Schistosomiasis caused by a parasitic blood fluke of the genus Schistosoma afflicts over 200 million people worldwide. Schistosoma mansoni cathepsin B1 (SmCB1) is a gut-associated peptidase that digests host blood proteins as a source of nutrients. It is under investigation as a drug target. To further this goal, we report three crystal structures of SmCB1 complexed with peptidomimetic inhibitors as follows: the epoxide CA074 at 1.3 Å resolution and the vinyl sulfones K11017 and K11777 at 1.8 and 2.5 Å resolutions, respectively. Interactions of the inhibitors with the subsites of the active-site cleft were evaluated by quantum chemical calculations. These data and inhibition profiling with a panel of vinyl sulfone derivatives identify key binding interactions and provide insight into the specificity of SmCB1 inhibition. Furthermore, hydrolysis profiling of SmCB1 using synthetic peptides and the natural substrate hemoglobin revealed that carboxydipeptidase activity predominates over endopeptidolysis, thereby demonstrating the contribution of the occluding loop that restricts access to the active-site cleft. Critically, the severity of phenotypes induced in the parasite by vinyl sulfone inhibitors correlated with enzyme inhibition, providing support that SmCB1 is a valuable drug target. The present structure and inhibitor interaction data provide a footing for the rational design of anti-schistosomal inhibitors.

Schistosomiasis (bilharzia) is a chronic infectious disease caused by trematode blood flukes that infect over 200 million people in tropical and subtropical areas (1). Of the five species of schistosomes infecting humans, Schistosoma mansoni is a major etiological agent of disease in parts of Asia, the Middle East, Africa, and South America. Morbidity associated with the disease arises from immunopathological reactions to parasite eggs that accumulate in various tissues, including the liver, intestinal tract, and bladder (2). Treatment and control of schistosomiasis now relies on just one drug, praziquantel, a perilous situation should drug resistance emerge and become established (1, 3). Accordingly, there is continued impetus to identify new schistosomal protein targets and chemotherapeutically active anti-schistosomals (4–6).

Adult schistosomes live in the cardiovascular system, and host blood proteins are a nutritious source for growth, development, and reproduction. In the schistosome gut, a network of peptidases (proteases) contributes to the digestion of host proteins, predominated by hemoglobin, to absorbable peptides and amino acids (7, 8). For S. mansoni, the component digestive peptidases thus far characterized include the following: (i) cysteine peptidases of the Clan CA (papain family), namely cathepsin B1, cathepsins L1–L3, dipeptidyl peptidase I (cathepsin C), and a Clan CD asparaginyl endopeptidase (legumain); (ii) the Clan AA aspartic peptidase, cathepsin D; and (iii) the Clan MF metallopeptidase, leucine aminopeptidase (7–11). This study focuses on S. mansoni cathepsin B1 (SmCB1),2 which is the most abundant cysteine peptidase in the parasite gut (12, 13) and is necessary for normal parasite growth (14). SmCB1 is synthesized as an inactivezymogen and is converted in vitro to a mature, active 31-kDa enzyme by proteolytic removal of the pro-peptide that can be catalyzed by legumain (12). SmCB1 is a molecular target for cure of schistosomiasis mansoni in a mouse model using the vinyl sulfone cysteine peptidase inhibitor K11777 (15). Inhibition of SmCB1 therefore represents an attractive option for anti-schistosomal drug development; however, target-based rational design of lead compounds has been hampered by a lack of structural information for the enzyme.

2 The abbreviations used are: SmCB1, cathepsin B1 from S. mansoni; VSPh, phenyl vinyl sulfone; RP-HPLC, reverse phase HPLC; AMC, aminomethylcoumarin; Cbz, carboxbenzyl; Hph, homophenylalanine; NTS, newly transformed schistosomula; r.m.s.d., root mean square deviation; Abz, amibenzoic acid; Np, 4-nitrophenylalanine; EDDnp, ethylenediamine 2,4-dinitrophenyl; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; N-Mpip, N-methylpiperoxycarbonyl; Mu, morpholinylcarbonyl; Cbz, benzoxycarbonyl.
Recently, we designed reversible inhibitors of SmCB1 based on the pro-peptide scaffold. These were effective in vitro in the low micromolar range (16). Here, we identify covalent nonomolar inhibitors of SmCB1 and analyze their binding mode by structural analysis. The inhibitors include the following: (i) epoxide inhibitor CA074, a specific inhibitor of cathepsin B-type peptidases (17) that has been previously structurally characterized in complex with mammalian cathepsins B (18), and (ii) vinyl sulfone inhibitors K11017 and K11777 that have not been crystallographically studied so far in complex with cathepsins B. Vinyl sulfones are effective against papain-like cysteine peptidases and were originally investigated in the context of inhibiting human cathepsins (19, 20). Later, they were demonstrated to inhibit cysteine peptidases from a variety of protozoan pathogens such as Trypanosoma and Plasmodium, and provide either parasitological cure or a temporary remission of parasitemia (21–23). As a chemotype, vinyl sulfones have acceptable pharmacokinetic attributes and in vivo safety profiles (24, 25). Currently, K11777 is in pre-clinical development as an anti-chagasic compound (26).

