Azanitrile Inhibitors of the SmCB1 Protease Target Are Lethal to Schistosoma mansoni: Structural and Mechanistic Insights into Chemotype Reactivity

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ABSTRACT: Azapeptide nitriles are postulated to reversibly covalently react with the active-site cysteine residue of cysteine proteases and form isothiosemicarbazide adducts. We investigated the interaction of azapeptide nitriles with the cathepsin B1 drug target (SmCB1) from Schistosoma mansoni, a pathogen that causes the global neglected disease schistosomiasis. Azapeptide nitriles were superior inhibitors of SmCB1 over their parent carba analogs. We determined the crystal structure of SmCB1 in complex with an azapeptide nitrile and analyzed the reaction mechanism using quantum chemical calculations. The data demonstrate that azapeptide nitriles, in contrast to their carba counterparts, undergo a change from noncovalent and covalent complex formation. Finally, azapeptide nitriles were considerably more lethal than their carba analogs against the schistosome pathogen in culture, supporting the further development of this chemotype as a treatment for schistosomiasis.

KEYWORDS: azapeptide inhibitors, cysteine proteases, protein structures, structure–activity relationships, schistosomiasis

Azapeptides, peptides in which the CαH of at least one amino acid has been replaced with nitrogen, have emerged as particularly important peptidomimetic structures (Figure 1). Compared to their parent carbapeptide analogs, bioactive azapeptides can possess improved potency and target selectivity as well as superior pharmacokinetics.1−5 Azapeptide nitriles were introduced as a class of efficient inhibitors of human cysteine cathepsins.6−9 This chemotype supported the successful development of activity-based probes, modified for organelle-specific delivery to lysosomal cysteine proteases and applied as PET-imaging agents for tumor-associated cathepsin activity.10−12 These reports have highlighted that azapeptide nitriles can enrich the portfolio of inhibitors of cysteine proteases suitable as activity-based probes13,14 and potential therapeutics against parasitic and protozoal infections.15−18

Similar to the well-established dipetide nitriles, azapeptide nitriles are thought to undergo a covalent, reversible interaction with the target proteases by forming a stabilized isothiosemicarbazide adduct (Figure 1).6−12

Here, we investigated the interaction of azapeptide nitriles with cathepsin B1 (SmCB1) from the human blood fluke, Schistosoma mansoni.19,20 Trematode flatworms of the genus Schistosoma cause schistosomiasis (bilharzia), a chronic disease that infects over 200 million people in tropical and subtropical areas.21 As treatment and control of schistosomiasis rely on just one drug, praziquantel, there is impetus to identify new antischistosomals.22−26 The cysteine protease SmCB1 is a central digestive enzyme of the parasite and has been validated as a chemotherapeutic target for the cure of schistosomiasis.22,23 Previously, we determined the crystal structure of SmCB1 as a footing for rational drug development.23,27,28

In this study, we compared the functional properties of dipetides with azanitrile and carbanitrile warheads and found azanitriles to be superior. The azapeptide nitriles were potent inhibitors of SmCB1 with antischistosomal activity and, thus, represent a new class of potential drug leads. We present the first crystallographic analysis of a protease–azanitrile inhibitor complex and quantum chemical calculations to provide
mechanistic insight into the phenomenon of azanitrile warhead reactivity.

■ RESULTS AND DISCUSSION

SAR Analysis of Aza- and Carbadipeptide Nitriles Reveals a High Potency of SmCB1 Inhibitors with an Azanitrile Warhead. We have evaluated a set of 18 azadipeptide and 50 carbadipeptide nitriles in vitro as potential inhibitors of the SmCB1 protease. The compound scaffold is defined by positions P3 through P1 (Schechter and Berger nomenclature), and the substitutions were selected to provide a high diversity in all positions. The compounds were screened against recombinant SmCB1, and their $K_i$ values were determined using a kinetic inhibition assay with the fluorogenic substrate Cbz-Phe-Arg-AMC. The data for seven representative pairs of both chemotypes are shown in Table 1.

Analogs with the azanitrile warhead (1a−7a) were more potent than those with the carbanitrile warhead (1c−7c). This general trend was confirmed for the entire set of test compounds (Tables S1 and S2). Most of the aza compounds were effective in nanomolar concentrations with slow-binding kinetics, whereas only 10% of the carba compounds had $K_i$ values <1 μM and had fast-binding behavior with linear progress curves (Figure 2), a typical feature of peptidic (carba)nitrile inhibitors of cysteine proteases. Using the example of the Cbz-capped phenylalanine pair (2a and 2c), additional variations in the carbanitrile warhead by the stepwise introduction of methyl groups at the amino-acetonitrile unit (i.e., the incorporation of alanine, sarcosine, or N-methylalanine-derived nitrile building blocks in 8c−10c) did not improve the inhibition of SmCB1 (Table 2).

