Molecular Recognition

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Water-Restructuring Mutations Can Reverse the Thermodynamic Signature of Ligand Binding to Human Carbonic Anhydrase

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Abstract: *This study uses mutants of human carbonic anhydrase (HCAII) to examine how changes in the organization of water within a binding pocket can alter the thermodynamics of protein–ligand association. Results from calorimetric, crystallographic, and theoretical analyses suggest that most mutations strengthen networks of water-mediated hydrogen bonds and reduce binding affinity by increasing the enthalpic cost and, to a lesser extent, the entropic benefit of rearranging those networks during binding. The organization of water within a binding pocket can thus determine whether the hydrophobic interactions in which it engages are enthalpy-driven or entropy-driven. Our findings highlight a possible asymmetry in protein–ligand association by suggesting that, within the confines of the binding pocket of HCAII, binding events associated with enthalpically favorable rearrangements of water are stronger than those associated with entropically favorable ones.*

Biomolecular recognition is a process influenced as much by rearrangements in the molecules of water that solvate interacting species as it is by the interactions between those species.[1–3] A detailed understanding of the mechanisms by which these rearrangements contribute to the thermodynamics of association between solutes is, thus, essential for predicting (and manipulating) the energetics of binding in biological systems.[4–6]

Many studies have investigated the role of water in protein–ligand interactions by examining the association of model proteins with sets of structurally varied ligands.[7–15] Such studies have revealed how the thermodynamic influence of water can differ between binding processes (for example, the entropy-driven association of nonpolar ligands with the well-hydrated S3/4 pocket of thrombin,[7] or the enthalpy-driven binding of nonpolar molecules to the poorly hydrated cavity of mouse major urinary protein[8]); they have not, however, illuminated the thermodynamic consequences brought about by systematic changes in the organization of water within a single pocket. An examination thus focused, could reveal how different hydration/rehydration processes alter the thermodynamic mechanisms by, and the overall affinities with, which proteins and ligands associate.

In this study, we used site-directed mutagenesis to rearrange water molecules within the binding pocket of human carbonic anhydrase II (HCAII, EC 4.2.1.1), a structurally rigid protein.[16] We combined isothermal titration calorimetry (ITC), X-ray crystallography, and molecular dynamics simulations to determine, and subsequently rationalize, the repercussions of those perturbations for the thermodynamics of HCAII–ligand association. We carried out our analysis with two arylsulfonamide ligands (Figure 1), 1,3-thiazole-2-sulfonamide (**TA**) and benzo[*d*]thiazole-2-sulfonamide (**BTA**). The additional benzo ring of **BTA** increases its binding affinity (relative to **TA**) through an enthalpically favorable hydrophobic interaction with the nonpolar wall of HCAII.[17] By examining the binding of **TA** and **BTA** to mutants of HCAII (with mutations localized in the binding pocket), we sought to establish the thermodynamic influence of mutations on protein–ligand association in this system. A comparison of binding between these two ligands, in turn, enabled us to examine how mutations affect the thermodynamics of hydrophobic association. (This study relies on the benzo-extension strategy, which we have described and exploited previously[17]).

To construct variants of HCAII with different organizations of water in their binding pockets, we used site-directed mutagenesis to make amino acid substitutions that increased or decreased the size or polarity of residues in both its polar wall and its nonpolar wall (Figure 1; Supporting Information, Methods). We chose residues within 5 Å of **BTA** in the HCAII–**BTA** complex that, when mutated, yielded titers of HCAII ($>100\,mg\,L^{-1}$) sufficient for calorimetric studies, which require large amounts of protein. To examine effects over large distances, we also mutated one residue (N67) located over 6 Å from **BTA**. We reasoned that these substitutions might alter the thermodynamics of protein–ligand association by reducing or enhancing the total amount of water in the binding pocket, and/or by changing the thermodynamic properties of the water filling it.