Here, we report the crystallographic structure of SmCB1, the first for a schistosomal proteolytic enzyme. A comprehensive analysis of structure-activity/inhibition relationships is provided to describe the active site of SmCB1. We demonstrate that SmCB1 is an efficient exopeptidase/endopeptidase against both synthetic peptide substrates and the physiologically relevant protein substrate, hemoglobin. Also, inhibition of SmCB1 by various vinyl sulfone inhibitors correlates with the severity of phenotypes induced in the parasite in culture. This study therefore provides both evidence that SmCB1 is a relevant drug target and the necessary structure-ligand data with which the design of anti-schistosomal SmCB1 inhibitors can be continued.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis of SmCB1**

The pPICZαA plasmid containing SmCB1 insert was constructed as described previously (12). A nonglycosylated mutant of SmCB1 was generated from the plasmid construct using site-directed mutagenesis performed by PCR according to QuikChange® system (Stratagene). A two-step PCR procedure was employed for disruption of consensus glycosylation sites Asn-His-Thr to Asn-His-Ala (residues 166–168) and Asn-Lys-Thr to Asn-Lys-Ala (residues 281–283) using forward 5'-AGTTGAAAGGAAATCAGCACGTTGGTGAAACCATATC-3' and reverse 5'-GATATGGTTCAACACCTGCGTTAGGTCTCTTCTTCAAGC-3' primers (for Thr to Ala-168 mutagenesis) and forward 5'-TTGGGTGTGGGAAACAGGTTCTTTATGTTGTAGTGGG-3' and reverse 5'-CCATCAGCAGTGGACCTTTTCCACACCTC-3' primers for Thr to Ala-283 mutagenesis. Constructs were sequenced to verify desired mutations.

**Recombinant Expression and Purification of SmCB1**

The nonglycosylated SmCB1 zymogen was expressed in the X33 strain of the methylo trophic yeast Pichia pastoris, purified and activated by S. mansoni legumain (27), as described previously (16). All purification steps were maintained under reducing conditions in the presence of 3.5 mM β-mercaptoethanol and 1 mM EDTA to prevent the active site cysteine from oxidation. The expressed nonglycosylated SmCB1 exhibited analogous activity properties as the wild-type SmCB1 produced in the Pichia expression system (12). The nonglycosylated SmCB1 was used in all experiments described here.

**Preparation of Substrates and Inhibitors**

Fluorogenic fluorescence resonance energy transfer (FRET) substrates Abz-Phe-Arg-Xaa-Nph-OH and Abz-Phe-Arg-Xaa-Nph-OH (the Xaa position contains one of the 19 proteinogenic amino acids except Cys) were synthesized by N-(9-fluorenyl)methoxycarbonyl (Fmoc) solid phase chemistry in an ABI 433A peptide synthesizer (Applied Biosystems) as described previously (28). Peptides were purified by RP-HPLC over a C18 column using a TFA/acetonitrile system and characterized by electrospray ionization mass spectrometry on an LCQ Classic Finnigan MAT device (Thermo Finnigan). FRET substrates Abz-Gln-Val-Val-Ala-Gly-Ala-EDDnp and Abz-Ala-Phe-Arg-Phe-Ser-Gln-EDDnp, fluorgenic substrate Cbz-Phe-Arg-AMC, and CA074 inhibitor were purchased from Bachem. Vinyl sulfone inhibitors (Table 1) with prefix K were synthesized as described previously (19, 29), with prefix WRR as described previously (30–33), and with prefix AR as described previously (34) and were kindly provided by James T. Palmer, William R. Roush, and Adam R. Renslo.

**Preparation of SmCB1-Inhibitor Complexes**

The freshly activated SmCB1 was incubated (10 h, 18 °C) with a 5-fold molar excess of the inhibitor in 0.1 mM sodium acetate, pH 5.5, containing 15 mM cysteine and 1 mM EDTA. The enzyme inhibition was monitored with Cbz-Phe-Arg-Nph substrate. The complex was rechromatographed on an FPLC Mono S column (16), concentrated, and buffer-exchanged into 2.5 mM sodium acetate, pH 5.5, using Amicon Ultra 10k centrifugal units (Millipore).