Table 1 shows the SAR analysis of the P1 to P3 positions of the azanitrile scaffold. The presence of the azanitrile warhead was not sufficient for strong potency, as compound 1a, which lacks a residue side chain for the P2−S2 interaction, was not active. Larger hydrophobic and aromatic residues at the P2 position strengthen binding to the protease (1a versus 2a, 11a). Also, modification of the $\alpha$-nitrogen substituent in the aza-amino nitrile unit of 2a by extended hydrophobic P1 substituents could improve inhibition (2a versus 8a, 9a). In the P3 position, the introduction of a benzyl urea moiety as present in 3a markedly increased the inhibitory potency. A comparison of P3 triaryl compounds, each with leucine in the P2 position (4a, 5a, 17a), showed that the amide linkage and the associated triaryl group (5a) was preferred over the urea and methylurea linkages (4a and 17a, respectively). The amide bond contributes to the binding affinity because its methylation decreased potency 300-fold (5a versus 6a). When the entire structure of 5a was maintained but leucine was replaced with a less appropriate P2 amino acid, i.e., homocycloleucine in 7a, a 40-fold decrease in potency was measured. Also, we analyzed the second-order rate constants of azadipeptide nitriles ($k_{2nd}$, Table S1). The $k_{2nd}$ values of the five most potent compounds with single-digit nM $K_i$ values (from 4.6 to 6.2 nM) were particularly high for 3a, 5a, and 11a.

![Figure 1. Carba- and azadipeptide nitriles and their reaction with cysteine proteases. Isoelectronic CαH/N exchange in the warhead (cyan/magenta) of dipeptide nitriles (left) leads to azadipeptide nitriles (right). Two carbohydrazide nitrogens in azanitriles need to be alkylated to circumvent spontaneous heterocyclization. R1 and R2 are substituents in amino acid residues at the P1 and P2 positions (binding in the enzyme subsites S1 and S2), respectively; PG is a protecting group. The depicted azadipeptide bears an aza-alanine nitrile at the P1 position. Reactive warheads of both chemotypes form a covalent, reversible bond with the thiol of the catalytic cysteine residue (orange) of papain-family cysteine proteases (represented by SmCB1).](https://dx.doi.org/10.1021/acsinfecdis.0c00644)
substituents in 8a and 9a (R1 = Bn–CH2– and CH3–(CH3)–, respectively).

Phenotypic Effects of Azanitriles on *Schistosoma mansoni*. A panel of 35 azanitrile and carbanitrile inhibitors that were effective against SmCB1 (Tables 1–3 and S2) was phenotypically screened in *vitro* at 10 μM against *S. mansoni* newly transformed schistosomula (NTS), the postinvasive stage of the parasite that feeds on host blood. The resulting phenotypic responses were graded from 0 through 4, i.e., from the least to the most severe. Figure 3 compares the severity scores for the azanitrile and carbanitrile analog pairs (for complete phenotyping data for the panel, see Table S3). We found that both inhibitor chemotypes substantially differed in their bioactivities.

The azanitrile inhibitors caused rapid and severe phenotypes (scores of 3 and 4) except for 1a, which was ineffective against NTS and SmCB1. In contrast, the incubation of NTS with the carbanitrile analogs resulted in more occasional and lower severity scores. The correlation between the severity scores generated by the azanitriles against the parasite and their potency of inhibition of SmCB1 was highly significant (Table S3, Spearman correlation test with nonzero value coefficients, 20,000 permutations, p < 0.001). The most inhibitory azanitriles (with Kᵢ values in the single-digit nM range) were also tested for their cytotoxicity against two human cell lines. They displayed low cytotoxicity at the same concentrations used in the NTS assay (Table S4), indicating that the observed phenotypic changes were specific to the parasite. In conclusion, azadipeptide nitriles that target SmCB1 were demonstrated to be effective antischistosomal compounds.

