Drug Design

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Thermodynamic Inhibition Profile of a Cyclopentyl and a Cyclohexyl Derivative towards Thrombin: The Same but for Different Reasons**

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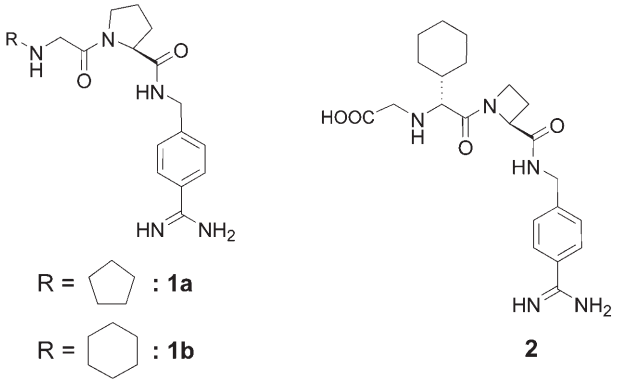
In the course of a drug development project prospective lead structures are optimized by systematically changing functional groups or side chains on a given core skeleton. If available, the crystal structure of the target protein is consulted to guide the selection of appropriate substituents. Medicinal chemists follow some basic rules collected for intrinsic functional group contributions that allow one to estimate by how much the binding affinity is expected to improve once a particular functional group is attached or modified.[1–3] For example, the group contribution assigned to a methylene group is estimated to about 3–4 kJ mol^{-1} in terms of the Gibbs free energy of binding.[1,2] Thus, within a congeneric series it is assumed that an additional methylene group will increase binding affinity by this amount if this substitution can be accommodated by the receptor.

A prerequisite for the incremental increase in hydrophobic binding per added methylene group is the analogous binding behavior of ligands within a congeneric series. To probe this concept we studied the homologous pair of thrombin inhibitors (**1a** and **1b**, Scheme 1) which differ only by one CH_2 unit in the cycloalkyl substituent designed to bind in the S3/S4 specificity pocket. In our design of these two compounds, we were guided by the known binding mode of melagatran (**2**), which nicely places a cyclohexyl moiety into the hydrophobic S3/S4 pocket.[4] Consequently, we selected a cyclohexyl moiety and the smaller cyclopentyl moiety for binding in the S3/S4 pocket.

The synthesis of **1a** and **1b** has been described elsewhere.[5] Their binding constants for thrombin were determined by a kinetic photometric assay.[6] Surprisingly, the two inhibitors had virtually the same binding affinity rather than the expected 3–4-kJ mol^{-1} increase in binding affinity for



Scheme 1. Structures of the derivatives (**1a** and **1b**) studied by isothermal titration calorimetry, X-ray crystallography, and MD simulations, and the structure of melagatran (**2**) for comparison.

analogue **1b** compared to **1a** due to the additional CH_2 group (Table 1). To further study this surprising result we determined the crystal structures of the cyclopentyl and cyclohexyl derivatives in complex with thrombin (Figure 1). The difference electron density clearly showed the expected binding geometry of the cyclopentyl derivative with a well-defined density assigned to the five-membered ring positioned in the S3/S4 pocket. However, to our surprise, the cyclohexyl derivative did not show a well-defined difference electron density for the six-membered ring in the S3/S4 pocket. The remaining portions of ligand **1b** were nicely defined and in the same binding orientation as the analogous portions of **1a**.

Typically, ill-defined difference electron density of molecular portions of a ligand indicates either enhanced residual mobility or scattered static orientation over multiple conformational states. In both cases, the atomic arrangement prevents constructive contribution to the diffraction experiment, and no electron density can be observed.

Table 1: Binding constants K_b and isothermal calorimetry data for **1a** and **1b**.[a]

Ligand	K_b [10^6 L mol^{-1}]	$\Delta G°$ [kJ mol^{-1}]	$\Delta H°$ [kJ mol^{-1}]	$-T\Delta S°$ [kJ mol^{-1}]
1a	1.54 ±0.41	−35.40 ±0.55	−16.88 ±0.58	−18.52 ±0.56
1b	2.18 ±0.62	−36.16 ±0.58	−10.50 ±0.28	−25.66 ±0.43

[a] All measurements were performed at 298 K.

