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Investigations on inhibitors of human 17α-hydroxylase-17,20-lyase and their interactions with the enzyme

Molecular modelling of 17α-hydroxylase-17,20-lyase, Part II

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Dedicated to Prof. W. Fleischhacker on the occasion of his 70th birthday

New methods in treatment of hormone-dependent diseases like prostate or breast cancer have become a major subject in medical and pharmaceutical research. Because of the direct correlation of cancer growth and hormone concentration, inhibition of hormone biosynthesis presents a promising strategy in cancer therapy. The key enzyme in androgen biosynthesis is the 17α-hydroxylase-17,20-lyase a cytochrome P450 system, which specifically converts gestagens to androgens. Because the 3D-structure of the enzyme is still unknown most recently a ligand-based design was used to gain deeper insights into protein structure and function. In this paper we present molecular modelling studies on compounds acting as competitive inhibitors of the human 17α-hydroxylase-17,20-lyase. The compounds developed by Hartmann et al. belong to two different structural classes and show a wide range of inhibitory potency. The physico-chemical properties of the molecules were investigated and compared by studying structural flexibility and by calculating molecular interactions fields. The superimposition of all inhibitors in a low energy conformation yielded in the common pharmacophore. In the second part of the paper individual inhibitors were docked into the active site of the enzyme model of CYP17 developed in our group. The dynamic behaviour and stability of the protein-inhibitor-complexes was studied. The protein ligand interactions observed in course of the molecular dynamics simulations correspond well with the experimental data.

1. Introduction

The cytochrome P450 superfamily comprises a large number of hemoproteins, of which about 500 individual members have been sequenced until now. More than 60 different enzymatic reactions are known to be catalysed by P450s, most of them are involved in the metabolism of a huge variety of endogenous and exogenous substrates. The remaining P450 families however are involved in specific biosyntheses of steroid hormones. The general reaction for the majority of cytochrome P450s is activation and cleavage of molecular dioxygen. During the catalytic cycle a single oxygen is inserted into the substrate while the other oxygen atom leaves the reaction forming a water molecule [1].

The 17α-hydroxylase-17,20-lyase is a microsomal cytochrome P450 system anchored to the membrane of the endoplasmatic reticulum [2]. The enzyme specifically converts progesterone and pregnenolone to androgens via 17α-hydroxylation (which is a starting point for glucocorticoid production also) followed by the cleavage of the side-chain [3, 4]. Because of its key role in biosynthesis of androgens enzyme inhibition results in a total blockade of androgen production. Thus the enzyme has become an interesting target in treatment of prostate cancer. Development of potent enzyme inhibitors requires a more detailed understanding of enzyme structure and function. Because the 3D-structure of this enzyme is still unknown, we constructed a theoretical 3D-model of the protein by homology modelling. This work was described in a preceding publication [6]. In this paper we report on the examination of the molecular properties of two classes of enzyme inhibitors as well as on studies of the dynamic behaviour of the enzyme-inhibitor-complexes. The behaviour of the compounds during the dynamics simulation exhibits a good correspondence with the experimental data of the ligands and enables us to explain the differences in inhibitory activities.

2. Investigations and results

2.1. Inhibitors of 17α-hydroxylase-17,20-lyase

Hartmann et al. [5] have developed two series of steroidal and non-steroidal compounds acting as competitive inhibitors of human 17α-hydroxylase-17,20-lyase. Common features of these molecules are the rather hydrophobic skeletons and at least one sterically accessible nitrogen atom. The non-steroidal compounds are derived from tetrahydronaphthaline (THN), substituted with a methoxyl-group at different positions of the aromatic ring (Table 1). The aliphatic ring is connected directly or via a methylene-spacer with a nitrogen-containing aromatic system like imidazole or pyridine. The smaller compounds directly linked to the THN-system show a reduced inhibitory activity. Introduction of electronegative elements like carbonyl groups in the THN sytem also reduces potency [7].

The steroidal compounds (Table 2) represent analogues of the natural substrate pregenolone, substituted in C17-position by a nitrogen containing group like oxime or aziridine. The oxime group either can be connected directly or by a double bonded ethylene spacer to the steroidal skeleton, the latter leads to E- and Z-isomers showing striking differences in inhibitory strenghts. Substitution with an aziridinyl ring in 17β-position leads to much higher activities than derivation in 17α-position because the lone pairs of the aziridine groups are pointing in different directions [8].

2.2. Mechanism of inhibition

All inhibitors show a competitive binding mechanism, so only one single ligand-binding-site can be assumed where the inhibitor competes with the natural substrate. The inhibitor directly interacts with the heme-iron forming a coordinative bond via the nitrogen lone pair. Thus the position of the molecular oxygen is occupied and the substrate will be displaced from its binding-site nearby the heme.