**Crystallographic Analysis Identifies the Binding Mode of Azanitriles to SmCB1.** The crystal structure of SmCB1 in complex with the single-digit nanomolar active site inhibitor 3a was determined at a resolution of 1.3 Å (PDB ID: 6YI7; Table S5). The binding mode of 3a in the active site is presented in Figure 4. The inhibitor’s P1 to P3 residues occupy the S1 to S3 binding subsites of SmCB1. Through the nucleophilic attack of the thiol group of the catalytic Cys100 residue, an isothiosemicarbazide adduct is formed that incorporates the C₄₅ atom of the nitrile moiety (Figures 4D and S1). The N₄₅ atom of the nitrile moiety is stabilized by two hydrogen bonds to the backbone amide of Cys100 and the side chain amide of Gln94 (Figure 4D). Analogous interactions were observed in the structure of rat cathepsin B complexed with a carbanitrile inhibitor.32

Azadipeptides are atropochiral molecules due to the restricted rotation around the methylated N–N axis. The induction of chirality was demonstrated by methylation of the hydrazine fragment in model azapeptides, which led to the E-configuration of the respective CO–N bond and, hence, to atropisomerism.33 However, E-configured peptidomimetic ligands should possess a weaker affinity for the active site of a protease than the Z-configured isomers. This is also supported by data for cathepsin inhibition involving E- and Z-locked azadipeptide nitriles.34 The configuration of nonlocked azadipeptide nitriles bound to the protease active site is so far unknown. Noteworthy, in this study, we were able to demonstrate the Z-configuration of the C(O)–Nₛₒₐₜ(Nₛₒₐₜ) fragment for the enzyme-bound prototypical inhibitor 3a (Figure 4).

Insights were also gained into the binding with the S2 and S3 subsites. At the P2 position, 3a contains a leucine substituent making nonpolar interactions with residues
Ala271 and Leu146 of SmCB1 (Figure 4C). Residue Glu316 in the S2 subsite has been shown to rotate out of the binding pocket to avoid steric clash with bulky ligand substituents.20 In the SmCB1−3a complex, the flexibility of Glu316 was demonstrated by its dual conformation with one of the conformations making contact with the P2 leucine. The P3 position of the inhibitor is formed by a benzylaminocarbonyl group. The lower value of the electron density of this substituent suggests its static or dynamic disorder (Figure S2). In the crystallographic model, one conformation of the benzylaminocarbonyl moiety was modeled into the electron density map, representing the major position. Nevertheless, it is obvious that the P3 substituent can acquire several alternative positions within the S3 subsite, which is rather wide and less engaged in ligand binding.20

Quantum Chemical Calculations Demonstrate the Highly Favorable Energetics of the Azanitrile Reaction Mechanism. Computational approaches based on quantum mechanics have proven powerful in exploring catalytic mechanisms of cysteine proteases and designing their covalent inhibitors.20−39 In the present study, quantum chemical calculations were employed to analyze the “free” energy profile of the binding reaction of 3a to SmCB1, proceeding via a noncovalent intermediate to the final covalent complex (Figure 5A; “free” energies refer to the sums of gas phase energies and solvation free energies). We observed different conformations of the planar amide group C(O)−N(N) at the warhead (Figure S2). The bound 3a adopts the Z-configuration as seen in the SmCB1−3a crystallographic complex; however, the modeling of unbound 3a reveals that the E-configuration is more stable in solvent (by 1.9 kcal mol−1) (Table S6 and

Table 3. Inhibition of SmCB1 and Antischistosomal Activity of Azadipeptide Nitriles

| Compound | Substituent position | SmCB1 inhibition | Antischistosomal effect |
|----------|----------------------|------------------|------------------------|
|          | R₁ | R₂ | R₃ | Kᵢ (nM) | Day 1/Day 2 (Severity score) |
|          |     |     |     |         |                           |
| P1 substitution |
| 2a      | Cbz | Phe | CH₃ | 19.4 ± 1.4 | 4/4 |
| 8a      | Cbz | Phe | Bn-CH₃ | 4.6 ± 0.2 | 4/4 |
| 9a      | Cbz | Phe | CH₂(CH₃)₂ | 6.2 ± 0.5 | 4/4 |
| 10a     | Cbz | Phe | Bn- | 48.9 ± 4.7 | 2/4 |
| P2 substitution |
| 1a      | Cbz | Gly | CH₃ | 31 ± 100 ± 1500 | 0/0 |
| 2a      | Cbz | Phe | CH₃ | 19.4 ± 1.4 | 4/4 |
| 11a     | Cbz | Cha | CH₃ | 4.6 ± 0.5 | 2/4 |
| 12a     | Cbz | Ile | CH₃ | 71.7 ± 4.4 | 4/4 |
| 13a     | Cbz | MetTy | CH₃ | 80.8 ± 7.1 | 4/4 |
| 14a     | Cbz | Val | CH₃ | 88.3 ± 10.4 | 3/4 |
| 15a     | Cbz | Ala | CH₃ | 714 ± 58 | 2/4 |
| P3 substitution |
| 3a      | Bn-NH-CO- | Leu | CH₃ | 6.1 ± 0.7 | 3/4 |
| 4a      | Triaryl-NH-CO- | Leu | CH₃ | 36.7 ± 2.6 | 3/4 |
| 5a      | Triaryl-CO- | Leu | CH₃ | 4.8 ± 0.3 | 2/4 |
| 6a      | Triaryl-CO- | Leu | CH₃ | 1540 ± 250 | 4/4 |
| 7a      | Triaryl-CO- | HomocycloLeu | CH₃ | 229 ± 7 | 3/4 |
| 16a     | Bzaryl-Ch₂-NH-CO- | Leu | CH₃ | 30.0 ± 4.1 | 0/2 |
| 17a     | Triaryl-Ch₂-NH-CO- | Leu | CH₃ | 58.3 ± 2.8 | 0/0 |
| 18a     | Ph-NH-CO- | Leu | CH₃ | 112 ± 11 | 2/4 |