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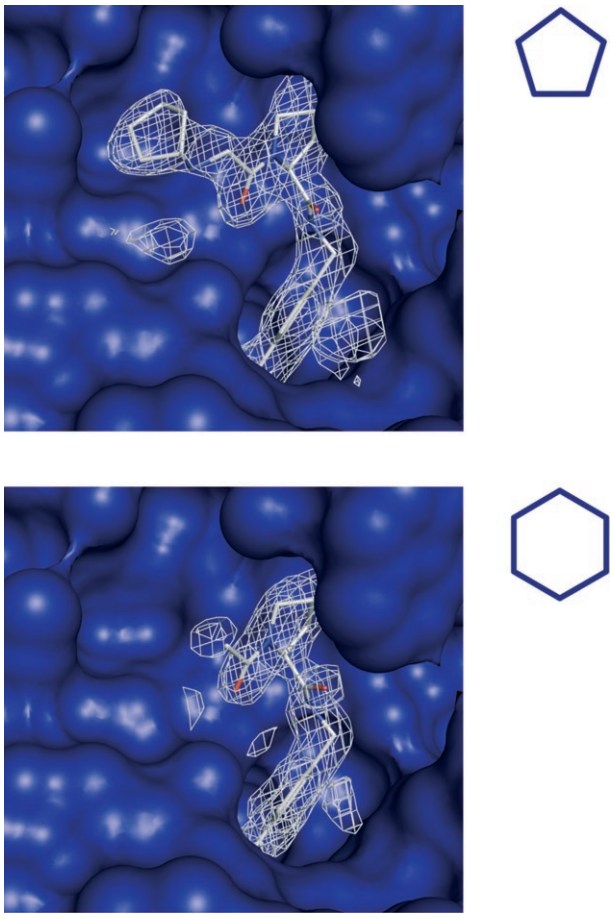

Figure 1. Fo-Fc difference electron density of the cyclopentyl derivative **1a** (top) and cyclohexyl derivative **1b** (below) in the binding pocket of thrombin. The difference electron density has been contoured at a level of 2 σ. Whereas the five-membered ring of **1a** is well-defined by the electron density, the six-membered ring of **1b** is ill-defined, most likely because of either a strong dynamic residual mobility or extensive static scatter over multiple conformational states. The solvent-accessible protein surface is shown in blue.

In order to better define the differences between the binding properties of **1a** and **1b** we utilized isothermal titration calorimentry (ITC).[7] Although ITC provided virtually the same Gibbs free energy of binding for **1a** and **1b** (Table 1), a dramatic difference was observed for the factorization of free energy into enthalpic and entropic contributions: for the cyclopentyl derivative binding enthalpy (ΔH) and entropy (expressed as $-T\Delta S$) adopt nearly the same values, whereas for the cyclohexyl derivative a strong entropic advantage was observed, compensating for a significantly reduced enthalpic contribution (Table 1).

If we assume enhanced residual mobility of the cyclohexyl derivative in the bound state, or scattering over multiple binding orientations, as indicated by crystallography, then the different factorization into enthalpy and entropy appears reasonable. The cyclohexyl derivative experiences an entropic advantage as it does not lose as many conformational degrees of freedom as the cyclopentyl derivative.

To further study the very different residual mobility properties of **1b** and **1a**, we performed molecular dynamics simulations of the bound ligands in complex with thrombin.[9] Analyzing the trajectory recorded over 1 ns reveals an interesting difference between the two ligand complexes. In Figure 2 and Figure 3 a,b the distances between C11 in the