We examined the influence of amino acid substitutions on the thermodynamics of protein–ligand association by using ITC to determine the enthalpy, entropy, and free energy of

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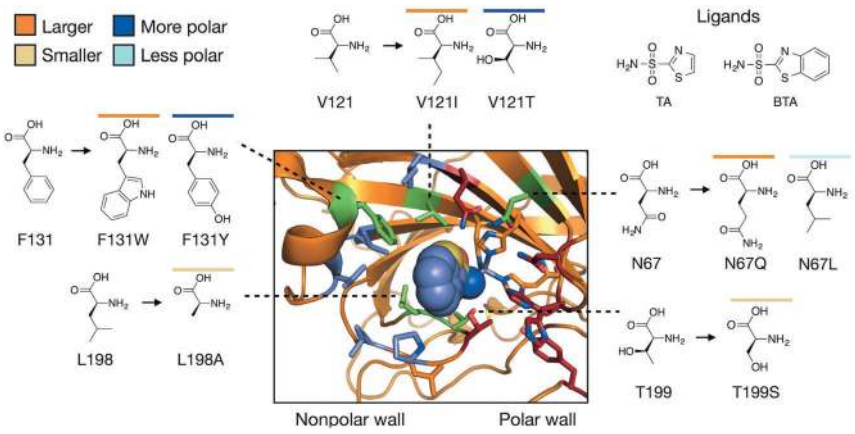

Figure 1. Experimental design. The center image depicts the structure of the active site of HCAII complexed with **BTA** (PDB ID: 3S73). Residues are colored as follows: nonpolar wall (purple), polar wall (red), and mutation sites (green). Ligands appear on the upper right: 1,3-thiazole-2-sulfonamide (**TA**) and benzo[*d*]thiazole-2-sulfonamide (**BTA**), a benzo-extended variant of **TA**.

binding ($\Delta H°_b$, $-T\Delta S°_b$, and $\Delta G°_b$) for each combination of ligand and mutant (Supporting Information, Table S2). Figure 2A plots the difference in thermodynamic binding parameters between mutant and wild-type proteins ($\Delta\Delta J°_{b\text{-mut}} = \Delta J°_{b\text{-mutant}} - \Delta J°_{b\text{-WT}}$, where $J = G$, H, or TS). Most mutations brought about nearly compensating changes in enthalpy and entropy of binding, a phenomenon termed enthalpy/entropy (H/S) compensation;[18] they caused $\Delta H°_b$ to become more positive (more unfavorable) and $-T\Delta S°_b$ to become more negative (more favorable). When we combined four of the mutations for which these changes were most pronounced (N67Q, L198A, V121T, and F131Y) into five double mutants (N67Q/L198A, N67Q/V121T, N67Q/F131Y, L198A/F131Y, and V121T/F131Y) and one triple mutant (N67Q/V121T/F131Y), H/S compensation remained the same or increased. This conservation/additivity confirms that H/S compensation in our system is not the result of experimental error (Supporting Information, Appendices 1–2).

A plot of $-T\Delta\Delta S°_{b\text{-mut}}$ against $\Delta\Delta H°_{b\text{-mut}}$ for **TA** and **BTA** indicates that these two parameters are linearly correlated for both ligands and fall onto the same line (slope $= -0.71$ and $R^2 = 0.98$; Supporting Information, Figure S3). This line suggests that all mutations influence the thermodynamics of protein–ligand association through a similar mechanism, one that 1) has the same relative influence on enthalpy and entropy, regardless of its overall magnitude of influence, and that 2) can be exerted from all sides of the binding pocket. One possible mechanism that satisfies (1) and (2) involves perturbations of water-mediated networks of hydrogen bonds. Several studies have suggested that hydrogen bonds, within the range of bond strengths likely to be encountered in hydrated proteins, have enthalpies and entropies of formation (relative to bulk water) that are linearly correlated.[19–21]

To examine how mutations might alter the thermodynamic properties of water in the binding pocket, we used X-ray crystallography to collect crystal structures of a subset of mutant–**BTA** complexes, and we used WaterMap (Schrödinger Inc.[22–24]) to calculate the enthalpy and entropy of water (that is, the change in enthalpy and entropy associated with the transfer of a molecule of water from the bulk) in crystallographically determined binding pockets with and without **BTA** bound.[25,26] Figure 2B compares values of $\Delta\Delta J°_{b\text{-mut}}$ (where $J = H$ or TS) determined using ITC with values estimated using WaterMap for the nine mutants for which we collected crystal structures of HCAII–**BTA** complexes (Supporting Information, Table S5). The two sets of parameters follow similar trends, suggesting that the influence of mutations on the thermodynamic properties of water in the binding pocket (the only influence for which WaterMap calculations account) causes these trends.

