Supporting Information

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Exploring the Flap Pocket of the Antimalarial Target Plasmepsin II: The “55% Rule” Applied to Enzymes

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1 Assay of in vitro Enzyme Activity
The assay was carried out at Actelion Pharmaceuticals Ltd, Allschwil. Purified enzyme (plasmepsin II or IV; 1 mM) was incubated with a fluorescently labeled peptide substrate (1 mM) and varying concentrations of inhibitor (0 – 2800 nM) in pH 5.0 acetate buffer (50 mM) containing glycerol (12.5%), Me₂SO (10%), and BSA (0.1%). The amount of peptide cleavage, as determined by fluorescence measurements, at each concentration of inhibitor was fitted to the Hill equation to give the IC₅₀ value for each inhibitor/enzyme combination.

2 Determination of the Flap Cavity Volume
The volume of the flap pocket was estimated using the two different programs Spartan 06 for Windows[1] and HOLE.[2] The software package HOLE was initially written for the analysis and visualization of the pore dimensions of ion channels such as gramicidin A.[3] In this case, HOLE 2.2.002 was used.[4]

2.1 HOLE

2.1.1 Modus Operandi
The modus operandi of HOLE has previously been reported[2] and is therefore only briefly summarized below. The user supplies a PDB file of the structure to be analyzed. Atom records are read, and the van der Waals radius of each atom is set up. In this case, the radii specified by Bondi were chosen.[5] HOLE then guesses an initial point in the channel cavity and a vector that roughly points in the direction of the channel. The program proceeds to move a point on the plane normal to the channel vector so as to find the largest sphere that can be accommodated without overlap with the van der Waals surface of any atom. The center of the sphere with maximum radius is identified by a Monte Carlo simulated annealing procedure.[6,7] This method was implemented to avoid becoming trapped in local ‘minima’. Once the largest sphere that can be accommodated on a particular plane is found, a small displacement s is taken in the direction of the channel vector (here s = 0.1 Å) and the process is repeated for the next plane. The net result of the routine is a series
of sphere positions that can be thought of a flexible sphere "squeezing" through the cavity. Figure 1SI illustrates the process.

![Diagram of sphere positions](image1.png)

**Figure 1SI:** *Illustration of the principal modus operandi of the software HOLE.*

The procedure described above gives a rough estimate of the cavity dimensions, but the results are not very accurate yet at this point. Therefore, in a next step, in each plane orthogonal to the channel vector \( v \), a grid with a certain spacing (here 0.6 Å) is set up. The procedure then finds points on this grid that can accommodate a spherical probe of a given radius (here 0.9 Å). The additional space accessible on the plane is then considered by applying a correction to the radius obtained in the first part of the run. The second part of the procedure is illustrated in Figure 2SI.

![Diagram of grid setup](image2.png)

**Figure 2SI:** *The second part of the HOLE-process. A 2D-grid is set up for each plane orthogonal to the channel vector and points on that grid, which can accommodate a smaller spherical probe, are identified.*

### 2.1.2 Input

The input files submitted to the program were fragments of the three published X-ray crystal structures of PM II in the flap-open conformation (PDB codes 2BJU, 2IGX, 2IGY). Since the software failed to find the cavity of interest in the complete structure, only the region around the analyzed area was considered. The cavity was manually terminated by the addition of P-atoms in the plane that was pre-assigned to be the boundary of the cavity (Figure 3SI).
Figure 3SI: a) Fragment of the X-ray crystal structure 2BJU, lined by Trp41, Val42, Pro43, Ser44, Cys57, Gly51, Cys52, Lys55, Met75, Asn76, Tyr77, Gly80, Thr81, Val82, Ser83, Gly84, Val105, Ile106, Asp107, Thr108, Asn109, Gly110, Phe111, Tyr115, Phe120, Asp121, Gly122, and Ile123. P-atoms (purple) were added to terminate the cavity. Color code: C-atoms: grey, O-atoms: red, N-atoms: blue, S-atoms: yellow. The picture was generated with the software VMD.\(^8\) For the two other X-ray crystal structures, 2IGX and 2IGY, the same amino acid residues were used for the calculation. b) Overlay of the fragments of the three X-ray crystal structures 2BJU (blue), 2IGX (red), and 2IGY (yellow). c) The same overlay as in a), but from a slightly different perspective, looking into the flap pocket. The amino acids lining the “beginning” of the pocket are colored in green.
2.1.3 Results

The output of HOLE is a text file containing a protocol of the run and the following parameters:

- cenxyz.cvec: coordinate in the direction of the channel vector
- radius: pore radius in that direction after the first part of the procedure
- cen.line.di: distance measured along the pore center line
- Requiv_esti: radius taking into account the results from the second part of the procedure