Table 1: Structures and IC50 values of the non-steroidal inhibitors of the enzyme

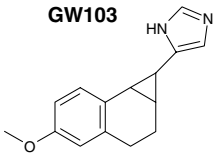
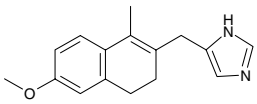
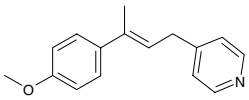
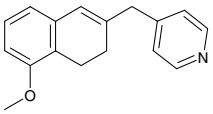
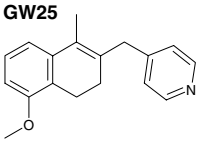
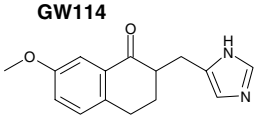
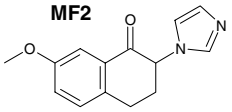
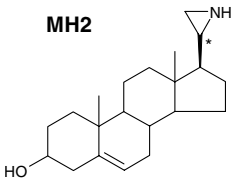
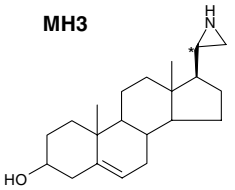
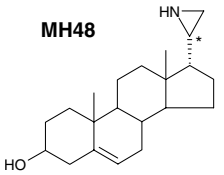
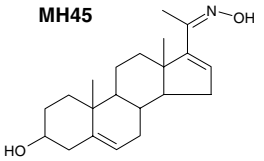
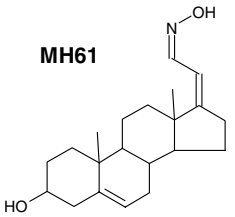
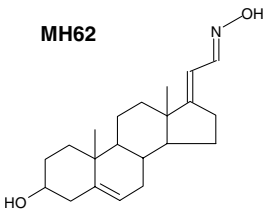
Inhibitors of 17α-hydroxylase-17,20-lyase	IC_{50} (μM)
GW103 	0.06
BW19 	0.11
BW35 	0.38
BW55 	0.081
GW25 	0.57
GW114 	7.5
MF2 	2.9
MF6 	9.3

Table 2: Structures and IC50 values of the steroidal inhibitors of the enzyme

Inhibitors of 17α-hydroxylase-17,20-lyase	IC_{50} (μM)
MH2 	1.52
MH3 	0.54
MH48 	>100
MH45 	0.17
MH61 	0.077
MH62 	>100

2.3. Study of the molecular properties of the inhibitors

2.3.1. Generation of the structures

Based on crystal structures taken from the Cambridge Structural Database (CSD) [9] all substances were generated using the Sybyl software [10]. Most of the calculations were performed using the Tripos force field because it offers a large set of well defined parameters especially for small molecules. Only in a few cases of ambiguous geometries and for the calculation of electronical properties quantum chemical methods were used.

The construction of the molecular structures was followed by extensive energy minimizations in order to optimize the geometry and to reduce the internal energy of the structures. The minimizations were done using steepest descent and conjugate gradient procedures.

Structural flexibility of the compounds was studied by molecular dynamics and systematic search procedures. The THN derivatives were subjected to simulated annealing calculations to determine the existence of different ring conformations of the THN system. During the dynamics simulation the system was heated up to 700 K for a period of 5 ps, then it was slowly cooled down and frozen to 0 K. This procedure was repeated for 30 cycles. The frozen conformers were sampled and energy minimized. Only two ring conformations showing identical internal energy could be observed during the molecular dynamics study. The two conformers represent halfchair conformations differing only in the upwards or downwards position of one carbon atom in the THN ring system, leading to axial or equatorial orientation of the imidazol heterocyclus. In the following step of the study both

conformers had to be considered, because arguments for the preference of one or the other did not exist in this stage. During the development of the pharmacophore it was revealed that only the ring conformer with equatorial orientation of the imidazol ring fits the pharmacophoric superposition of all inhibitors.
Conformational analysis of rotatable bonds was done by systematic search procedures. The search was concentrated on the important rotors. These bonds were rotated in steps of 5°, after each step the potential energy was calculated and the actual conformer was stored. With the help of the automatic subroutine IXGROS developed in our group [12] the immense number of conformers obtained was reduced to a few conformers showing minimal potential energy. Thus finally only a small number of low energy structures was obtained.

