High-Affinity Inhibition of a Family of *Plasmodium falciparum* Proteases by a Designed Adaptive Inhibitor†

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Received January 22, 2003; Revised Manuscript Received April 15, 2003

ABSTRACT: Drug development against viral or microbial targets is often compounded by the existence of naturally occurring polymorphisms or drug resistant mutations. In the case of *Plasmodium falciparum*, the etiological agent of malaria, four related and essential proteases, plasmepsin I, II, and IV and the histo-aspartyl protease (HAP), have been identified in the food vacuole of the parasite. Since all of these enzymes are involved in the hemoglobin degradation of infected victims, the simultaneous inhibition of the four enzymes can be expected to lead to a faster starvation of the parasite and to delay the onset of drug resistance, since four enzymes will need to mutate in a concerted fashion. This study describes the design of an adaptive inhibitor intended to inhibit the entire plasmepsin family. Adaptive inhibitors bind with extremely high affinity to a primary target within the family and maintain significant affinity against the remaining members. This objective is accomplished by engineering the strongest and most specific interactions of the inhibitor against conserved regions of the binding site and by accommodating target variations by means of flexible asymmetric functional groups. Using this approach, we have designed an inhibitor with subnanomolar affinity (0.5 nM) against the primary target, plasmepsin II, and with no loss or a very small loss of affinity against plasmepsin IV, I, and HAP (K_i ratios of 0.4, 7.1, and 17.7, respectively). The core of the inhibitor is defined by an allophenylnorstatine scaffold. Adaptability is provided by an asymmetric amino indanol functional group facing one of the key variable regions in the binding site. Adaptive inhibitors, which display high affinity against several variations of a primary target, are expected to play an important role in the chemotherapy of infectious diseases.

A significant obstacle to the efficacy of drugs directed against viral, bacterial, or parasitic targets is the presence of amino acid polymorphisms in the targeted molecules. Amino acid polymorphisms may arise as a result of different strains of the infectious agent, different versions of the same target within a single microorganism, or the appearance of drug resistant mutations. An ideal drug will be one that is extremely effective against a primary target and maintains its efficacy against the most important variants of the target molecule. In the case of *Plasmodium falciparum*, the etiological agent of malaria, a family of aspartyl proteases, the plasmepsins (Plms),[1] are involved in the degradation of hemoglobin, the main foodstuff of the parasite (*1*, *2*). Four plasmepsins in the genome of *Plasmodium* have been localized in the food vacuole of the parasite, Plm I, Plm II, HAP, and Plm IV, and shown to be involved in hemoglobin degradation (*1*, *2*). Plm I, II, and IV are classical aspartic proteases with two aspartates in the catalytic site, whereas in HAP, one of the catalytic aspartates is replaced with a histidine (*2*, *3*). A drug that simultaneously inhibits the four plasmepsins will lead to a faster starvation of the parasite and retard the appearance of drug resistant mutants, since the parasite will have to mutate several proteins to overcome inhibition. Drug molecules with the ability to inhibit several members of a protein family with high affinity have been termed adaptive drugs (*4*, *5*). Usually, one member of the family is considered the primary target (e.g., the wild type, the most abundant, the most active, etc.). Among the plasmepsins, Plm I and II have the highest catalytic efficiency with substrates that mimic the initial cleavage site of the natural substrate, hemoglobin (*1*). Plm II is preferred as the primary target because its high-resolution structure and active site specificity are known (*6*, *7*). In addition, it has been shown that Plm II from *P. falciparum* has unique specificity and binding characteristics compared to the same enzyme from other *Plasmodium* species (*8*). The practicality of this approach requires that the primary target and the remaining

† Supported by National Institutes of Health Grants GM 57144 (E.F.) and AI 47798 (D.E.G.) and National Science Foundation Grant MCB-9816661 (E.F.). D.E.G. is a recipient of the Burroughs Wellcome Fund Scholar Award in Molecular Parasitology.

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[1] Abbreviations: Plm, plasmepsin; ITC, isothermal titration calorimetry; HAP, histo-aspartyl protease.

10.1021/bi034131z CCC: $25.00
Published on Web 06/21/2003

members of the family share a common three-dimensional structure and a high degree of sequence similarity within the binding cavity. The sequences of the plasmepsins are 60−70% identical (*2*), and they have similar tertiary structures. Superposition of the crystallographic structures of Plm II (PDB entry 1sme) and Plm IV (PDB entry 1ls5) reveals that their backbones deviate from each other by only 1.05 Å.