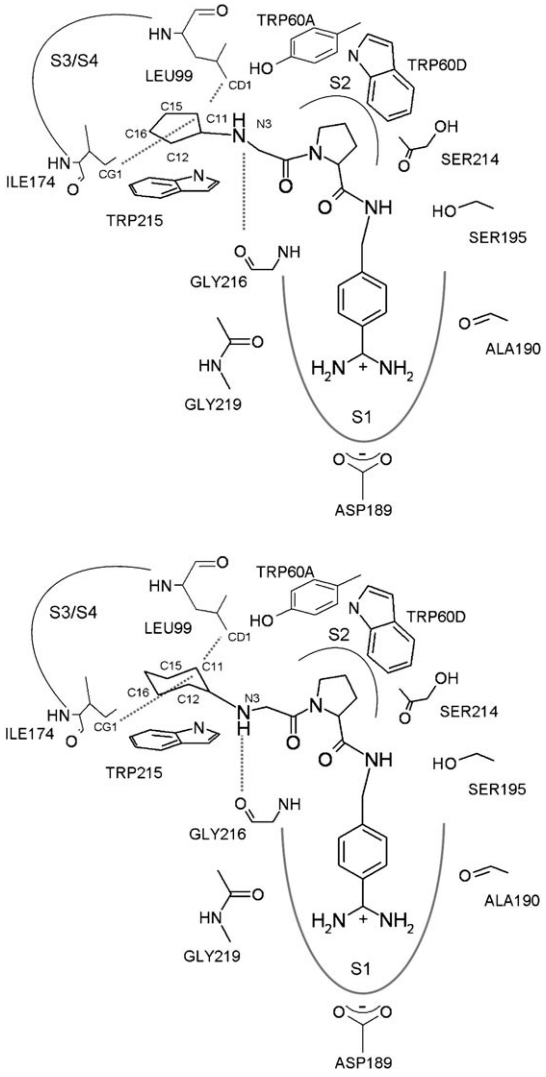

Figure 2. Schematic drawing of the binding mode of cyclopentyl derivative **1a** (top) and cyclohexyl derivative **1b** (below). The distances recorded in Figure 3 along a molecular dynamics trajectory are shown as dotted lines.

cyclopentyl ring and CD1 of the adjacent Leu 99 residue and CG1 of Ile 174 are shown. Along the trajectory the two distances fluctuate between 4 and 6.5 Å in a concerted manner. This obviously corresponds to a jump rotation of the cyclopentyl moiety which flips the ring by 180° about the bond axis from N3 to the pivotal carbon atom of the five-membered ring. In contrast, for the cyclohexyl moiety in **1b**, the corresponding distances measured between the flanking residues and C11 show different trends (Figures 2 and 3 a,b). First of all, one distance (C11–CG1) remains above 5 Å, whereas the other (C11–CD1) fluctuates in an uncorrelated manner between 4.5 and 7 Å. This indicates a motion of the six-membered ring in and out of the S3/S4 pocket. Further-

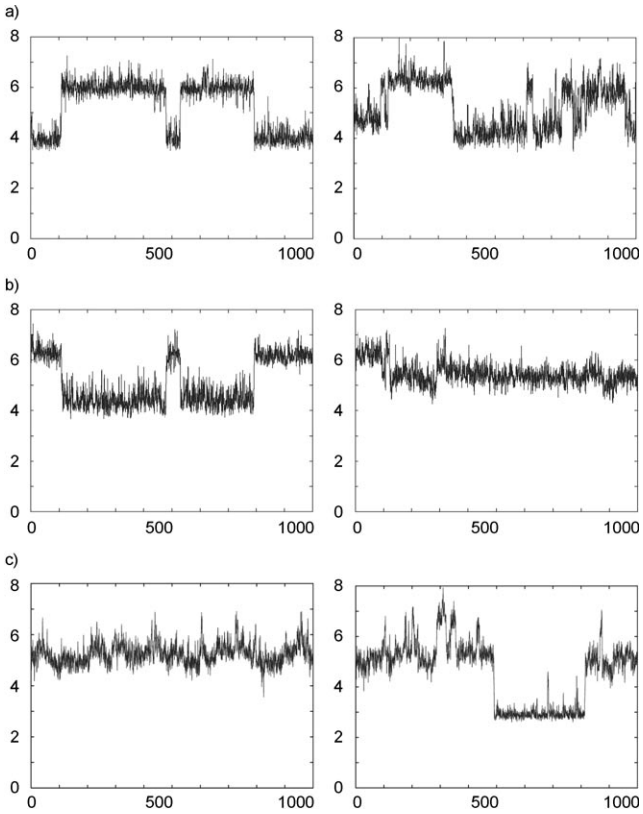

Figure 3. Fluctuations of representative distances [Å] in the complexes of the cyclopentyl derivative **1a** (left) and cyclohexyl derivative **1b** (right) along the trajectory (t [ps]). The following distances are shown: a) C11 to CD1 of Leu99, b) C11 to CG1 of Ile174, c) N3 to Gly216 CO.