2.3.2. Bio-active conformations

Common molecular characteristics and physico-chemical properties of the ligands essential for bioactivity are called the pharmacophoric elements. The pharmacophore is constructed by superimposing specific, so called "bioactive" conformations of all ligands (which must represent low energy forms) according to their common structural features. The molecule chosen as template structure for the superposition should fulfil the following criteria: Firstly the compound should represent a very potent inhibitor because then its molecular structure contains all features necessary for activity. Secondly the structure should be rigid or semi-rigid to facilitate the search for the bioactive conformer.
The highly potent compound MH3, a semi-rigid 17β-substituted aziridinyl-steroid was chosen as template in the case under study. As mentioned before, the identification of low energy conformers was rather easy since only two minimum conformations were detected in the systematic search procedure. For both conformations almost an identical potential energy was calculated in the Tripos force field, the lone pair of the aziridine nitrogen, however, points into opposite directions. Because the orientation of the lone pair is the critical parameter for the superposition, both conformers were studied in more detail using a quantum mechanical approach. Applying the ab initio 3-21G* basis set (program SPARTAN, [13]) a geometry optimization revealed a significant energy difference between the two conformations. Consequently, the resulting minimum energy conformation was chosen as the template structure.
In the case of the non-steroidal compounds the existence of two different NH-tautomers of the imidazole system had to be taken into consideration. In order to determine which tautomer is preferred here, both NH-tautomers of GW25 as an example were generated and superimposed onto the 2-pyridyl-substituted compounds which are known to be completely inactive. (The 2-pyridyl-substituted compounds cannot undergo coordination with the heme-iron because the nitrogen atom is sterically not accessible). In the superposition the nitrogen lone pair of the NτH-tautomer revealed a perfect overlap with the one of the inactive 2-pyridyl-compound. This indicates that the NπH-imidazol-tautomer represents the bioactive conformation. Correspondingly only NπH- tautomers were used in the following steps of our study (Fig. 1).

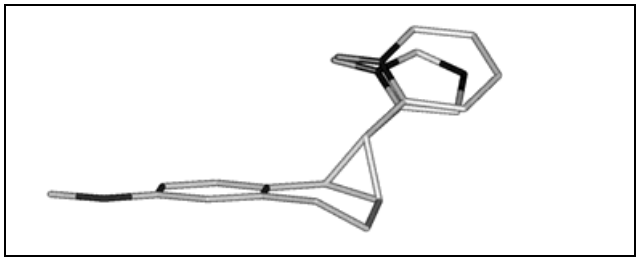


Fig. 1: Superimposition of the inactive 2-pyridyl-compound with the imidazol NτH-tautomer of GW25

2.3.3. Calculation of MEPs

A very important point in the study of molecular interactions is the determination of the MEPs because the first contact of two approaching molecules is governed by long-range electrostatic forces. The molecular electrostatic potentials (MEPs) visualise the impact of molecular electron distribution onto the surrounding space. MEPs can be determined directly from wave functions. For the purpose of molecular mechanics or dynamics simulations MEPs can be represented as interaction energies calculated between molecular partial charges located at atoms and a positive point charge located at grid points in a 3D grid surrounding the molecule. The molecular distribution of electron densities as well as the partial charges were calculated using a quantum mechanical ab-initio method. The molecular electrostatic potential based on the electron density distribution was determined using the ESP approach in the program SPARTAN [13].
Visualisations of the molecular electrostatic potentials calculated for each inhibitor show regions of high electron density focused on the sterically accessible nitrogen atom and on the oxygen atom in case of compounds containing a hydroxyl-group. In contrast the MEPs of the steroidal compounds containing an oxime-group are marked by two areas of high electron density one located around the oxygen and the other close to the nitrogen atom of the oxime. Therefore in principle the coordination to the heme-iron can be established by both, the oxygen or the nitrogen atom. The examination of structures of heme-oxime-complexes in the CSD database [9] however yields that most of the complexes are formed via the nitrogen atom (Fig. 2).

2.4. Construction of the pharmacophore

The heme molecule was included in the construction of the pharmacophore. Based on the heme-ligand-complexes archived in the CSD database the template structure MH3

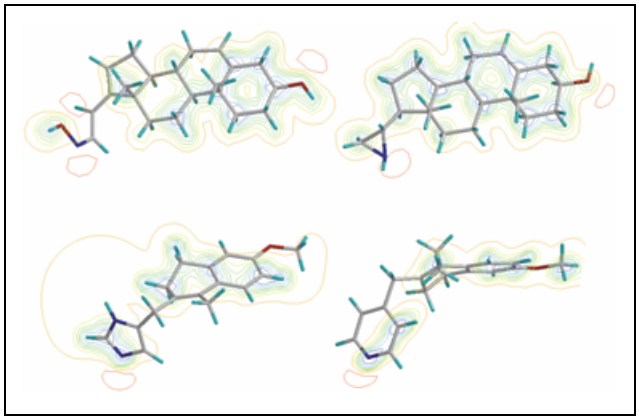


Fig. 2: Visualisation of the MEPs of some active compound; top from left to right: MH45, MH3; bottom from left to right: BW19, GW25

was orientated with the nitrogen lone pair in orthogonal direction above the heme plane. The nitrogen-iron-distance was set to 2.0 Å. Low energy conformers of the strong and medium inhibitors were superimposed with the template MH3 by fitting the lone pair of the nitrogen but also taking into account an optimal overlap of the hydrophobic skeletons of the ligands.

In this way the spatial orientation of the essential structural elements of the ligands was manually constructed. It can be described as follows: All inhibitors are positioned with the lone pairs in orthogonal direction above the heme plane, the hydrophobic volumes are located in almost parallel orientation to the plane. The methoxyl or hydroxyl groups of all inhibitor molecules point into the same direction (Fig. 3).