Current drug design paradigms are based upon the lock-and-key hypothesis, which emphasizes shape complementarity as a way to attain specificity and improved binding affinity. Shape complementarity is accomplished by the introduction of conformational constraints in the drug molecule. While highly constrained molecules do well against a unique target, they lack the ability to adapt to target variations like those originating from naturally occurring polymorphisms or drug resistant mutations. Targeting a protein family rather than a single family member, while still maintaining high affinity and specificity, requires a different approach. The overall goal is to design a molecule that inhibits the primary target with extremely high affinity while still retaining substantial activity against the remaining members of the family. Adaptive drug molecules must attain high affinity and specificity by establishing strong interactions with conserved regions of the target and be able to accommodate existing variations without losing significant binding affinity. In this study, we describe the design and structure of an inhibitor that displays high potency against the four plasmepsins found in the food vacuole of *P. falciparum*.

EXPERIMENTAL PROCEDURES

Plasmepsin Purification. Plm II was purified and activated by following a protocol described previously (*9*). Briefly, BL21(DE3) pLysS *Escherichia coli* was transformed with pET3a containing pro-Plm II, and protein expression was induced with 1 mM IPTG. The protein was expressed as inclusion bodies and purified by multiple rounds of centrifugation. The purified inclusion bodies were subsequently denatured in 8 M urea, 5 mM CAPS (pH 10.5), 5 mM EDTA, and 200 mM 2-ME, and then refolded to the native state by extensive dialysis against 20 mM Tris (pH 8.0). The folded protein was then concentrated and applied to an equilibrated anion exchange Q-Sepharose column (Q-Sepharose, HP, Pharmacia). After being extensively washed, the protein was eluted with a linear gradient of 0 to 1 M NaCl in 20 mM Tris (pH 8.0). Pro-plasmepsin II is activated by autocatalysis under acidic conditions. Autocatalysis was achieved by dialyzing pro-plasmepsin overnight against 10 mM sodium formate buffer (pH 4.0) at 4 °C. The completion of autocatalysis under these conditions was confirmed by SDS−PAGE.

Plasmepsins I and IV and HAP. Plasmepsin I was generated by a procedure similar to that of Moon et al. (*10*) as described in detail by Siripurkpong et al. (*11*). Briefly, pro-Plm I was expressed from pET3a as inclusion bodies in BL21(DE3) pLysS; inclusion bodies were solubilized, and the enzyme was purified, refolded, and autoactivated. Plasmepsin IV was generated by the procedure of Banerjee et al. (*1*). Briefly, pro-Plm IV was expressed from pET15b as inclusion bodies in BL21(DE3) pLysS; inclusion bodies were solubilized, and the enzyme was refolded, purified, and autoactivated. The full coding region of the HAP gene starting with the first methionine was cloned into pET22b, using the C-terminal hexahistidine tag. The plasmid was transformed into *E. coli* BL21(DE3) pLysS. Culture was induced at an OD of 0.7 with 0.4 mM IPTG for 4 h. Bacteria were harvested, and inclusion bodies were prepared with extensive Triton X-100, NaCl, and 2 M urea washes. The protein was solubilized overnight in 20 mM Tris (pH 8.0) containing 8 M urea. HAP was partially purified by Ni affinity chromatography (*12*). The eluate was refolded by dilution into water and was further purified by DEAE chromatography. The final preparation was active at acidic pH. The active enzyme was quantitated by pepstatin titration. The recombinant enzyme thus prepared had kinetics that could not be distinguished from those of the native enzyme using the fluorogenic peptide substrate (*1*).

Spectrophotometric Enzymatic Assays. The specific activity of plasmepsin II was determined by assessing the hydrolysis of varying concentrations of the chromogenic substrate Ala-Leu-Glu-Arg-Thr-Phe-nPhe-Ser-Phe-Pro-Thr-OH (California Peptide Research Inc., Napa, CA) at 25 °C. The decrease in absorbance was measured using a Cary 100 spectrophotometer (Varian Instruments). An extinction coefficient of 1736 M^{-1} cm^{-1} at 300 nm was used to convert the absorbance change to reaction rates. Inhibition constants (K_i) were estimated by fitting the data obtained at varying concentrations of inhibitor to standard equations for tight binding competitive inhibitors (*9*).