“Compounds 1a to 7a are also presented in Table 1. The abbreviations used are as follows:

R₁: Bn-CH₂- | CH₂(CH₃)₂- | Bn | R₂: Cha | MetTy | HomocycloLeu
R₃: Cbz | Triaryl-CO- | Bzaryl-NH-CO- | Bzaryl-CH₂-NH-CO- | Triaryl-NH-CO- | Triaryl-Ch₂-NH-CO- | Ph-NH-CO-

“The Kᵢ values were determined using a kinetic activity assay with the fluorogenic peptide substrate Cbz-Phe-Arg-AMC. Phenotypic changes in the parasite in the presence of 10 μM inhibitor are indicated for 24 and 48 h (see Table S3 and converted to a severity score on a scale from 0 (no effect) to 4 (severe). “2a is listed in both P1 and P2 sections. N-Methylated leucine.
This is in line with the preferred E-conformation determined experimentally for other free azanitriles and trisubstituted hydrazides in general. Thus, 3a undergoes a conformational change upon binding to the enzyme. The activation energy for the E- to Z-conversion calculated using the implicit solvent model was approximately 15 kcal mol\(^{-1}\) (Table S6). This rotational barrier is a possible explanation for the slow binding of azanitriles to SmCB1 observed in the inhibition kinetics (Figure 2). On the contrary, for the carbanitrile, 3c, the Z-configuration of the C(O) – N(C) moiety was more stable than the E-configuration for both the modeled SmCB1–3c complex and the uncomplexed 3c (by 3.1 and 2.0 kcal mol\(^{-1}\), respectively) (Figure S3 and the Supplementary Results). This agrees with the Z-configuration reported for various protease-bound and free carbanitriles. The fact that no conformational change is required for the interaction of 3c with the enzyme determines the fast-binding kinetics of carbanitriles to SmCB1 (Figure 2).

We next investigated the intermediate noncovalent complexes of 3a and 3c with SmCB1. In optimized noncovalent models, the catalytic residue His270 is positively charged, and the thiolate sulfur atom of Cys100 is separated by about 3.2 Å from the C\(_{\text{AB}}\) atom of the inhibitor (Figure S4 and the Supplementary Results).

Conversion to the covalent complex is associated with the formation of a covalent bond between the S atom of Cys100 and C\(_{\text{AB}}\) atom of the inhibitor and a concomitant water-mediated proton transfer from His270 to the N\(_{\text{AA}}\) atom of the nitrile group (Figure 5C). The transformation proceeds via a transition state with the S···C\(_{\text{AB}}\) distance of about 2.3 Å and a proton localized on H3O\(^+\) (crystallographic water molecule 628). The transition barrier is 11.1 and 19.2 kcal mol\(^{-1}\) for 3a and 3c, respectively (Table S6). This agrees with the Z-configuration reported for various protease-bound and free carbanitriles.32,33 The stronger noncovalent interaction of 3a might be the result of a distinct electrostatic pattern of the azanitrile warhead (Figure S4 and the Supplementary Results).

Figure 3. Correlation of the antischistosomal activity with the inhibition of SmCB1 by azanitrile and carbanitrile analogs. (A) Pairs of analogs with azanitrile and carbanitrile warheads were phenotypically screened against *S. mansoni* newly transformed schistosomula (NTS) and the data arising was compared to those for the inhibition of SmCB1. Phenotypic changes in the parasite (Table S3) were observed every day for 4 days in the presence of 10 μM inhibitor. Changes were converted to a severity score on a scale from 0 (no effect) to 4 (severe; red heat map). \(K_i\) values for SmCB1 inhibition (from Table 1) are shown in the blue heat map. (B) An example of an inhibitor-induced degenerated phenotype in NTS of *S. mansoni* versus untreated controls (for details, see Table S3).