Using the same fitting procedure also inactive compounds were added to the superposition of the active congeners. The comparison of the volumes of active (atom-type coloured) and inactive compounds (green) revealed two different only scarcely overlapping areas and an intermediate region which is occupied by the medium potent inhibitors (yellow) (Fig. 4).

The pharmacophore was characterised in more detail by calculating molecular interaction fields for each ligand in the pharmacophoric conformation obtained. The calculations were performed using the program GRIN/GRID [13]. Different probes were applied to localise fields of

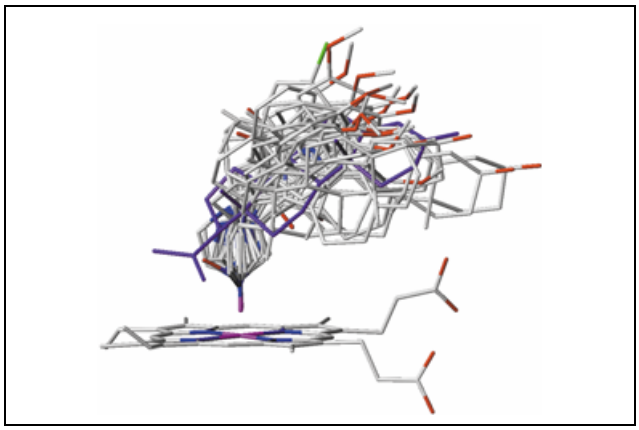


Fig. 3: Superposition of the active inhibitors. The inhibitors are atom-type coded, the physiological ligand progesterone is coloured violet

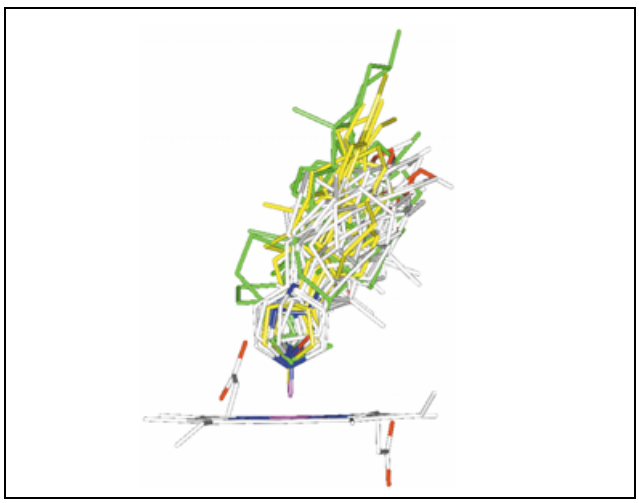


Fig. 4: Orthogonal view of the pharmacophore with the medium active (yellow) and inactive (green) compounds included

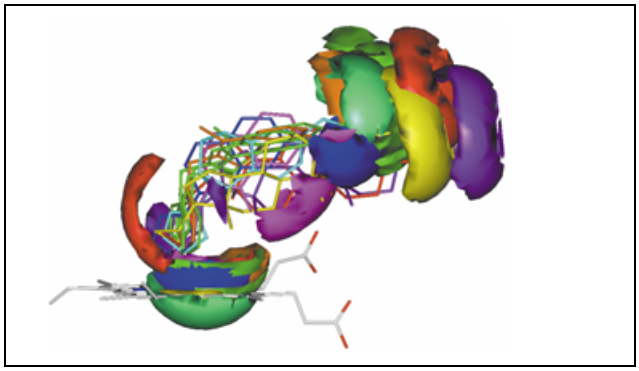


Fig. 5: GRID interaction fields calculated with a hydroxyl-group for active and medium active compounds in their pharmacophoric superposition. Fields are coloured according to the colours of the corresponding ligands

attractive hydrophobic or electrostatic interactions. For localisation of hydrophobic fields the methyl probe was used, regions of attractive electrostatic interactions were determined using the water or the carbonyl probe.

The mainly lipophilic skeleton of the ligands provokes large and diffuse hydrophobic fields. Polar fields exist at both ends of the molecules caused at one side by the methoxyl or hydroxyl group and at the opposite side by the nitrogen-containing substituent. In Fig. 5 are presented the electrostatic molecular interaction fields superimposed with the pharmacophore. The electronegative fields located at the nitrogen lone pair show a very good overlap for all the different ligands whereas the interaction fields produced by the methoxyl or hydroxyl groups form a rather large semicircle. It is worthwile to mention already at this point of the study that the interaction fields found for the smaller non-steroidal inhibitors (nitrogen containing substituent directly connected to the THN bicyclus) cannot be superimposed simultaneously with all fields of the larger steroidal compounds. This partial misfit of the electrostatic fields gives a first hint for the explanation of the reduced activity of this type of non-steroidal inhibitors (Fig. 5).

2.5. Protein-inhibitor-complexes – the protein model of CYP17

For calculation of protein-ligand-interactions a model structure of CYP17 was used. The CYP17 model developed in our group was constructed by homology modelling using InsightII/Homology [14] based on the crystal structure of CYPeryF [15]. Determination of conserved segments in the model sequence was based on a 3D-structural alignment of four crystallized P450 proteins in combination with results of different secondary structure predictions [16–17].