Isothermal Titration Calorimetry. ITC experiments were carried out using a high-precision VP-ITC titration calorimeter system (Microcal Inc.). Because of plasmepsin II aggregation under stirring conditions in the calorimetric cell, standard titrations could not be performed. Instead, binding enthalpies were obtained by injecting plasmepsin II into the reaction cell containing an excess of inhibitor. The heat evolved after each injection was obtained from the time integral of the calorimetric signal. The heat of dilution was obtained by injecting plasmepsin II into the same buffer solution [10 mM sodium formate (pH 4.0) and 2% DMSO] without the inhibitor. The heat due to the binding reaction between the inhibitor and enzyme was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Under these conditions, the binding enthalpy is equal to the binding reaction heat divided by the amount of protein injected.

Docking Procedures. The structure of KNI-10006 was generated using CS Chem3D Pro (Cambridge Software) and minimized with the MM2 force field implemented in the same program. The minimized KNI-10006 was docked in the Plm II binding site using the structure of Plm II in complex with the statine-based inhibitor IsoVal-Val-Val-Sta-OEt (PDB entry 1ME6). First, the statine inhibitor was replaced with KNI-10006 by aligning the hydroxyl group of allophenylnorstatine within hydrogen bonding distance of the catalytic residues, Asp34 and Asp214, and keeping the orientation of the allophenylnorstatine group similar to that of the statine moiety of the original inhibitor in the complex. Second, constraints were removed from the residues in the Plm II binding site (a 5 Å perimeter around the bound inhibitor), while the rest of the molecule was kept in a fixed position. Third, the allophenylnorstatine and dimethylthi-

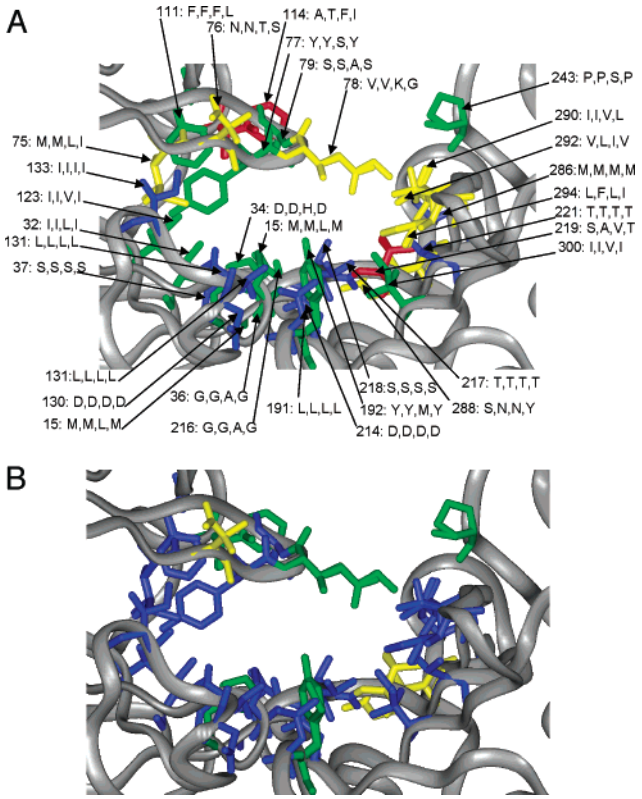

FIGURE 1: Distribution of amino acid heterogeneity within the binding site of the plasmpesins. The side chains of residues within the binding cavity of Plm I, II, and IV and HAP are shown using the backbone of Plm II as a common scaffold. (A) Residues that are identical in all four plasmepsins are blue. Residues identical in at least three plasmpesins are green and two plasmepsins yellow, and residues different in all plasmepsins are red. The labels indicate the identity of the residues in the following order: Plm I, Plm II, HAP, and Plm IV. (B) Residues in the binding cavity are color coded according to the nature of the polymorphism at each position. Residues shown in blue have similar polarity in all four plasmepsins. Residues shown in green have conserved polarity among at least three plasmepsins, while residues shown in yellow have conserved polarity among only two plasmepsins. There was no change in polarity or charge among all plasmepsins.

oproline scaffold were put under constraint. Finally, the P2 and P2′ moieties were then allowed to energy minimize using the Discover module of Insight II (Accelerys, San Diego, CA). The minimization took place using 100 000 iterative steps of the conjugate gradient algorithm.