The values for \( \text{Requiv}_{\text{esti}} \) that fell within the pre-assigned cavity limits were used to calculate the void volumes of the resulting slices of 0.1 Å thickness \((s)\) each. Addition of the slice volumes gave the total cavity volume \( V_{\text{Cavity}} \).

\[
V_{\text{Cavity}} = \sum \text{Requiv}_{\text{esti}}^2 \cdot \pi \cdot s
\]

(1)

The calculated volumes are 246.6 Å\(^3\) (2BJU), 258.3 Å\(^3\) (2IGX), and 251.5 Å\(^3\) (2IGY), which leads to a mean value of 252 ± 6 Å\(^3\) (error given as standard deviation). The result for 2BJU is visualized in Figure 4SI.

**Figure 4SI:** Visualization of the results obtained for 2BJU by use of HOLE. The calculated cavity volume inside the protein fragment is shown in black. The amino acids shown are those listed in the caption of Figure 3SI. The picture was generated with VMD.\(^8\)
2.2 Spartan 06

In this procedure, known as the ‘cavity filling method’, a hydrocarbon framework was built into the protein fragment in a way that the framework overlapped with the surrounding amino acid residues. The volume of the protein was then subtracted from the volume of this construct. Spartan does not count overlapping areas twice (this was verified in control experiments), so the cavity volume was obtained by this operation. Obviously and unlike this is the case with HOLE, in the Spartan method also tiny arborizations of the cavity, which are not relevant for hosting an inhibitor, are considered. Therefore, the obtained cavity volume is very slightly overestimated. As a consequence of these considerations, we have used the more accurate results from HOLE, but double-checked the plausibility of the obtained value with Spartan for one structure fragment (2BJU). A volume of 268 Å³ resulted for 2BJU: a value that lies well in the range of the volumes calculated by HOLE. Overall, the agreement between the two approaches is excellent and conclusions drawn from both are the same.

3 Calculation of Packing Coefficients

Packing coefficients were determined relative to the average volume of 252 Å³. The inhibitors were optimized in MOLOC (see paragraph 4), and the volume of the flap substituent of the optimized structures was calculated by Spartan 06, using again the Bondi van der Waals radii as with HOLE. By calculating the volume of the optimized structure, potential folding of the chains, which leads to a smaller volume compared to the staggered (all-anti) alkane conformation, was considered.

4 Modeling/Optimization of Inhibitors Using MOLOC

Structures of potential inhibitors were manually docked within a flap-open structure of PM II (PDB code 2BJU). The enzyme structure was fixed, and the energy of the system was minimized using the MAB force field as implemented in the program MOLOC.
5 Uncertainty Analysis

The assay used for measuring the inhibition of in vitro enzyme activity was described in section 1. IC$_{50}$ values resulting from these measurements carry an estimated error of ± 50%. This error is very small for a protease assay and does furthermore not affect the validity of the core statements, namely that (1) inhibition of PM II shows a broad maximum, (2) this maximum is achieved, when volume occupancy lies in the range of 55 ± 9%, and (3) the maximum is less broad for PM IV.

Figure 5SI illustrates the relative size of the 50% uncertainty: The blue curve is the original plot of the IC$_{50}$ values as a function of the number of carbon atoms in the alkyl chain for inhibition of PM II (inhibitors (±)-1 and (±)-3–(±)-9), the yellow one shows all IC$_{50}$ values reduced by 50%, the green one all enhanced by 50%, the purple one alternating 50% reduction and 50% enhancement across all data points, and the orange one alternating 50% enhancement and 50% reduction. It is clear, that the uncertainty of the data points does not affect the validity of the conclusions. The differences in binding affinity between the inhibitors that fit (nM affinities) and the ones being too large or too small (µM affinities) are by several orders of magnitude larger than the uncertainty.

![Figure 5SI: Illustration of the relative magnitude of the uncertainty associated with the IC$_{50}$ values. Assuming maximum errors for each data point does not significantly change the shape of the binding curves.](image-url)
6 Contorted Alkyl vs. Cycloalkyl Binding

When the \( n \)-alkyl chains of the inhibitors fold to reduce their length, the resulting conformation at the position, where the gauche dihedral angle is observed, closely resembles the geometry of a cycloalkyl ring. This becomes apparent from molecular modeling experiments (Figure 6SI).

![Figure 6SI: Overlay of two inhibitors with a contorted octyl chain flap vector (blue) and an alkyl vector containing a cyclopentyl ring (yellow) exactly at the position, where the gauche dihedral angle is observed in the folded chain. The figure shows MOLOC-optimized structures inside the active site of PM II (PDB code 2BJU). It is apparent, that the vector bearing the cyclopentyl ring closely resembles the geometry of the contorted \( n \)-octyl chain.](image)

7 References