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Computational Design and Discovery of Conformationally Flexible Inhibitors of Acetohydroxyacid Synthase to Overcome Drug Resistance Associated with the W586L Mutation

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Acetohydroxyacid synthase (AHAS, also known as acetolactate synthase, EC 2.2.1.6 (formerly EC 4.1.3.18)) has attracted attention for many years as a potential target for inhibitors to be used as herbicides and antibiotics.[1] Despite the great success of commercial AHAS inhibitors over the past decades, drug resistance has become one of the most serious problems to overcome.[2] In most cases, resistance to AHAS-inhibiting products has been shown to be caused by an alteration in the AHAS enzyme itself. Single point mutations that confer resistance to AHAS inhibitors include A117T, P192A, P192S, P192E, P192L, A200V, and W586L (*Saccharomyces cerevisiae* AHAS residue numbering).[2a] Among these, W586L is the most comprehensively characterized mutation, which results in at least 10-fold resistance to all types of AHAS inhibitors.[2d] Therefore, the design of novel compounds that block the activity of the W586L mutant form has become one of the biggest challenges in this field. Herein we report the first computational design that has led to the discovery of 2-aroxyl-1,2,4-triazolo[1,5-*c*]pyrimidines that have the same level of inhibitory activity against both wild-type AHAS and its W586L mutant. The present study demonstrates that the computational design approach based on the analysis of ligand conformational flexibility in the binding pocket holds promise for the rational design of conformationally flexible inhibitors of enzymes to overcome drug resistance.

A general understanding of the molecular mechanisms behind some representative commercially available AHAS inhibitors provides useful clues for further molecular design against drug resistance. Three structurally diverse AHAS inhibitors, chlorsulfuron (CS), bispyribac (BP), and flumetsulam (FS) (Figure 1) were considered for this purpose.

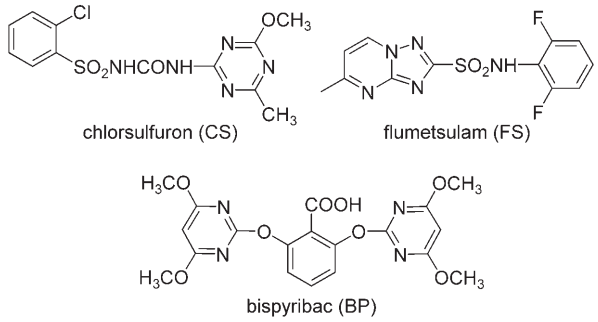

Figure 1. Some representative commercial AHAS inhibitors.

It has been reported that the W586L mutation confers ~1000-fold greater resistance toward CS and FS, whereas the same mutation gives a ~10-fold increase in resistance against BP.[2a,d–f] We were interested in comparing the conformational changes of these ligands upon binding to the W586L mutant form relative to wild-type. Although several crystal structures of sulfonylurea–AHAS complexes have been reported,[3] there is no crystal structure available for sulfonamide–AHAS or salicylate–AHAS complexes. Therefore, we established three-dimensional models for the BP–AHAS and FS–AHAS complexes by integrating molecular docking and dynamics simulations (MD). The reported crystal structure[3c] (2.19 Å) of yeast AHAS in complex with CS was used as the initial structure for the MD simulation, whereas the initial structures of the BP–AHAS and FS–AHAS complexes were obtained by respectively docking BP and FS into the binding pocket of the crystal structure of the yeast CS–AHAS complex after removal of CS. The resulting docking complexes with the highest scores were subjected to MD simulations[4] for 1 ns or longer to ensure that a stable MD trajectory for each simulated wild-type complex was obtained. The mutant complexes were then obtained by directly mutating residue 586 of the equilibrated wild-type complexes from Trp to Leu. The structures associated with the mutant were then subjected to energy minimizations and MD simulations to ensure that the simulated systems reached equilibrium. To examine the reliability of our theoretical models, we carried out kinetics studies according to the reported method[1f] for CS, BP, and FS with *Escherichia coli* wild-type and W464L mutant AHAS II (corresponding to the W586L mutant of *S. cerevisiae* AHAS) under the same conditions (Table 1). The respective K_i

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Table 1. Results of experimental kinetics characterization and computational binding free energies.