RESULTS AND DISCUSSION

The feasibility of finding or designing an inhibitor capable of targeting several proteins with high affinity requires that the binding sites in all members of the target family share conserved regions against which the strongest interactions can be directed. Accordingly, the first step in the design of an adaptive inhibitor is the specification of a composite target site containing a precise description of the location and characteristics of conserved and variable regions in the target family. This composite site becomes the template for drug design. Figure 1 shows the composite plasmepsin binding cavity constructed by using the backbone of Plm II as a template and placing the side chains of the four plasmepsins at their corresponding site within the binding cavity. The composite plasmepsin site is characterized by the presence of regions that are identical in the four proteins, regions that

Table 1: Polymorphisms in the Binding Site of Plasmepsin I, II, and IV and HAP

Protein	Binding site residue number									
	15	32	34	36	37	75	76	77	78	79
Plm I	M	I	D	G	S	M	N	Y	V	S
Plm II	M	I	D	G	S	M	N	Y	V	S
HAP	L	L	H	A	S	L	T	S	K	A
Plm IV	M	I	D	G	S	I	S	Y	G	S
	111	114	123	130	131	133	191	192	214	216
Plm I	F	A	I	D	L	I	L	Y	D	G
Plm II	F	T	I	D	L	I	L	Y	D	G
HAP	F	F	V	D	L	I	L	M	D	A
Plm IV	L	I	I	D	L	I	L	Y	D	G
	217	218	219	221	243	286	288	290	292	294	300
Plm I	T	S	S	T	P	M	S	I	V	L	I
Plm II	T	S	A	T	P	M	N	I	L	F	I
HAP	T	S	V	T	S	M	N	V	I	L	V
Plm IV	T	S	T	T	P	M	Y	L	V	I	I

are identical in some of them, and regions that are different in each enzyme (Figure 1A). Furthermore, some regions within the binding site include very conservative amino acid polymorphisms that alter only the shape but not the chemical polarity or charge of those regions (Figure 1B and Table 1). Only a small area of the binding site contains polymorphisms with different polarities and none of them with opposite charge. The design rules are dictated by these characteristics. The most conserved regions should be targeted with a constrained molecular moiety capable of establishing strong and highly specific interactions. The inhibitor must also have the built-in capability to adapt to the variations found in the four plasmepsins. Two situations are possible. (1) If the amino acid polymorphisms are conservative and elicit only a shape distortion in the binding site, adaptation can be achieved by introducing asymmetric functional groups linked to the inhibitor core by rotatable bonds. (2) If the polymorphisms also change the polarity or charge, then asymmetric groups carrying different chemical functionalities can be introduced.

Once conserved and variable regions within the binding site are identified, the next step in the design of an adaptive inhibitor is the identification of a molecular scaffold that establishes strong interactions with the most conserved regions of the target site. Previously, we identified a series of allophenylnorstatine-based compounds with inhibitory activity against Plm II (*9*). The allophenylnorstatine scaffold (Figure 2) contains four different positions (R1−R4) where different chemical functionalities can be introduced. The core itself corresponds to the P1 position in the enzyme and contains three potential H-bond donors and three H-bond acceptors. R1 corresponds to the P2 position. The thioproline group together with R2 and R3 corresponds to the P1′ position. R4 corresponds to the P2′ position.

At the thermodynamic level, the interactions that contribute to the binding affinity are either enthalpic or entropic ($\Delta G = \Delta H - T\Delta S$). Favorable enthalpic contributions to the binding affinity originate mainly from van der Waals or hydrogen bonding interactions and are preferred over nonspecific hydrophobic interactions that contribute mostly to the binding entropy change (*4*, *5*, *13*−*15*). If the allophenylnorstatine core is docked into the structures of Plm II

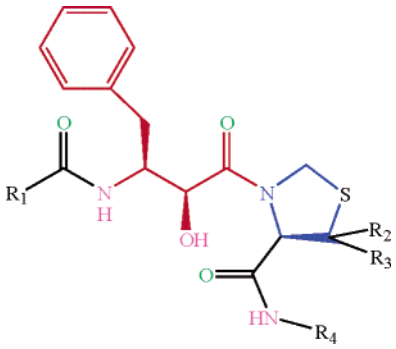

FIGURE 2: Common molecular scaffold of all KNI compounds which consists of an allophenylnorstatine moiety (red) and a thioproline ring (blue). This scaffold can be decorated at positions R1−R4 with different chemical functionalities. Potential core hydrogen donors are shown in magenta, while potential hydrogen acceptors are shown in green.