The model was refined by extensive geometry optimisation and molecular dynamics simulations. Evaluation of the stability and dynamic behaviour of the protein was achieved via molecular dynamics simulations using the program GROMACS [18]. The calculation was performed in a waterbox under almost physiological conditions i.e. 310 K, pH 7.4, 300 ps. The calculations yielded a reasonable and stable model structure with good protein geometry.

In the active site of the enzyme molecular interaction fields were calculated applying GRIN/GRID using the methyl, the water and the carbonyl probe. A large field of hydrophobic character was detected extending above the

heme plane in parallel as well as in orthogonal direction. The endpoints are marked by smaller regions favoured for polar interactions. All ligands superimposed in their bioactive conformations were docked into the active site of the CYP17 model according to the calculated molecular fields of the protein. At this state of the study the docking was performed manually with the aim to generate rough but acceptable starting geometries for further dynamics calculations. Surprisingly however, the strong and medium active inhibitors fit remarkably well into the favourable interaction fields whereas the inactive compounds mostly protrude from the fields (Fig. 6).

In the following step each inhibitor was individually docked into the active site in its pharmacophoric conformation in order to examine the binding circumstances of each protein ligand complex in more details. First the ligand was located in a distance of 2.0 Å to the heme-iron to form a coordinative bond and then the structure was orientated according to the hydrophilic and hydrophobic interaction fields calculated for the protein. In some cases the pharmacophoric conformation of the ligand had to be slightly modified in order to obtain a better fit with the interaction fields.

Subsequently molecular dynamics simulations were performed in order to examine the stability and dynamic behaviour of each protein-inhibitor-complex in a waterbox as described above. Molecular dynamics simulations of the heme-ligand-complexes are very problematic because the generally used force fields do not include parameters for an adequate description of the coordinative heme-ligand bond which is composed of two components. One is the attractive electrostatic interaction between the ligand heteroatom and heme-iron, this part can be easily described. The second is the directional force of the hybrid orbitals of the iron cation leading to an optimal octahedral complex-geometry. A correct description of the coordinative bond only can be achieved by using quantum chemical methods as they are incorporated nowadays in hybrid quantum mechanics/molecular mechanics approaches, the so-called "embedding methods". This type of programs, however, was not available for us. In order to overcome the lack of directional force parameters the heme-ligand coordination had to be simulated applying a special procedure realised with the help of NMR distance restraints [19] implemented in the GROMACS program. Two distances between the heme iron and the coordinating structural element of the ligand ($Fe-N_{coord.}$, $Fe-C_{ligand}$) were restricted to a range between 2.0–4.5 Å and 3.5–6.5 Å, respectively (to keep the complex geometry) while the rest of the system (protein, ligand, water) was allowed to move freely. Subsequently each protein-ligand complex was set in a pre-equilibrated waterbox and a 150 ps molecular dynamics simulation was performed at physiological temperature (310 K), constant pressure and pH 7.4.

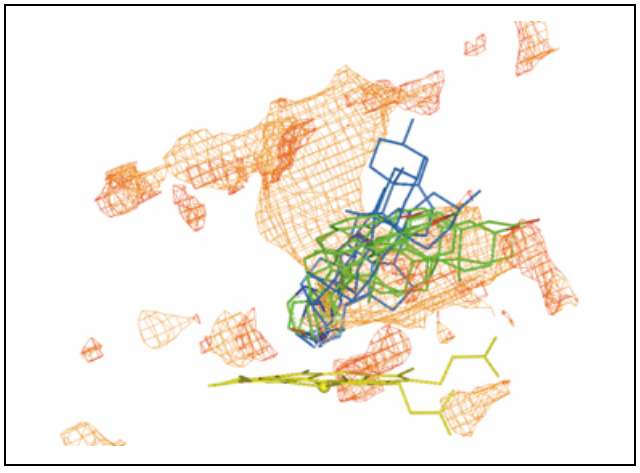


Fig. 6: Position of active (green) and inactive (blue) enzyme inhibitors in the active site of the enzyme.The interaction fields calculated with the program GRID are coloured orange (hydrophobic) and red (hydrophilic)

2.6. Molecular dynamics simulations of protein-ligand complexes

The molecular dynamics calculations were studied by analysing the following criteria:

1. The time needed for complex-equilibration (equilibration period).
   After equilibration of the system every 1000 fs the actual complex conformation was stored and the average structure was calculated.
2. The mobility of the ligand in the active site.
   The movements of the ligand were determined by calculation of the rms deviation by comparing the starting position of the ligand with its position in the average structure.
3. The stability and the number of hydrogen bonds formed between ligand and protein or ligand and water molecules in course of the simulation period.
4. The geometry of heme-ligand coordination was studied measuring the ligand-heme distance and the orthogonal angle ($N_{ligand}-Fe-N_{porphyrine}$), which is a measure for the conservation of the octahedral complex geometry.