A formal comparison of ITC- and WaterMap-based estimates of mutation-derived changes in enthalpy and entropy appears in the Supporting Information, Figure S6. Correlations are slightly stronger for enthalpy ($r = 0.89$) than for entropy ($r = 0.66$), but both sets of parameters are linearly correlated with $P < 0.01$. Interestingly, measured and estimated values of $\Delta\Delta G°_{b\text{-mut}}$ are weakly correlated with $P = 0.06$, suggesting a link between discrepancies in enthalpy and entropy. These comparisons suggest that computational approaches such as WaterMap are better able to predict the enthalpic contributions (as opposed to the entropic contributions) of water to binding, and they show that errors associated with both contributions can sum (rather than cancel) to create significant errors in predicted free energies.

Previous studies have shown that mutations can alter the thermodynamics of protein–ligand association by altering the conformation of the protein and/or protein–ligand complex.[27,28] In this study, by contrast, three observations suggest that mutations do not bring about major changes in protein structure or dynamics: 1) Crystal structures of mutant–ligand complexes, when aligned, have root-mean-square deviations of 0.21–0.23 Å throughout the protein and 0.10–0.16 Å within the active site (Supporting Information, Table S9), suggesting that mutations do not cause major changes in protein conformation. 2) The orientation of the sulfonamide group of **BTA** and **TA** is unperturbed across mutants, suggesting that mutations do not alter direct hydrogen bonds between the protein and ligand (Supporting Information, Appendix 4). 3) WaterMap results, which do not take changes in protein conformation into account, reveal trends in thermodynamic parameters similar to those observed in our experimental measurements.

The mechanisms by which mutations reorganize water are apparent in the WaterMap-predicted hydration sites near the amino acid substitution for which H/S compensation was most pronounced: L198A (Figure 2C). Figure 2C suggests that the leucine-to-alanine mutation enlarges and stabilizes a network of water molecules near the nonpolar wall. During HCAII–**BTA** association, **BTA** distorts but does not fully displace this network, triggering a rearrangement of water that is more