Compd	K_i [$\times 10^{-5}$ M][a]		ΔG kcal mol^{-1}][b]		$\Delta(\Delta G)$	
	WT	W464L	WT	W586L	Calcd[c]	Exptl[d]
CS	0.032	36.40	−9.20	−5.06	4.14	4.17
BP	2.40	24.60	−7.40	−5.91	1.49	1.38
FS	0.19	140.00	−5.60	−2.21	3.39	3.93
1	5.51	694.00	−6.24	−3.81	2.43	2.87
2	5.47	7.45	−5.75	−5.44	0.31	0.18
3	6.25	6.44	−5.90	−5.61	0.29	0.02

[a] Determined with *E. coli* AHAS II; the W464L mutant of *E. coli* AHAS II is equivalent to the W586L mutant of *S. cerevisiae* AHAS. [b] The ligand binding free energy was calculated using the MM-PBSA method. [c] $\Delta(\Delta G) = \Delta G_{W586L} - \Delta G_{WT}$. [d] Obtained according to $\Delta\Delta G_{exp} = -RT\ln(K_{i\,W464L}/K_{i\,WT})$.

values of CS, BP, and FS are 3.22×10^{-7} M, 2.40×10^{-5} M, and 1.85×10^{-6} M with the wild-type enzyme, and 3.64×10^{-4} M, 2.46×10^{-4} M, and 1.40×10^{-3} M with the mutant. According to the equation of $\Delta\Delta G_{exp} = -RT\ln(K_{i\,mutant}/K_{i\,WT})$, the experimental binding free energy changes for CS, BP, and FS were then calculated to be 4.17, 1.38, and 3.93 kcal mol^{-1}, respectively. The corresponding binding free energy changes predicted for these three inhibitors using the MM-PBSA protocol used in our previous studies[5] are respectively 4.14, 1.49, and 3.39 kcal mol^{-1}. The qualitative agreement between the computational and experimental results confirms the reliability of our computational models.

The resistance mechanism could be rationalized according to the MD-simulated structural models. As shown in Figure 2, CS and FS did not undergo any clear conformational change with the W586L mutation, and the atomic root-mean-square deviation (RMSD) values of the conformational superposition are 0.67 for CS and 0.81 for FS (see Figure 3s in the Supporting Information). As a result, the π–π stacking interactions between residue W586 and the heterocyclic plane disappeared, which explains the high level of resistance against sulfonylurea and sulfonamide compounds. In contrast, BP underwent clear a conformational change with the W586L mutation, and the RMSD value (6.48) of the conformational superposition was much higher than that of CS and FS. At the same time, some interactions with residues K251, M354, and G658 were improved significantly to partly compensate the loss of π–π stacking interactions with W586 (see Figure 4s in the Supporting Information), which explains why resistance against BP is much lower than that against CS and FS. Additionally, the interaction analysis shown in Figure 2 indicates that residue R380 forms strong hydrogen bonds with the bridges ($SO_2NHCONH$ and SO_2NH) of CS and FS. However, no H-bonding interaction was observed between R380 and the bridging oxygen atom of BP. Thus, we hypothesized that the H-bonding interactions with the bridge decreases the flexibility of the ligand, thus limiting its ability to adapt to the conformational change of the binding pocket to form new interactions. Therefore, from the standpoint of combating resistance, an oxygen atom might be a more flexible bridge than the $SO_2NHCONH$ and SO_2NH groups.