Figure 4. Binding mode of the azadipeptide nitrile inhibitor 3a in the SmCB1 active site. (A) Overall crystal structure of the SmCB1–3a complex in surface (enzyme) and stick (inhibitor) representation. In the SmCB1 active site (boxed), the catalytic residues Cys100 (yellow) and His270 (pink) and major subsite residues (cyan) are highlighted. (B) Chemical structure of 3a forming a covalent bond with the S atom of the catalytic Cys100. The azanitrile warhead is boxed in gray, and atom labeling is indicated (hydrogen atoms are omitted). (C) Zoomed view of (A) showing the active site residues that form nonpolar interactions (green) with 3a (in stick representation with carbon atoms in magenta); the catalytic residues are also indicated. (D) The P1 to P3 positions of the inhibitor bind the corresponding S1 to S3 subsites of the SmCB1 active site. Dashed lines indicate hydrogen bonds formed between SmCB1 residues (gray) and 3a (magenta); heteroatoms have standard color coding (O, red; N, blue; S, yellow). Coordinates are deposited under PDB code 6YI7.
and 3c, respectively, and the transition state has lower “free” energy than the initial separated reactants (Table S6). The final covalent complexes with a S−C_{AB} separation of 1.8 Å are more stable by 9.2 and 5.4 kcal mol\(^{-1}\) for 3a and 3c, respectively, compared to the noncovalent complexes, thus indicating that the “free” energy gain is higher for 3a also in the last step of the reaction (Table S6).

To conclude, the azanitrile 3a has more favorable thermodynamics of its reaction with SmCB1 by 10.2 kcal mol\(^{-1}\) compared to its carbanitrile analog (Table S6), which corresponds to their inhibitory potencies differing by several orders of magnitude (Table 1). The computational analysis showed that the carbanitrile inhibitor has the Z-configuration both in solution and in covalent complex with SmCB1, whereas the azanitrile inhibitor undergoes the E- to Z-transformation. Further, the Z-configuration of the azanitrile in the SmCB1 noncovalent complex is more stable than the E-configuration (by 3.5 kcal mol\(^{-1}\), Supplementary Results).

Figure 5. Computational analysis of the binding reaction of azanitrile and carbanitrile inhibitors to the active site of SmCB1. (A) The “free” energy profile of the binding of azanitrile 3a and carbanitrile 3c was determined using quantum chemical calculations. Individual states along the reaction pathway (indicated by numbers) are defined by their relative “free” energies (Table S6). (B) The unbound azanitrile inhibitor has the E-configuration in solution (with minimum “free” energy) and undergoes a conformational change to the Z-configuration that was also demonstrated crystallographically in the SmCB1−3a complex. (C) Modeled states upon binding of the azanitrile inhibitor to the active site include an initial noncovalent complex (4), a transition state with proton transfer from His270 to H\(_2\)O (5), and a final covalent complex after proton transfer to the nitrile group (6). The distance (sulfur−carbon) of the catalytic Cys100 and the inhibitor’s C_{AB} atom is 3.2, 2.3, and 1.8 Å, respectively.
the physicochemical reaction scheme. On the basis of however, composed of several distinct mechanistic events in transition barrier TS2 of azanitrile is even lower than that of the supposed to be attributed to the slow binding because the controlling step, while the covalent bond formation is not fast-binding carbanitrile. The slow binding observed for chemotypes of covalent inhibitors of cysteine proteases.35,44 concentrations (Figure 2) indicates a one-step kinetic reaction activity.6,7

potent against human cysteine cathepsins with endopeptidase selectivity of azanitrile inhibitors that are, in general, highly azanitrile- and carbanitrile-binding reactions with SmCB1, we demonstrated that the azanitriles alone undergo an azanitrile warhead provide a mechanistic insight into the distinct conformational dynamics and binding energetics of the azanitrile warhead. The slow binding observed for azanitriles is a known kinetic feature of several other chemotypes of covalent inhibitors of cysteine proteases.35,44-46

■ CONCLUSIONS

We investigated the inhibition potency of dipeptidomimetics with azanitrile and carbanitrile warheads against the cysteine protease SmCB1, a drug target from the human blood fluke S. mansoni. A screen of 68 compounds, including analog pairs of both chemotypes, demonstrated the nanomolar inhibition potency of the azanitriles and their general superior potency over their carbanitrile counterparts. Furthermore, the azanitriles were more quickly lethal to the schistosome parasite in vitro and, accordingly, represent a new class of compounds for the development of schistosomiasis drugs. The study provides a platform for further improvement of the azanitrile scaffold based on, for example, the introduction of a P3 triaryl moiety that is connected via an amide bond to a hydrophobic P2 amino acid and that proved particularly advantageous for SmCB1 inhibition. Further research will also address the selectivity of azanitrile inhibitors that are, in general, highly potent against human cysteine cathepsins with endopeptidase activity.6,7