The dynamic behaviour and stability of the protein-ligand complexes shall be discussed in more detail using a very potent, a medium active and an inactive ligand as examples. For the case of the very strong inhibitor the molecular interaction fields of the active site of the protein were calculated twice. Once for the starting position of the complex and second for the average structure of the protein ligand complex after the molecular dynamics simulation. This was done in order to find out whether the ligand-receptor interaction in fact does improve in course of the dynamics simulation.

The group of very potent inhibitors is represented by the steroid MH61 ($IC_{50} = 0.08$ µmol/ml) a pregnenolone analogue with a conjugated C17-oxime substituent (Z-isomer). The starting geometry of the protein-ligand complex was constructed as described in chapter 2.5. As can be observed in Fig. 7 (left part) this molecule fits quite well into the calculated hydrophobic fields of the active site. However the agreement found for electrostatically attractive regions is far from being optimal. In the starting position the 3-hydroxyl-group forms a hydrogen bond to Ser70 in the B′-C turn of the protein. In the right part of Fig. 7 the interaction fields of the protein after the dynamics are displayed. During the simulation the ligand moves away from Ser70 and forms new permanent hydrogen bonds with Thr54, and with a water molecule which simultaneously interacts with the 3-hydroxyl-group of the ligand. For a limited period of time the hydroxyl-group of the oxime additionally interacts with Thr259 in helix I. The coordinative geometry of the complex, $Fe-N_{ligand}$ distance and the orthogonal angle $N_{ligand}-Fe-N_{porphyrine}$, are kept for the complete period of the simulation. The new position attained in course of the molecular dynamics allows the inhibitor to perfectly interact with hydrophobic as well as with electrostatic fields in the active site of the

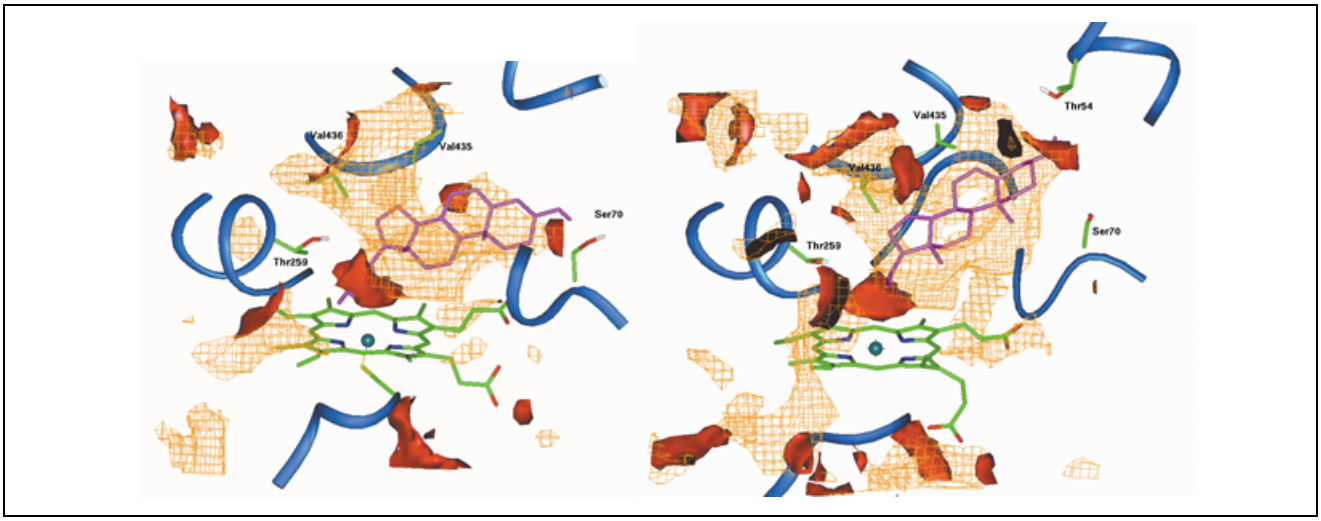


Fig. 7: Interaction geometries of the strong inhibitor MH61 before (left) and after (right) molecular dynamics simulations of the complex. Colour code: inhibitor MH61 is coloured magenta, the protein is shown atom-type coded, the hydrophobic interaction fields are contoured in orange, the electrostatic fields in solid red

enzyme. It can be concluded that starting from an acceptable but not optimal location of the ligand in the protein active site, the dynamical treatment of the complex did yield a significant improvement of the interaction geometry leading to an enhanced stabilisation of the complex.

MF2 ($IC_{50} = 2.9$ µmol/ml) is a medium active inhibitor. The non-steroidal compound belongs to those THN derivatives which are directly connected with the imidazole ring. As was already mentioned the regions of the interaction fields computed for compounds of this type cannot be completely aligned with the pharmacophore and accordingly small sized molecules like MF2 do not satisfy all the binding contacts offered by the active site of the protein. During the dynamics simulation the ligand moves from the starting position and from time to time the ligand-protein-coordination is lost. The deviation from the orthogonal angle decreases to 76°. Hydrogen bonds between ligand and protein are only formed but with different partners in course of the simulation. Therefore no permanent stabilisation via hydrogen bonds can be observed (Fig. 8).