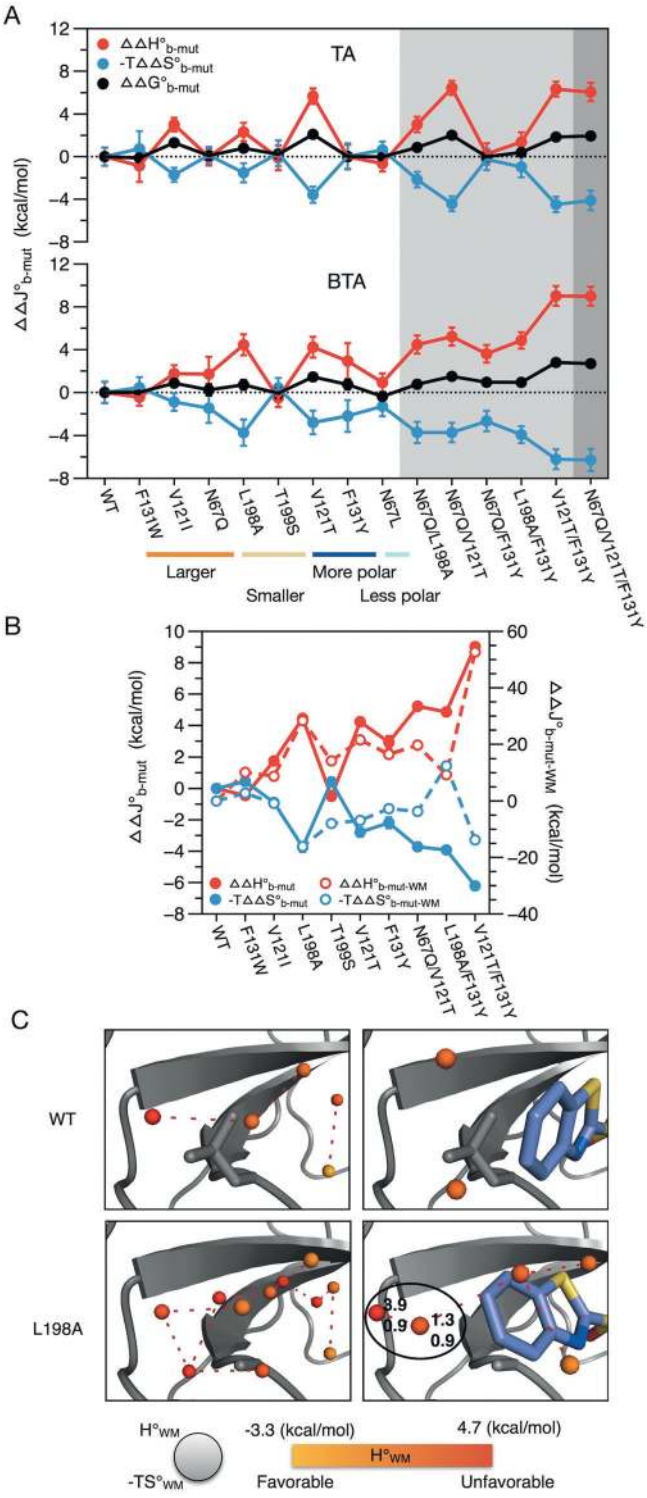

Figure 2. The effect of mutations. A) Differences in the thermodynamic binding parameters of mutants and wild-type HCAII, $\Delta\Delta J°_{\text{b-mut}} = \Delta J°_{\text{b-mutant}} - \Delta J°_{\text{b-WT}}$. Most mutations cause $\Delta H°_{\text{bind}}$ to become more positive and $-T\Delta S°_{\text{bind}}$ to become more negative in a nearly compensating fashion. B) A comparison of values of mutation-derived changes in enthalpy and entropy of binding determined using ITC (left axis) and WaterMap calculations (right axis). Similar trends between the two sets of parameters suggest that the influence of mutations on the thermodynamic properties of water causes these trends. (Error bars in (A) and (B) represent standard error, $n \geq 7$). C) The influence of L198A on the thermodynamic properties of water. Colors are as follows: Protein (gray) and **BTA** carbon (purple), nitrogen (blue), and sulfur atoms (yellow). Molecules of water appear as spheres colored according to their enthalpies ($H°_{\text{WM}}$), relative to bulk water. Plausible hydrogen bonds (intermolecular distances ≤ 3 Å) appear as dashed red lines. The leucine-to-alanine mutation strengthens a network of water near the nonpolar wall. During HCAII–**BTA** association, this network undergoes an enthalpically unfavorable rearrangement (circle). (The circle highlights two enthalpically unfavorable waters). We used X-ray crystal structures for the WaterMap calculations depicted in (B) and (C).

enthalpically unfavorable (and more entropically favorable) than the rearrangement of water associated with the binding of **BTA** to wild-type HCAII. Results from WaterMap calculations, thus, suggest that mutations can enhance the enthalpic cost (and entropic benefit) of rearranging water-mediated networks of hydrogen bonds during protein–ligand association by strengthening those networks in the unliganded binding pocket.

Our mechanism analysis highlights an important asymmetry in our system; namely, that mutations tend to make $\Delta H°_{\text{b}}$ less favorable and $-T\Delta S°_{\text{b}}$ more favorable but not the reverse. Our results suggest that a reversed form of H/S compensation (more favorable $\Delta H°_{\text{b}}$ and less favorable $-T\Delta S°_{\text{b}}$) could result from mutations that weaken networks of water molecules in the unliganded binding pocket. The two mutations most likely to cause such an effect (N67L and F131W, which by making the polar wall or the nonpolar wall, respectively, less hydrophilic, could weaken networks of water solvating them), however, have essentially imperceptible thermodynamic influences. The nearly unidirectional nature of H/S compensation in our system, thus, suggests that mutations can strengthen networks of water over the nonpolar wall more easily than they can disrupt networks over either wall. Future work examining the applicability of this generalization to different ligands, especially those involving charged or nonplanar functionalities, would be enormously interesting.