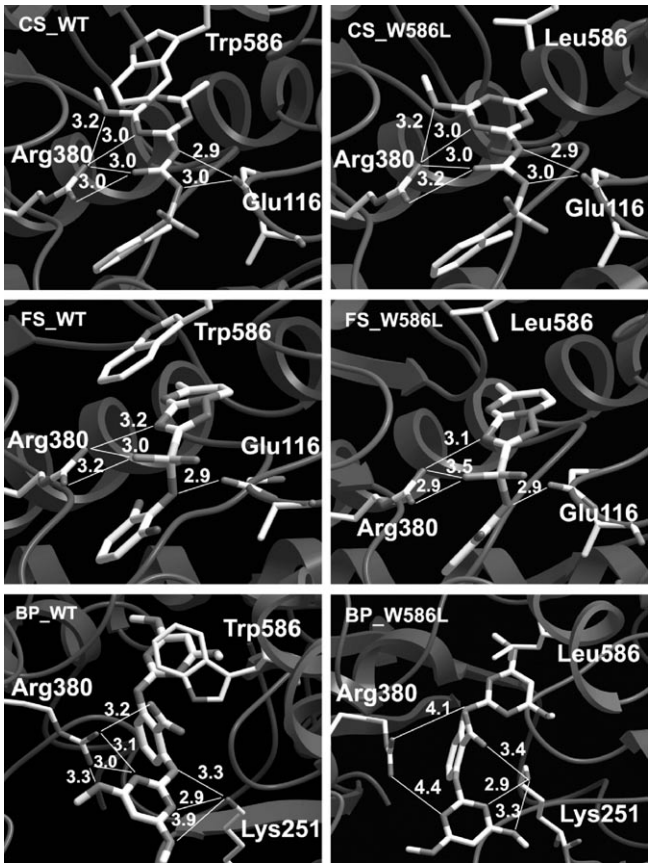

Figure 2. Comparison of the MD-simulated models of the wild-type and W586L mutant in complex with CS, BP, and FS.

We proposed that improving the flexibility of the ligand in the binding pocket should result in a higher binding affinity for the mutant enzyme. We initially designed compound **1** by bridging the phenyl and triazolopyrimidinyl moieties with an oxygen atom,[6] and the result was expected to be less prone to resistance than CS and FS (Figure 3). As shown in Table 1,

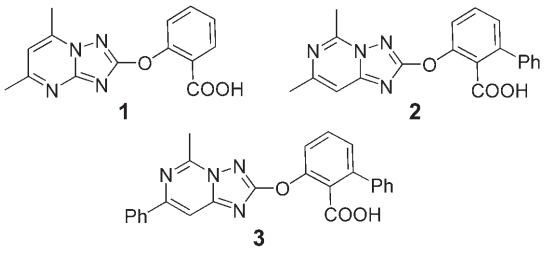

Figure 3. Structures of newly designed compounds **1**–**3**.

the K_i values of compound **1** against wide-type and W586L AHAS were determined to be 5.51×10^{-5} M and 6.94×10^{-3} M, respectively. These results indicate that for compound **1**, the mutant is only ~126-fold more resistant than the wild-type, which is much less than the case for CS and FS (~1000-fold). As shown in Figure 4, the interaction analysis revealed that there is no H-bonding interaction between R380 and the

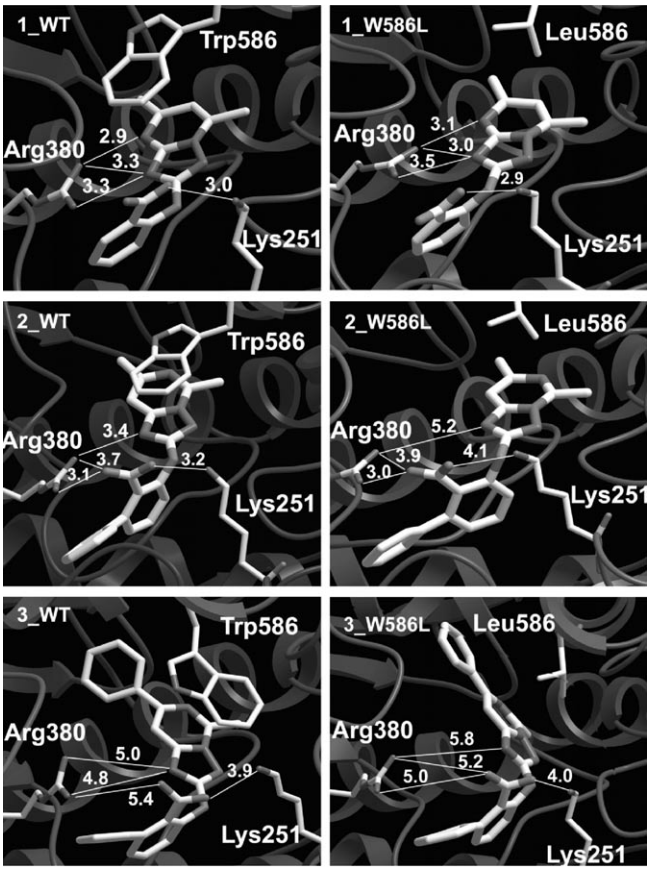

Figure 4. Comparison of the MD-simulated models of the wild-type and W586L mutant in complex with compounds **1**, **2**, and **3.**

bridging oxygen atom, but unfortunately, a new H bond between R380 and N8 is formed, which is still present in the binding pocket of W586L mutant in complex with **1**. Therefore, compound **1** was observed to be locked and unable to change its conformation to adapt to the new binding environment, eventually leading to resistance.