The atomic-level azanitrile—target interaction has not been elucidated up to now. In this study, we solved the first crystal structure of SmCB1 in complex with an azadipeptide nitrile and structurally analyzed the azanitrile-binding mode. By then analyzing the quantum chemical “free” energy profiles of the azanitrile- and carbanitrile-binding reactions with SmCB1, we demonstrated that the azanitriles alone undergo an E- to Z-conformational change upon binding to the enzyme, which we propose as an explanation for the different inhibition kinetic behavior of both chemotypes. Furthermore, azanitriles have a significantly higher “free” energy gain in three consecutive steps along the reaction coordinate, including formation of the initial noncovalent inhibitor–enzyme complex, the transition state, and the final covalent complex with the inhibitor warhead linked to the enzyme catalytic site. The data on the distinct conformational dynamics and binding energetics of the azanitrile warhead provide a mechanistic insight into the reactivity of azanitrile peptidomimetics.

■ METHODS

Synthesis of Inhibitors. General Conditions. Except for compound 3c, the inhibitors used in this study have been synthesized as described.6-8,47 Thin-layer chromatography was carried out on Merck (Darmstadt, Germany) aluminum sheets, silica gel 60 F254. Detection was performed with a UV light at 254 nm. Preparative column chromatography was performed on Merck silica gel (0.063–0.200 mm, 60 Å). Melting points were determined on a Büchi (Essen, Germany) 510 oil bath apparatus. 1H NMR (500 MHz) and 13C NMR (125 MHz) spectra were recorded on a Bruker Avance DRX 500 spectrometer and 1H NMR (600 MHz) and 13C NMR (150 MHz) spectra, on a Bruker Avance III 600 NMR spectrometer. Chemical shifts δ are given in ppm referring to the signal center using the solvent peaks for reference: DMSO-d6, 2.49/39.7 ppm. LC-MS analyses were carried out on an API2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer coupled to an Agilent (Santa Clara, CA, USA) 1100 LC system using a Phenomenex Luna C18 column (Phenomenex, Aschaffenburg, Germany; 50 × 2.0 mm, particle size 3 µm). The purity of the compounds was determined using the diode array detector (DAD) of the LC-MS instrument between 196 and 400 nm. HRMS spectra were recorded on a microTOF-Q (Bruker, Köln, Germany) mass spectrometer connected to a Dionex (Thermo Scientific, Braunschweig, Germany) Ultimate 3000 LC via an ESI interface using a Nucleodur C18 Gravity column (50 × 2.0 mm I.D., 3 µm, Macherey-Nagel, Düren, Germany). The compounds were of at least 95% purity. All compounds passed the PAINS filter using a false positive remover.48

(S)- tert-Butyl 1-(Cyanomethylamino)-4-methyl-1-oxopen- tan-2-ylcarbamate (II).

(S)-2-((tert-Butoxycarbonyl)amino)-4-methylpentanoic acid hydrazide (I, 5.39 g, 21.6 mmol) was dissolved in anhydrous THF (30 mL) and cooled to −25 °C. N-Methylmorpholine (2.61 mL, 2.41 g, 23.8 mmol) and isobutyl chloroformate (3.10 mL, 3.25 g, 23.8 mmol) were consecutively added to the stirred solution. Aminoacetonitrile monosulfate (6.65 g, 43.1 mmol) was suspended in H2O (3 mL). The resulting suspension was warm to room temperature within 0.5 h and stirred for a further 1.5 h. After evaporation of the solvent, the resulting aqueous residue was extracted with EtOAc (3 × 30 mL). The combined organic layer was washed with aq. 10% KHSO4 (30
(S)-2-(3-Benzyleido)-N-(cyanomethyl)-4-methylpenta-namide (3c).

(S)-tert-Butyl 1-(cyanomethylamino)-4-methyl-1-oxopentan-2-ylcarbamate (II, 2.02 g, 7.50 mmol) was dissolved in anhydrous CH₂Cl₂ (200 mL) and treated with TFA (50 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was then evaporated, and the residue was diluted with CH₂Cl₂ (4 × 50 mL) and evaporated to remove the excess TFA. The crude product was dissolved in anhydrous CH₂Cl₂ (30 mL) and cooled to 0 °C. Subsequently, benzyl isocyanate (2.01 mL, 21.8 g, 16.3 mmol) was added, followed by triethylamine (5.15 mL, 3.76 g, 37.1 mmol). Stirring at 0 °C was prolonged for 15 min, and it then continued at room temperature overnight. After evaporation of the solvent, the residue was suspended in H₂O (50 mL) and extracted with EtOAc (3 × 75 mL). The organic layer was washed with saturated KHSO₄ (50 mL), H₂O (50 mL), a sat. aq. NaHCO₃ solution (50 mL), H₂O (50 mL), and brine (50 mL). The solvent was dried over Na₂SO₄, filtered, and evaporated to dryness. The crude residue was purified by recrystallization from EtOAc/n-hexane to obtain II as a white solid (4.01 g, 69%); mp 118–120 °C (lit. mp 114–116 °C (Scheme 1).