**Protein Crystallization and Data Collection**

Crystals were obtained by vapor diffusion in hanging drop. Drops consisting of 1 μl of the protein solution and 1 μl of the reservoir solution were equilibrated over 1-ml reservoir solution at 20 °C. The reservoir solutions were 0.2 mM ammonium acetate, 0.1 mM sodium citrate, 30% PEG 1500, pH 6.2, for SmCB1-K11777 and SmCB1-K11017 complexes, and 0.1 mM sodium citrate, 0.2 mM ammonium acetate, 30% PEG 1500, pH 6.1, for SmCB1-CA074 complex. Protein concentrations of the stock solutions of the complexes were 2.5–5 mg/ml (in 2.5 mM sodium acetate, pH 5.5). Rectangle-shaped crystals reached their final size within 10 days and were flash-cooled by plunging into liquid nitrogen. Diffraction data at 100 K were collected at beamline 19-BM of the Structural Biology Center at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL. All diffraction data were processed using the HKL-3000 suite of programs (35). Crystal parameters and data collection statistics are given in supplemental Table S1.

**Structure Determination, Refinement, and Analysis**

The structure of SmCB1 was determined by molecular replacement with the program Molrep (36) using the structure...
of human cathepsin B (Protein Data Bank code 1HUC) (37) as
the search model. The sequence alignment of SmCB1 with
human cathepsin B is shown in supplemental Fig. S1. Model
refinement was carried out using the program REFMAC 5.2
(38) from the CCP4 package (39), interspersed with manual
adjustments using Coot (40). The final steps included TLS
refinement (41). The quality of the final models was validated
with Molprobity (42). Final refinement statistics are given in
supplemental Table S1. Atomic coordinates and experimental
structure factors have been deposited with the Protein Data
Bank with the codes 3QSD, 3S3R and 3S3Q for SmCB1-CA074,
SmCB1-K11777, and SmCB1-K11017, respectively. The fol-
lowing services were used to analyze the structures: PISA server
(43) and CONTACTS (39). All figures showing structural rep-
resentations were prepared with the program PyMOL (44).

Interaction Energy Calculations

The subsite interaction energy between inhibitors and
SmCB1 was calculated using the quantum chemical approach.
The strategy consisted of the following two steps: optimization
of the crystallographic complexes and calculation of interaction
energies in the individual subsites.

Model Setup and Geometry Optimization—Hydrogen atoms
were added to the crystallographic complexes of SmCB1 and
inhibitors to correspond to pH of ~6 and were optimized using
the AMBER 10 software (45). Further optimization of the inhibi-
tor in the active site (residues within 6 Å) was carried out using
the corrected semi-empirical quantum chemical method PM6-
DH2 (46, 47), including implicit water environment. The alter-
native conformation of VSPh in P1’ position of inhibitors was
modeled using PyMOL (44).

Subsite Interaction Energies—The inhibitor structures were
fragmented into the P3 to P2’ segments, with separated side
chains and the main chains, and capped by hydrogens. The
reactive centers of the inhibitors originating from the vinyl and
epoxide moieties (located between P1 and P1’) as well as the
catalytic Cys-100 that form the covalent linkage were not cal-
culated. The subsite interaction energies were obtained as the
difference between the energy of the fragment noncovalently
bonded to the enzyme and the sum of energies of the enzyme
and the inhibitor fragment calculated separately. The PM6-
DH2 quantum chemical calculations in implicit water were
applied.

SmCB1 Activity and Inhibition Assays

Activity measurements were performed in a microplate for-
mat (100-μl assay volume) at 37 °C. The reaction mixture con-
tained enzyme (0.6 nM) and fluorogenic substrate Cbz-Phe-
Arg-AMC (20 μM) in 0.1 M sodium acetate, pH 5.5, containing
2.5 mM dithiothreitol and 0.1% PEG 1500 (16). The kinetics of
product release was continuously monitored in an Infinite
M200 microplate reader (Tecan) at excitation and emission
wavelengths of 320 and 420 nm, respectively. The screening of
libraries of FRET carboxypeptidase substrates was performed with
40 μM substrates in the reaction mixture. The Michaelis-
Menten kinetic parameters (supplemental Table S5) were
determined by measuring the rate of hydrolysis in the substrate
concentration range of 0–200 μM, and Km and kcat values were
obtained by nonlinear regression using GraFit software. In all
assay systems, the final concentration of DMSO did not exceed
1.5%. Each measurement was performed in triplicate. The con-
centration of SmCB1 was determined by active site titration
with E-64; the peptide solutions were quantified by amino acid
analysis.