In one emerging theory, the organization of water within the binding pocket of a protein dictates the thermodynamic signature of the hydrophobic effect.[11,12,17,29,30] Hydrophobic interactions associated with large favorable changes in entropy, for example, might involve the expulsion of well-ordered molecules of water from the binding pocket, while those associated with large favorable changes in enthalpy might result from the expulsion and/or reorganization of poorly-ordered water molecules. To examine the influence of mutations on the thermodynamic signature of the hydrophobic effect in our system, we calculated the difference in thermodynamic binding parameters for **BTA** and **TA** ($\Delta\Delta J°_{\text{b-benzo}} = \Delta J°_{\text{b-BTA}} - \Delta J°_{\text{b-TA}}$), a difference associated with the hydrophobic interaction between the benzo ring of **BTA** and the nonpolar wall of HCAII. The results of this analysis show, surprisingly, that the thermodynamic signature of this interaction differs between mutants (Figure 3A); for most mutants, it is enthalpy-driven (that is, enthalpically favorable and entropically unfavorable), but for two mutants (N67Q/F131Y and L198A/F131Y), it is entropy-driven.

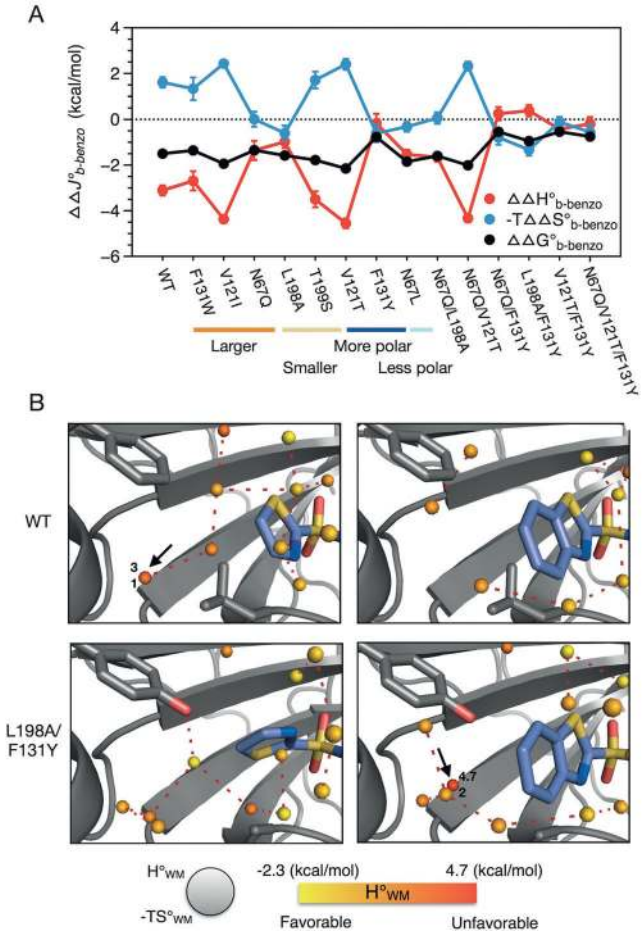

Figure 3. Hydrophobic association. A) Differences in the thermodynamic binding parameters for **BTA** and **TA** ($\Delta\Delta J°_{\text{b-benzo}} = \Delta J°_{\text{b-BTA}} - \Delta J°_{\text{b-TA}}$) reveal the thermodynamic signature of hydrophobic association between the benzo ring of **BTA** and the nonpolar wall of HCAII. B) Results of WaterMap calculations for L198A/F131Y (colored as in Figure 2C). In the wild-type, the binding of **TA** traps an enthalpically unfavorable water molecule near the nonpolar wall (arrow); when **BTA** binds, its benzo ring releases this molecule and brings about an enthalpically favorable and entropically unfavorable rearrangement of water. In L198A/F131Y, mutations stabilize a network of water over the nonpolar wall. The binding of **TA** leaves much of this network intact, but when **BTA** binds, the benzo ring forces an enthalpically unfavorable rearrangement of its constituent water molecules. (The arrow indicates a particularly enthalpically unfavorable water molecule). We used X-ray crystal structures for the WaterMap calculations depicted in (B).