The dynamic behaviour of an inactive ligand shall be demonstrated using MH48. The steroidal compound is substituted with an aziridine ring in 17α-position which leads to a dramatic decrease of inhibitory activity ($IC_{50} > 100$ µmol/ml).

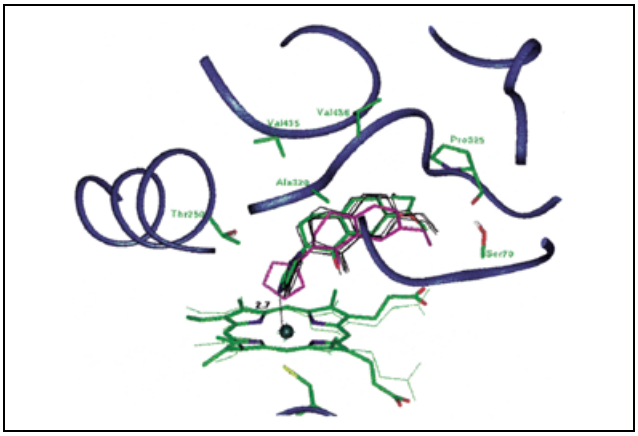


Fig. 8: Position of the medium active inhibitor MF2 in the active site during the molecular dynamics simulation The starting structure is marked magenta, the average structures of protein and inhibitor are atom-type coded

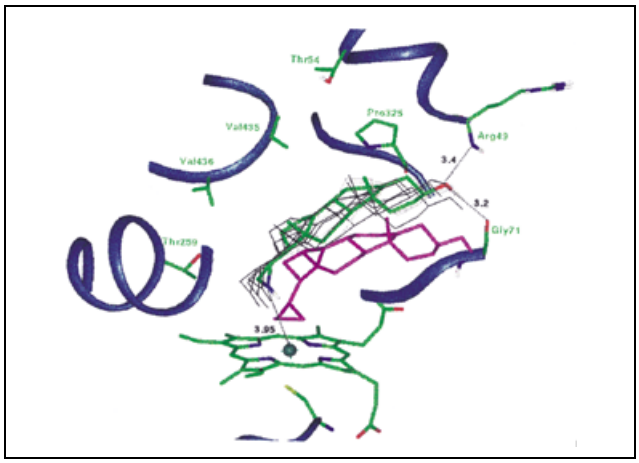


Fig. 9: Position of the inactive inhibitor MH48 in the active site during the molecular dynamics simulation. The starting structure is marked magenta, the average structures of protein and inhibitor are atom-type coded

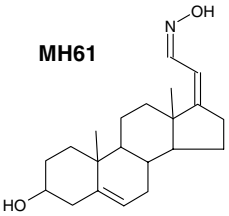
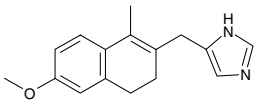
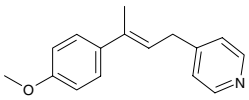
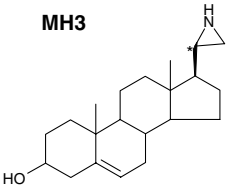
The construction of a reasonable starting geometry for this protein-ligand complex was rather difficult, because MH48 cannot be positioned in the limits of the interaction fields of the active site. Therefore the pharmacophoric conformation of MH48 was changed slightly in order to keep the correct coordination geometry and to adjust the position of the molecule inside the interaction fields as good as possible. Despite of a reasonable coordination geometry of the complex at the start, the ligand quickly moves away from the heme plane during the simulation so that the coordinative bond with the iron is completely lost. The N_{ligand}–Fe distance increases up to 3.9 Å, and the orthogonal angle shifts to 122° (Fig. 9).

3. Discussion

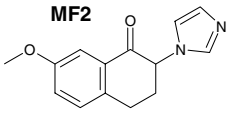
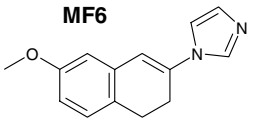
The behaviour of strong inhibitors is marked by a permanently well conserved octahedral geometry during the simulation (the values of $90 \pm 10°$ are also observed in heme-ligand complexes stored in the crystallographic database). The strong inhibitors can form at least one or more permanent hydrogen bonds with the protein which stabilize the position of the ligand. If the structure of the inhibitor does not allow a direct hydrogen bond to the protein,

Table 3: Analysis of the protein-ligand interactions during the molecular dynamics simulations

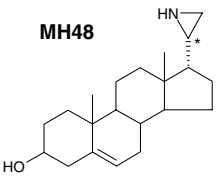
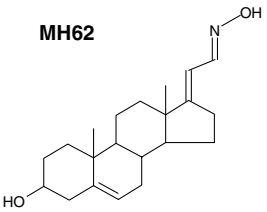
Strong inhibitors