Hemoglobin Degradation

Digestion of human hemoglobin (Sigma, H7379) with
SmCB1 was performed as described previously (48). Briefly,
hemoglobin (10 μg) was incubated with SmCB1 (0.25 μg) in 25
mM sodium acetate, pH 3–6, including 2.5 mM DTT in a total
volume of 35 μl for 1–4 h at 37 °C. Aliquots of the digest were
subjected to derivatization with fluorescamine to quantify the
newly formed N-terminal ends (49). The fluorescence signal
was measured using an Infinite M200 microplate reader
(Tecan) at 370 nm excitation and 485 nm emission wave-
lengths. All measurements were performed in triplicate. For
SDS-PAGE visualization, hemoglobin digests were separated in
Tricine gels (16% T, 6% C) containing 6 M urea (50). For RP-
HPLC analysis, hemoglobin (0.15 mg) was incubated with
SmCB1 (1.25 μg) in 50 mM sodium acetate, pH 4.5, containing
2.5 mM DTT in a total volume of 200 μl for 0–15 h at 37 °C. The
reaction mixture was treated with 10 μl of 10% TFA and sepa-
rated by RP-HPLC on a C4 Vydac column (Vydac) equilibrated
in 0.1% (v/v) TFA and eluted with a 1%/min gradient of a 99%
(v/v) acetonitrile solution in 0.1% (v/v) TFA. The collected peak
fractions were analyzed by FT-MS using an LTQ Orbitrap XL
mass spectrometer (Thermo).

Parasite Assay and Phenotype Scoring

 Newly transformed schistosomula (NTS) of S. mansoni were
prepared from infective larvae (cercariae) as described previ-
ously (5) and incubated in the presence of protease inhibitors.
Briefly, the assay was performed in a microplate 96-well format
(200 μl assay volume) with 200–300 NTS in Basch Medium 169
(51) containing 5% FBS, 100 units/ml penicillin, and 100 μg/ml
streptomycin (52). Final concentrations of 1 or 10 μM inhibitors
in 0.5% DMSO were added and incubations continued for 3
days at 5% CO2 and 37 °C. Phenotypes that arise as a function of
time and concentration were graded as follows: grade I, dead
NTS by 2 days of culture at 10 μM and dying/dead NTS by 3 days
at 1 μM; grade II, dead NTS by 3 days at 10 μM and round/dark/
dying by 3 days at 1 μM; grade III, round/dark/dying by 3 days at
1 and 10 μM concentrations.

RESULTS

Determination of Crystal Structures—Recombinant SmCB1
was produced as a nonglycosylated mutant in the methyl-
otrophic P. pastoris expression system. The enzymatically
active SmCB1 was obtained by activation processing of the
SmCB1 zymogen with legumain that removes the activation peptide (pro-peptide) (12). The activated SmCB1 contains 253 amino acid residues starting with N-terminal Val-70 (the SmCB1 zymogen numbering is used throughout the paper).

SmCB1 was crystallized in complex with three covalent active site inhibitors, namely CA074, K11017, and K11777. The structure of SmCB1 was determined by molecular replacement using the structure of human cathepsin B (37) sharing 59% sequence identity. The SmCB1-CA074 and SmCB1-K11017 complexes crystallized in the same orthorhombic space group $P_2_1 2_1 2_1$, with one molecule in asymmetric unit and solvent content of $\sim 23\%$. The structures were refined using data to resolution 1.3 and 1.8 Å and the final crystallographic model contains residues 71–323 and 70–323 for SmCB1-CA074 and SmCB1-K11017 complex, respectively. The electron density used for modeling of inhibitors was of excellent quality in both structures (Fig. 3). The SmCB1-K11777 complex crystallized in the orthorhombic space group $P_2_1 2_1 2_1$, with three molecules (named A, B, and C) in the asymmetric unit and solvent content of $\sim 47\%$ and was refined using data to resolution 2.64 Å. One C-terminal residue in molecule C as well as residues 118–122 of molecule B could not be located in the electron density map and were thus not included in the final model. All three molecules of the SmCB1-K11777 complex present in the asymmetric unit were very similar. The root mean square deviations (r.m.s.d.) for superposition of the three protein molecular backbones onto each other range from 0.34 to 0.48 Å, a value within the range observed for different crystal structures of identical proteins (53). Minor structural changes were localized in the surface exposed loops, and the substrate-binding sites are structurally almost identical. The electron density used to model K11777 was of excellent quality in all three protein chains in the asymmetric unit (Fig. 3). Mutual comparison of SmCB1 in complex with K11777, K11017, and CA074 did not reveal any significant differences in protein structure (backbone r.m.s.d. values are 0.18–0.54 Å).