We examined the mechanisms by which mutations reversed the thermodynamic signature of hydrophobic association by comparing WaterMap-predicted hydration sites in two variants of HCAII with **TA** and **BTA** bound, wild-type and L198A/F131Y (Figure 3B). (A full comparison of $\Delta\Delta J°_{\text{b-benzo}}$ and $\Delta\Delta J°_{\text{b-benzo-WM}}$ for all double mutants appears in the Supporting Information, Figure S5). When **TA** is bound to wild-type HCAII, there is an enthalpically unfavorable molecule of water near the nonpolar wall; when **BTA** binds, its benzo ring releases this molecule and brings about an enthalpically favorable rearrangement of water molecules. The resulting hydrophobic effect between the benzo ring and the nonpolar wall is enthalpy-driven. In L198A/F131Y, mutations stabilize a network of water over the nonpolar wall. The binding of **TA** leaves much of this network intact; when **BTA** binds, however, the benzo ring forces an enthalpically unfavorable and entropically favorable rearrangement of its constituent water molecules. The resulting hydrophobic effect is entropy-driven.

Enthalpy-driven hydrophobic effects are often attributed to van der Waals interactions between opposing nonpolar surfaces.[31–33] To determine if differences in van der Waals interactions between mutants and **BTA** are responsible for the differences in the thermodynamics of binding, we compared values of $\Delta G°_{\text{b-BTA}}$ with values of $\Delta SASA_{\text{bind-ligand}}$ ($\Delta SASA_{\text{bind-ligand}} = SASA_{\text{ligand-free}} - SASA_{\text{ligand-bound}}$; Supporting Information, Appendix 5), the loss in solvent-accessible surface area of the ligand that occurs during binding, for single mutants for which we had crystal structures. Interestingly, the values of $\Delta SASA_{\text{bind-ligand}}$ differed by less than 21 Å^2 between mutants and showed no correlation with $\Delta G°_{\text{b-BTA}}$ ($P < 0.01$), suggesting that differences in the thermodynamics of HCAII–**BTA** association do not arise primarily from differences in van der Waals contact area between the ligand and protein.

The results of this study suggest a possible strategy for enhancing the strength of biomolecular interactions in which water, or, more specifically, its reorganization during binding, exerts a dominant influence on the thermodynamics of association. The influence of mutations on free energy and enthalpy of binding is positively correlated (slope of 0.29 and $P < 0.01$; Figure 4). While one might expect free energy and enthalpy to be correlated in any system with H/S compensation, the nature of the correlation in this system (positive), and our ability to attribute it primarily to the influence of mutations on local water networks, has a specific implication; namely that enthalpically favorable rearrangements of water molecules within the binding pocket of HCAII give rise to stronger protein–ligand association than entropically favorable ones. (The differential influence of such rearrangements

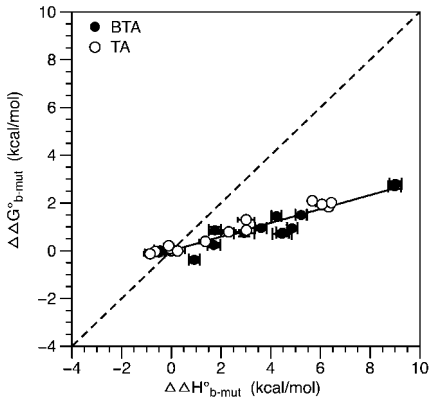

Figure 4. The influence of mutations on binding affinity. Plot showing the influence of mutations on the free energy of binding against their influence on the enthalpy of binding ($\Delta\Delta G°_{\text{b-mut}}$ against $\Delta\Delta H°_{\text{b-mut}}$ from Figure 2A). The black line through these points represents a linear fit (slope = 0.29 and $R^2 = 0.90$, $n \geq 7$; error bars represent standard error). Mutations that increase the enthalpic cost of binding lower binding affinity (that is, make $\Delta G°_{\text{b}}$ more positive).

on HCAII–ligand association rate, which, in light of recent evidence,[34] might correlate with ligand hydrophobicity, represents an interesting direction for a future study.)

Enthalpy-driven hydrophobic effects and entropy-driven hydrophobic effects, both of which occur in biology, are often treated as two different versions of the same phenomenon.[35] The results of this study, however, suggest that these two effects, at least within the confines of the binding pocket of HCAII, can have nonequivalent repercussions for free energy and, in fact, provide a thermodynamic rationale for evolution to favor one variety (in this case, enthalpic) in interactions for which tighter binding is advantageous, and for which water exerts a dominant influence on the strength intermolecular association. The applicability of this generalization to other binding pockets merits further investigation.

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Conflict of interest

The authors declare no conflict of interest.

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