Ligands	IC_{50}	RMS value of the ligand	Hydrogen-bond ligand–protein	Hydrogen-bond ligand–water	Angle between $N/O_{lig}–Fe–N_{heme}$	Distance $N/O_{lig}–Fe$
MH61 	0.077	0.5 to 1 Å	2 to 3 stable	1 stable after 50 ps MD	88° to 97°	2.2 to 2.5 Å
BW19 	0.11	0.5 to 1 Å	1 stable	1 stable	88° to 97°	2.2 to 2.5 Å
BW35 	0.38	0.5 to 1 Å	1 stable	no	88° to 97°	2.2 to 2.5 Å
MH3 	0.54	0.5 to 1 Å	2 stable	no	88° to 97°	2.2 to 2.5 Å

Medium active inhibitors

Ligands	IC_{50}	RMS value of the ligand	Hydrogen-bond ligand–protein	Hydrogen-bond ligand–water	Angle between $N/O_{lig}–Fe–N_{heme}$	Distance $N/O_{lig}–Fe$
MF2 	2.9	0.8 Å	1 discontinous	no	76° to 88°	2.2 to 2.5 Å
MF6 	9.3	0.8 Å	1 discontinous	1 discontinous	76° to 88°	2.2 to 2.5 Å

Weak inhibitors

Ligands	IC_{50}	RMS value of the ligand	Hydrogen-bond ligand–protein	Hydrogen-bond ligand–water	Angle between $N/O_{lig}–Fe–N_{heme}$	Distance $N/O_{lig}–Fe$
MH48 	> 100	> 0.8 Å	1 stable partially 2	no	122°	3.9 Å
MH62 	> 100	strongly variable	2 stable partially 3	no	70°	4.02 Å

The data were calculated from the average structures

this interaction can be mediated by a few water molecules of the active site.

The inhibitors of medium potency are characterised by a minor stable coordination geometry undergoing deviations from the orthogonal angle up to $\pm 20^{\circ}$. The structures only scarcely can undergo other stabilising interactions with the protein which is indicated by the rare and rather irregular formation of hydrogen bonds. Sometimes the structures are too small to coordinate and to interact with several amino acids of the active site simultaneously.

In the case of the inactive compounds the heme-ligand coordination is lost completely in course of the molecular dynamics simulations.

In Table 3 the important protein-ligand interactions found in course of the molecular dynamics simulations are summarised. The analysis of the dynamics simulations of all protein-inhibitor-complexes clearly demonstrates that there is a strong correspondence between the persistence of the correct octahedral coordinative complex geometry and a strong inhibitory activity of the ligand. If the structure of the ligand allows a conservation of the coordination with the heme iron then the ligand is a strong inhibitor. If the structure of the ligand does not allow the conservation of this contact during the simulation, then the ligand looses inhibitory activity.

According to this finding it may be postulated that the protein-ligand interaction predominantly is stabilised by the attraction of the heme iron. It could be argued against this hypothesis, that as in the well known and unique case of the 2-phenyl-imidazole inhibitor of P450 cam [20] the interaction of the imidazole nitrogen with the heme iron energetically can be overbalanced by two hydrogen bonds, if one of those is made up with an ionic partner (asp in the described complex). Two conditions, however, have to be fulfilled to make this modification possible: firstly the ligand has to be much smaller than the active site and therefore almost can move freely to find optimal contacts with other polar amino acids. Secondly the binding between nitrogen and heme iron has to be impaired by steric factors. Both arguments are not valid in the case studied here. As a consequence we believe that the proposed mechanism indeed is responsible for the modulation of inhibitory activities.

In summary the theoretical studies on the 17α-hydroxylase inhibitors and the evaluation of the protein-ligand-complexes reveal good agreement with the experimental data.

The superposition of the ligands according to their physico-chemical characteristics yields in a common pharmacophore, which allows a rough distinction of strong, medium and inactive inhibitors. The structural requirements for strong inhibitors can be characterised as follows:

- a sufficiently large hydrophobic skeleton with an overall size comparable to a steroid
- substitution by electronegative groups only at the external positions of the hydrophobic skeleton
- one nitrogen-containing group which can form a stable heme-coordination at one of the external positions
- another group that can accept and/or donate hydrogen bonds located at the opposite side.

The differentiation in active and inactive compounds is nicely possible by docking the pharmacophore into the active site of the protein model and by subsequently performing molecular dynamics simulations of the individual protein-ligand complexes. The evaluation of the complex stability in course of the molecular dynamics runs reveals that the experimental data can be unequivocally explained on the basis of these calculations.

Because of lacking force field parameters describing the coordinative ligand-iron bond correctly the determination of reliable interaction energies allowing for quantitative correlations with experimental binding affinities is impossible.

Another critical point which prevents quantitative correlation with the experimental data is the fact that water molecules in the active site participate in the protein-ligand interaction by mediating hydrogen bonds with the protein and/or the ligand. The role of these water molecules is difficult to evaluate in energetic terms. Therefore we confined our studies to a half quantitative description of the ligand-enzyme complexes, however, the procedure developed, very well may be used for predictive purposes.

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