and Plm IV in a position similar to the position of other statine-based inhibitors such that the hydroxyl group remains within hydrogen bonding distance of the two catalytic aspartic residues, it can be seen that it interacts mostly with conserved regions. Strong interactions are established with seven residues at the bottom of the active site: Asp34, Gly36, Ser37, Tyr192, Thr217, Asp214, and Gly216. Of these residues, Ser37, Asp214, and Thr217 are identical in the four plasmepsins. Asp34, Gly36, Tyr192, and Gly216 are identical in three plasmepsins (I, II, and IV). In HAP, these four positions are occupied by His34, Ala36, Met192, and Ala216, respectively. The favorable binding enthalpy originates from a significant number of polar interactions. The interactions of the allophenylnorstatine core with the plasmepsins are mostly polar, involving three hydrogen bonds in both Plm II and IV.

While the region corresponding to the catalytic area is highly conserved among the four plasmepsins, other regions within the binding pocket are not. In particular, the region corresponding to P1′ and P2, opposite the opening of the flap (Figure 1), shows significant variability. Residues 288, 290, 292, and 294 are different in two of the four plasmepsins. Residues 290, 292, and 294 are hydrophobic in the four plasmepsins and cause only a geometric change that can be compensated by an asymmetric group. Residue 288, on the other hand, is Asn in Plm II and HAP and Ser and Tyr in Plm I and IV, respectively. Another variable region is the flap itself (residues 75−79). Residues 75, 76, and 78 are conserved in Plm I and II but variable in Plm IV and HAP. The change in residue 78 in HAP (Lys) involves a bulkier side chain as well as the addition of a positive charge. Residues 77 and 79 are conserved in Plm I, II, and IV. The side chains of residues 75 and 77 point toward the interior of the binding cavity, contributing to the heterogeneity at positions P1 and P2′. As expected, most of the variability is found in the histo-aspartic protease (HAP).

Since the allophenylnorstatine core interacts with conserved regions, different functionalities at R1−R4 provide an opportunity to impart adaptability to these inhibitors. Toward that goal, a diverse set of 77 allophenylnorstatine-based compounds (KNI compounds) containing different functionalities at R1−R4 were assayed for inhibitory activity against Plm II (inhibition data for the entire set are included as Supporting Information). Quantitative structure−activity correlations identified preferred moieties at different positions. At the core of these compounds, the preference for allophenylnorstatine was confirmed by additionally assaying phenylnorstatine, allonorstatine, and allocyclohexylnorstatine. This result was expected since allophenylnorstatine resembles the natural substrate of plasmepsin at this position (Phe33 of the hemoglobin α-chain) (*9*). Methyl groups at positions R2 and R3 of the thioproline ring significantly increased the potency of the inhibitor. Therefore, a core composed of allophenylnorstatine and dimethylthioproline was used in the search for a high-affinity inhibitor.

At R1, the preferred moiety among a large subset of aliphatic, polar, charged, and aromatic moieties was 2,6-dimethylphenyloxymethyl. Other substitutions on this phenyl ring (such as hydroxyl, amine, chloride, or a combination of those) also yielded tight binding inhibitors with inhibition constants in the low nanomolar range. The preferred moiety at R4 was 2-aminoindanol. This large and asymmetric group is connected to the thioproline ring via a rotatable bond. The best inhibitor, KNI-10006, incorporates 2,6-dimethylphenyloxymethyl at P2, allophenylnorstatine at P1, dimethylthioproline at P1′, and 2-aminoindanol at P2′ and displays a K_i of 0.5 nM against Plm II.

KNI-10006 contains aromatic groups at positions P1, P2, and P2′. Since these groups are joined by rotatable bonds to the core of the inhibitor molecule, they provide a potential mechanism for adaptation to the binding site variations found among the plasmepsins. Docking of KNI-10006 into Plm II suggests important interactions for these aromatic groups. The phenyl group at P1 is predicted to interact with residues at the back of the flap and residues in the hinge region (Figure 1). This region shows only minor variability among the four plasmepsins. The 2,6-dimethylphenyloxymethyl moiety at P2 faces the tip of the flap (residues 78 and 79) as well as the conserved region comprised of residues 216−218. It also faces the highly variable region containing residues 290 and 292. Residue 294 in this same region is opposite P1′. Since all the polymorphisms in this region are hydrophobic, the variability in this region involves only changes in pocket geometry that can be accommodated by bond rotations. Finally, the aminoindanol group at P2′ is expected to interact with conserved residues at the opening and bottom of the binding cavity (37−39 and 130−133), with residue 192, and with variable residues (75−77) in the flap. In particular, and depending on its orientation, the hydroxyl group has the potential to establish hydrogen bonding interactions with the OH group of Tyr192 in Plm I, II, and IV, with the backbone of Asn76 (Plm I and II), or with the side chains of Thr76 (HAP) or Ser76 (Plm IV). In the first case, the hydrogen bond cannot be satisfied with HAP, which has a Met at position 192, partially accounting for the lower affinity of KNI-10006 for this enzyme.