1H NMR (600 MHz, DMSO-d₆) δ 0.83 (d, J = 6.5 Hz, 3H) and 0.86 (d, J = 6.6 Hz, 3H, CH(CH₂)₂), 1.33–1.39 (m, 1H, CH2–CH–), 3.16 (s, 9H, C(CH₃)₃), 1.43 (dd, J = 13.6 Hz, 3H, J = 10.1 Hz, J = 5.2 Hz, 1H, CH–CH₂–), 1.52–1.63 (m, 1H, CH₂–CH–), 3.95 (td, J = 9.1 Hz, J = 4.8 Hz, 1H, NH–CH), 4.08–4.12 (m, 2H, CH2–C=N–N), 6.96 (d, J = 8.2 Hz, 1H, O–CO–NH), 8.52 (t, J = 5.7 Hz, 1H, CH–CO–NH). 13C NMR (125 MHz, DMSO-d₆), 126.77 (C-4), 127.13 (C-2), 128.39 (C-3), 140.75 (C-1), 157.76 (NH–CO–NH), 174.07 (CH–CO–NH). LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 210–400 nm), 99% purity, m/z = 302.9 ([M + H]+). HRMS. calc. for C₁₆H₂₂N₄O₂: [M + H]+ m/z 303.1814; found: 303.1816.

Production of Recombinant SmCB1. A nonglycosylated mutant of the SmCB1zymogen (Uniprot accession Q8MN2Y) was expressed using the pPICZαA vector in the yeast Pichia pastoris, activated by S. mansonii legumain and purified as described previously. All purification steps were performed under reducing conditions in the presence of 2 mM dithiothreitol and 1 mM EDTA under an argon atmosphere to prevent the active site cysteine residue from oxidation.

Preparation of the SmCB1–Inhibitor Complex. The freshly activated SmCB1 (0.5 mg mL⁻¹) was incubated with a 4-fold molar excess of the inhibitor 3a in 10 mM sodium acetate, pH 5.5, containing 20 mM cysteine and 1 mM EDTA, for 3 h at room temperature under an argon atmosphere. The enzyme inhibition was monitored using a kinetic assay with the fluorogenic substrate Cbz-Phe-Arg-AMC. The complex was buffer-exchanged into 5 mM sodium acetate, pH 5.5, and concentrated to the final concentration of 4 mg mL⁻¹ using Amicon Ultragel-10k centrifugal units (Millipore, Burlington, USA); the inhibitor 3a was maintained in a 4-fold molar excess to SmCB1 in the mixture.

Protein Crystallization and Data Collection. Crystals of the SmCB1–3a complex were obtained by vapor diffusion in a hanging drop. The drop consisting of 1 μL of protein solution and 1 μL of reservoir solution was equilibrated over 1 mL of reservoir solution at 20 °C. The reservoir solution contained 180 mM ammonium acetate, 80 mM sodium citrate, and 27% PEG 1500, pH 6.1. The protein concentration of the stock solution of the complex was 4 mg mL⁻¹ (in 5 mM sodium acetate, pH 5.5). The obtained needle-shaped crystal was flash-cooled by plunging into liquid nitrogen without cryoprotection. Diffraction data were collected at 100 K on the beamline MX14.1 operated by the Helmholtz-Zentrum Berlin at the BESSY II electron storage ring (Berlin-Adlershof, Germany). Diffraction data were processed using the XDS suite of programs. Crystal parameters and data collection statistics are given in Table S5.