more, it is instructive to analyze the distance between N3 and the carbonyl group of the neighboring Gly216 (Figure 3c). In the cyclopentyl derivative this distance remains beyond 5 Å, clearly excluding formation of a hydrogen bond. For the cyclohexyl derivative the simulation passes temporarily through a geometry that has a hydrogen bond between N3 and the CO unit of Gly216. This temporary formation of the hydrogen bond is correlated with an enhanced residual mobility of the cyclohexyl moiety in and out of the S3/S4 pocket.

The crystallographic and thermodynamic evidence for the surprisingly different binding properties of **1a** and **1b** was confirmed by the MD simulations. The jump rotation of the five-membered ring in the S3/S4 pocket is consistent with its defined electron density. The system fluctuates between two distinct but geometrically identical states. This allows for constructive contribution to the diffraction experiment and a well-defined electron density is observed. For example, in crystalline benzene the molecules perform pronounced jump rotations perpendicular to the sixfold axis; however, the crystal structures show a well-defined highly resolved electron density.[10,11]

Entropically, the residual jump rotation of the cyclopentyl moiety in the binding pocket still corresponds to a significant loss in degrees of freedom compared to the situation in the unbound solvated state. In contrast, the cyclohexyl moiety shows high mobility with unconcerted motions resulting in a tumbling in and out of the binding site. Apparently the temporarily formed and rather solvent-exposed hydrogen bond does not contribute strongly to binding affinity. However, the substantial residual mobility is entropically beneficial for binding: the system does not sacrifice as many degrees of freedom as the cyclopentyl derivative does with respect to the unbound situation.

What lesson can be learned from this example? Usually ligands are optimized in congeneric series. The addition of functional groups is expected to enhance binding; for example, the change from a five- to a six-membered ring should augment binding affinity by approximately 3–4 kJ mol^{-1}.[1,2] However, even very similar ligands can exhibit very different binding properties that destroy a simple structure–activity relationship.

In our example the cyclopentyl ring is well accommodated in the S3/S4 pocket, and thus it can experience good enthalpic interactions with the protein. In this case, an increase of the hydrophobic contact interface would be expected to enhance binding. The cyclohexyl derivative shows a high residual mobility of its hydrophobic moiety. Thus, it does not establish strong contacts with the hydrophobic S3/S4 pocket. Having observed these surprising binding properties, one would predict that this ligand will not profit from a further expansion of its hydrophobic surface, for example, by the insertion of additional methylene groups in the ring. Nevertheless, its binding free energy is the same as that of the cyclopentyl derivative because the loss of good enthalpic contacts is compensated by an entropic advantage.[12] It is worthwhile to note in this context that cyclopentane and cyclohexane show the same hydration free energy.[13] While at first sight this could indicate that the virtually identical binding free energy of **1a** and **1b** is merely a consequence of the identical dehydration free energies, we tend to rule out this possibility because of the compounds' different behavior in the crystal structures and the MD simulations. Further calculations based on recently described methods for determining conformational and vibrational entropy changes upon complexation could help to clarify the contributions that lead to the observed thermodynamic data.[14]

This example of two closely related ligands shows that binding properties are a complex interplay of structure and dynamics. It also shows that some, on first glance, trivial and "obvious" structure–activity relationships do not necessarily display the anticipated straightforward correlation. Similar conclusions have recently been reported by Krishnamurthy et al. on a congeneric series of *para*-substituted benzenesulfonamides binding to carbonic anhydrase. The authors also come to the conclusion that protein–ligand interactions are still rather poorly understood with respect to the underlying biophysical phenomena.[15]

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