Microcalorimetric analysis (Figure 3) of a subset of the KNI compounds revealed that all of them bind to Plm II with favorable enthalpy, independent of the substitutions at positions R1−R4. This result is consistent with the conclusion of the docking simulation indicating that the KNI core establishes mostly polar interactions with conserved regions of the plasmepsins. KNI-10006 exhibited the highest binding affinity (0.5 nM) due to a combination of strong favorable contributions from the binding enthalpy (−5.4 kcal/mol) and

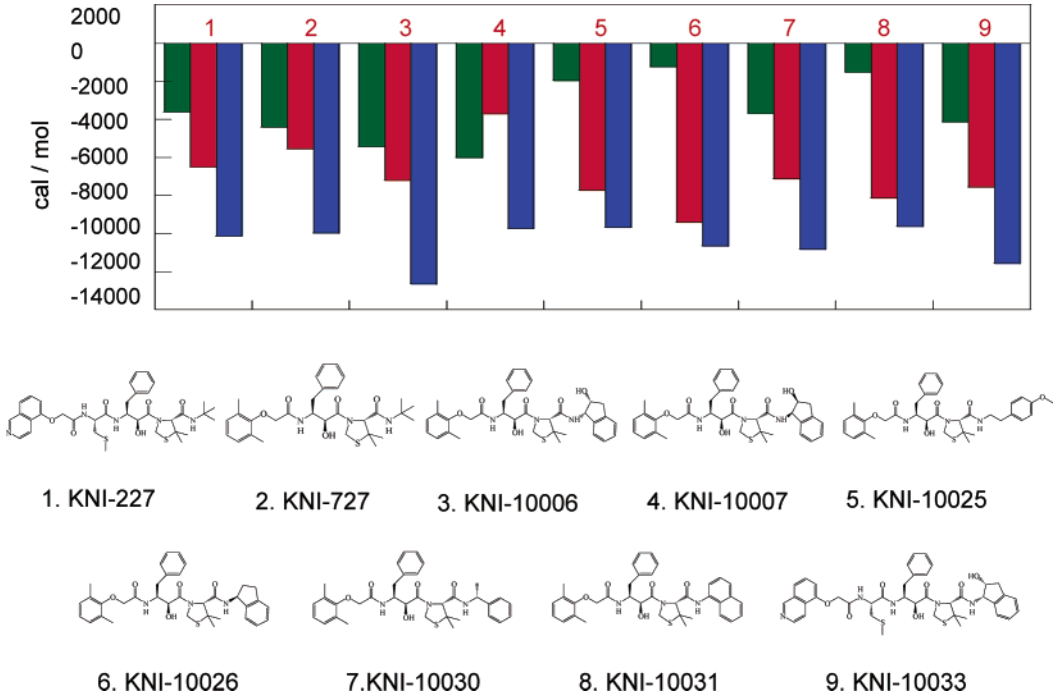

FIGURE 3: Binding energetics of a subset of KNI compounds to Plm II. The Gibbs energies of binding are shown with blue bars. The contributions of enthalpic forces (ΔH) are shown with green bars, and entropic contributions ($-T\Delta S$) are shown with red bars. All KNI compounds bind to Plm II with favorable (negative) enthalpic and entropic contributions.

binding entropy (−7.2 kcal/mol) to the Gibbs energy of binding. The hydroxyl group in the aminoindanol makes a significant contribution to the binding enthalpy and binding affinity (e.g., KNI-10026), consistent with its participation in a hydrogen bonding interaction.

