louis, MO 63144–2913, USA, **2000**.
[43] H. J. Bohm, *J. Comput.-Aided Mol. Des.* **1994**, *8*, 243 – 256.

Received: October 12, 2004