Crystal Structure Determination. The SmCB1–3a complex crystallized in the orthorhombic space group P2₁2₁2₁, containing one molecule in the asymmetric unit and a solvent content of ~41% (Table S5). The structure of the SmCB1–3a complex was determined by molecular replacement with the program Molrep from the CCP4 package using the structure of the mature SmCB1 (PDB code: 3S3Q) as the search model. Model refinement was carried out using the program REFMAC 5.2 from the CCP4 package, interspersed with manual adjustments using Coot. The structure was refined using data to a resolution of 1.3 Å. The final crystallographic model contains residues 70–323 (the SmCB1zymogen numbering is used throughout the paper). Anisotropic refinement of all atomic displacement parameters (ADPs; B-factors) was included in the refinement protocol. The geometric restraints for ligand 3a were constructed by the program Libcheck using a model optimized by the DPT-D3/B3LYP/DZVP method combined with the COSMO implicit solvent model. The optimization was performed by the Turbomole7.057 and Cuby458 programs. The 3a molecule was modeled with an occupancy factor of 1 into generally well-defined electron density. The final refinement statistics are...
Inhibition Assays. Inhibition measurements were performed in triplicates in 96-well microplates (100 μL assay volume) at 37 °C. SmCB1 (20–40 pM) was added to a mixture of the fluorescent substrate Cbz-Phe-Arg-AMC (20 μM) and an inhibitor (0–100 μM) in 0.1 M sodium acetate, pH 5.5, containing 2.5 mM dithiothreitol and 0.1% PEG 6000. The substrate hydrolysis was monitored in an Infinite M1000 microplate reader (Tecan, Männedorf, Switzerland) at excitation and emission wavelengths of 360 and 465 nm, respectively, for up to 15 and 60 min for fast- and slow-binding inhibitors, respectively. Fast-binding inhibitors showed linear progress curves, and the apparent inhibition constant \( K' \) was determined by nonlinear regression using the equation \( v/v_o = 1/(1 + [I]/K') \) by the GraFit software (Erithacus Software, East Grinstead, UK), where \( v \) is the steady-state velocity, \( v_o \) is the velocity in the absence of an inhibitor, and [I] is the inhibition constant. For slow-binding inhibitors, an observed first-order rate constant \( k_{obs} \) was calculated at each inhibitor concentration by fitting the progress curve to the equation \( P = v + (v - v_o)(1 - \exp(k_{obs}t))/k_{obs} + d \), where \( P \) is the product formation, \( v \) is the steady-state velocity, \( t \) is the reaction time, \( v_o \) is the initial velocity, and \( d \) is the offset. The inhibition constant \( K' \) was determined by nonlinear regression using the equation \( v/v_o = 1/(1 + [I]/K') \). The true inhibition constants \( K' \) were then calculated using the equation \( K = K'/\left(1 + [S]/K_m\right) \), where [S] is the substrate concentration and \( K_m \) is the Michaelis constant. The apparent second-order rate constant \( k_{2nd} \) was determined by fitting to the linear equation \( k_{obs} = k_{2nd}[I] + k_{off} \) where \( k_{off} \) is the first-order rate constant for the dissociation of the enzyme–inhibitor complex and the true constant \( k_{2nd} \) was calculated by the correction \( k_{2nd} = k_{off}(1 + [S]/K_m) \). The \( K_m \) value determined for Cbz-Phe-Arg-AMC and SmCB1 was 25 μM. In none of the assay systems did the final concentration of DMSO exceed 1.5%.

Interaction Energy Calculations. The crystal structure of SmCB1 in complex with 3a was used for molecular modeling. Hydrogen atoms were added to the protein by the Reduce and Leap programs in AMBER 14. Aspartates, glutamates, lysines, arginines, and histidines were charged (except for the neutral His270 in covalent complexes). Hydrogen atoms were relaxed by annealing from 1000 to 0 K at the MM level in AMBER 14. The FF14SB force field was used for the protein while the GAFF force field was used for the ligand. The cooling runs were 4 ps long with a 1 fs step and Berendsen thermostat used. Fourteen crystallographic water molecules were considered for the modeled complex. The 3c ligand was built manually using the PyMOL 1.7.6.

The QM part comprised residues within 2.5 Å of 3a (i.e., 300 atoms). The QM part was treated at the DFT-D3/BLYP/TZVPP level for single-point energy calculations. For gradient optimizations, we used the DFT-D3/BLYP/DZVP level. The rest of the system was treated at the PM6-D3H4X level. The environment was described by the COSMO implicit solvent model. The coupling between QM and SQM was done by Cuby4, which calls Turbomole 7.0 and Mopac.
binding reaction of the inhibitors 3a and 3c to SmCB1; four figures: (S1) atom labeling scheme for the inhibitor 3a, (S2) electron density maps for the inhibitor 3a, (S3) energy calculation of the conformation change of the inhibitors 3a and 3c, and (S4) the molecular surface of the electrostatic potential (ESP) of the inhibitors 3a and 3c (PDF)

Accession Codes
Atomic coordinates and experimental structure factors for the SmCB1–3a complex have been deposited in the Protein Data Bank with accession code 6Y7.

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Notes
The authors declare no competing financial interest.

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Abbreviations
AMC,aminomethylcoumarin; Bn, benzyl; Cbz, benzoylcarbonyl; Cha, cyclohexylalanine; Hph, homophenylalanine; NTS, newly transformed schistosomula; RMSD, root-mean-square deviation; SmCB1, cathepsin B1 from Schistosoma mansoni

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