The affinity of KNI-10006 for Plm II is ~2 orders of magnitude stronger than KNI-727, the best compound identified in a previous study (*9*). The only difference between these two compounds is that the aminoindanol group replaces the symmetric *tert*-butylamine group in KNI-727 at the same position. The enhanced affinity over KNI-727 is due to a combination of more favorable enthalpic and entropic contributions to the Gibbs energy of binding. The enthalpic advantage is due to better interactions with the target and the entropic advantage to a better desolvation of the complex. A required property of adaptive inhibitors is an extremely high affinity against the primary target and only a mild response to target heterogeneities. Unlike standard inhibitors, adaptive inhibitors do not rely on conformational constraints to achieve high binding affinity. In fact, the absence of conformational constraints at critical locations must provide the adaptation mechanism to target variations. KNI-10006 exhibits a subnanomolar affinity against Plm II despite having a conformationally flexible group at the R4 position. For those reasons, the inhibitory activity of KNI-10006 was measured against the entire plasmepsin family. Because of the limited amounts of the remaining plasmepsins, for comparison of relative inhibitory activity, IC_{50} values against Plm I, II, and IV and HAP were determined for KNI-10006 and KNI-727 under identical conditions (Figure 4). IC_{50} values are not directly comparable with the rigorously obtained K_i values and are used only to compare relative activities. IC_{50} values are larger in magnitude than K_i values because they are obtained without explicit consideration of free and total inhibitor concentrations. The results indicated that KNI-10006 inhibited Plm IV with a higher affinity than

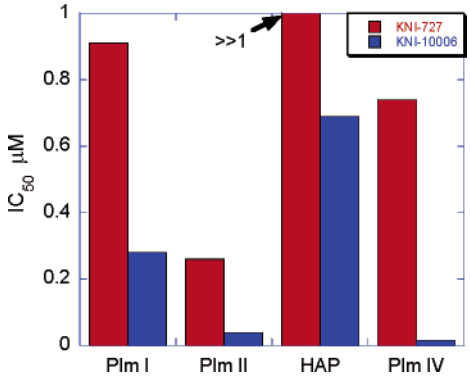

FIGURE 4: Inhibition of Plm I, II, and IV and HAP by KNI-10006 and KNI-727. The IC_{50} values for KNI-727 are 0.91, 0.26, 10.0, and 0.74 μM for Plm I, Plm II, HAP, and Plm IV, respectively. The IC_{50} values for KNI-10006 are 0.28, 0.039, 0.69, and 0.015 for Plm I, Plm II, HAP, and Plm IV, respectively. The assays were conducted in 0.1 M sodium acetate at pH 5.1 for Plm I, II, and IV and at pH 5.6 for HAP.

Plm II (IC_{50} ratio = 0.4) and that the inhibitory activity against Plm I was only decreased by a factor of 7 and against HAP by a factor of 17. KNI-727, on the other hand, performed relatively well against Plm I and IV (IC_{50} ratios of 3.5 and 2.8, respectively) but failed completely against HAP. KNI-10006 behaves well against all plasmepsins, including HAP. HAP is the most distantly related member of the family; less than 40% of its sequence is identical to that of Plm II at the active site (compared to 84% for Plm I and 68% for Plm IV). Interestingly, HAP is the only member of this family in which a bulky charged residue (Lys) has replaced the hydrophobic residue (Val in Plm I and II and Gly in Plm IV) at position 78. In addition, the substitution of one of the catalytic aspartates at position 34 with histidine, and Tyr192 with Met, might also contribute to the lower affinity of KNI-10006 against HAP.

Adaptability should be restricted to targets within a family and not against other enzymes, especially those of the same class. Additional experiments (T. Owens, unpublished data from this laboratory) indicate that the discrimination of KNI-10006 between Plm II and pepsin (0.5 nM for Plm II and 240 nM for pepsin) is comparable to that of ritonavir, a HIV-1 protease inhibitor in clinical use (0.1 nM for HIV-1 protease and 59 nM for pepsin). These results suggest the feasibility of designing ligands that bind with high affinity against closely related molecules but maintain a discrimination against more distant relatives comparable to that of compounds currently in clinical use.

CONCLUSIONS

The results presented in this paper provide strong experimental support to the proposition that high-affinity inhibitors can be developed against several members of a target family, provided that the inhibitor molecules have chemical functionalities that allow them to adapt to the variations existing in the target molecules. Since these adaptive functionalities necessarily contain rotatable bonds that allow them to adopt different conformations, high affinity is accomplished by strong enthalpic interactions between the inhibitor core and the target protein.

SUPPORTING INFORMATION AVAILABLE

Structures and data for compounds used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI034131Z