To eliminate this newly formed H bond, N8 of compound **1** was moved to position 6 to generate the new compound **2**,[6] which, as expected, has greater flexibility owing to loss of the H bond between R380 and the triazolopyrimidine ring. With the W586L mutation, compound **2** undergoes a clear conformational change, and the interactions of the heterocyclic ring of compound **2** with residues F201, G116, K251, P192, V191, D379, A117, Q202, A195, L119, G115, and A200 became much stronger, which in turn compensate the loss of π–π stacking interactions with W586. Therefore, compound **2** maintains nearly the same level of binding affinity against the mutant ($K_i = 7.45 \times 10^{-5}$ M) as it does for the wild-type enzyme ($K_i = 5.47 \times 10^{-5}$ M).

These results encouraged us to make a further structural modification to compound **2**. As shown in Figure 4, L586, having a hydrophobic side chain, is very close to the methyl group at position 7 of compound **2**. Thus, with the introduction of a phenyl group at this position, an additional hydrophobic interaction between L586 and the phenyl group at position 7 should further improve the binding affinity of the compound. Indeed, compound **3** interacts stronger with L586 than compound **2** (see Figure 4s in Supporting Information), and the K_i value (6.44×10^{-5} M) of compound **3** against the mutant AHAS is a little lower than that (7.45×10^{-5} M) of compound **2**. The experimental results of kinetics studies and the binding free energy calculations using the MM-PBSA method are summarized in Table 1. As shown in Figure 5, the correlation coefficient (R^2) between the computational and experimental $\Delta(\Delta G)$ values is 0.9887, suggesting that the present computational protocol for rational design is promisingly robust.

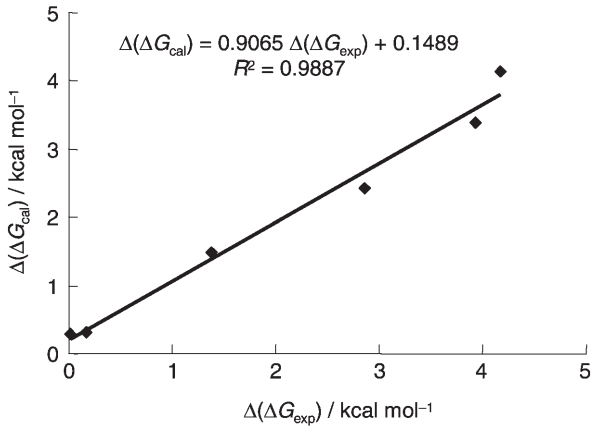

Figure 5. Correlation between the computational and experimental binding free energy changes ($\Delta(\Delta G)$).

In conclusion, we have successfully designed and synthesized a series of 2-aroxyl-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as conformationally flexible AHAS inhibitors by integrating the methods of molecular dynamics simulations, binding free energy calculations, and conformational flexibility analysis. The kinetic characterization experiments give K_i values of 5.47×10^{-5} M and 6.25×10^{-5} M against the wild-type enzyme, and 7.45×10^{-5} M and 6.44×10^{-5} M against the W586L mutant for compounds **2** and **3**, respectively. Notably, the K_i values of most commercial AHAS inhibitors range from 10^{-4} M (imidazolinone-type inhibitors) to 10^{-7} M (sulfonylurea-type inhibitors).[2b] Therefore, the very similar inhibitory activity data of compounds **2** and **3** against the wild-type AHAS and its W586L mutant shown that compounds **2** and **3** could be used as new leads for the future development of anti-resistance herbicides. Additionally, the excellent agreement between the computational and experimental data suggests that the computational design protocol based on conformational flexibility analysis is promising for the rational design of enzyme inhibitors to overcome drug resistance.

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[4] See the Supporting Information for details concerning the molecular dynamics simulations.

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[6] Detailed synthetic procedures for compounds **1**, **2**, and **3** are available in the Supporting Information.

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