**Overall Structure of SmCB1—**SmCB1 is a single polypeptide chain that adopts a classic papain-like fold in which the molecule is divided into L and R domains (37). The active site cleft with catalytic residues Cys-100, His-270, and Asn-290 is located between both domains (Fig. 1). The SmCB1 structure clearly resembles cathepsin B-type peptidases of papain superfamily with the characteristic “occluding loop” (Phe-175 to Pro-197) that restricts access to the primed region of the active site (37). A comparison of SmCB1 with the structure of human cathepsin B shows a high degree of similarity (r.m.s.d. 0.87 Å for 247 Ca atoms). The major differences in backbone superposition (with r.m.s.d. $>1$ Å) are located at the surface loop segments, including residues 117–125, 164–167, 175–194 (occluding loop), 247–268, and 281 (supplemental Fig. S2A). On the SmCB1 surface, there are several large basic patches; the major positively charged cluster is located along the edge of the occluding loop and is absent in mammalian structures (supplemental Fig. S2B). This is reflected in the pI of SmCB1 that is more than 3 units higher than that of human (and other mammalian) cathepsin B based on theoretical pI values of 8.7 and 5.2, respectively.

In the SmCB1 occluding loop, there are two important features of structural rearrangements compared with mammalian homologs. First, a conserved segment Gly-Glu-Gly-Asp is replaced by the sequence Lys-Ile-Tyr-Lys (residues 192–195) in SmCB1. The glycine-containing segment is flexible in mammalian structures and able to move into the active site (18, 54, 55). In SmCB1, this segment is located more distally from the active site, where it is stabilized by the stacking interaction formed between Tyr-194 and Phe-175 at the side of the occluding loop (Fig. 2A). Second, the flexibility of the occluding loop of SmCB1 is restrained by the presence of two salt bridges that stabilize the loop in the “closed” conformation (37). The ion pair His-180/Asp-93 is conserved, whereas the pair Arg-186/Asp-295 of mammalian structures (SmCB1 numbering) is rearranged in SmCB1. The arginine is substituted by Tyr-186, which interacts with Asp-295 through Lys-185 to form a cluster stabilizing the loop conformation in SmCB1 (supplemental Fig. S3).

**Mode of Binding of Inhibitors to SmCB1—**Based on the crystal structures of the SmCB1-inhibitor complexes, the binding mode of the inhibitors K11777, K11017, and CA074 was described. These irreversible inhibitors form a covalent adduct with the thiol group of the catalytic residue Cys-100 and differ substantially in their positions in the active site (Fig. 3). CA074 (l-trans-epoxysuccinyl(propylamide)-Ile-Pro-OH) occupies the S2 to S2′ subsites and is bonded via a C6 atom after opening the epoxide ring. K11777 (N-Mpip-Phe-Hph-VSPh) and K11017 (Mu-Leu-Hph-VSPh) occupy the S3 to S1′ subsites making covalent bond through the CB$^*$ atom of the vinyl sulfone moiety. In all complexes, there is a set of common interactions between the inhibitor backbone and the enzyme active site that involves predicted contacts of Gln-94, Gly-98, Gly-143, Gly-144, Gly-269 and His-270, Trp-101, Trp-292 (supplemental Table S2). Specific critical structural determinants include:...
nants of inhibitors and their interactions with SmCB1 subsites are as follows. In the SmCB1-CA074 complex, propyl and carbamoyl groups of CA074 are in the S2 and S1 subsites, and the -Ile-Pro-OH part mimics a substrate in the S1/H11032 and S2/H11032 subsites (Fig. 3). The C-terminal carboxyl group of the P2/H11032 Pro residue interacts with two His residues localized at the occluding loop of SmCB1; three charge-assisted hydrogen bonds in total were formed between carboxyl oxygens and imidazole nitrogens of His-180 and His-181 (Fig. 4). Thus, the binding mode of CA074 to SmCB1 is similar to that known in mammalian cathepsin B complexes with CA074 and related derivatives, which target the

FIGURE 2. Structural details of the occluding loop in SmCB1. A, Lys-Ile-Tyr-Lys (residues 192–195) segment in SmCB1 (salmon) is stabilized by a stacking interaction between Tyr-194 and Phe-175; this segment replaces a flexible segment Gly-Glu-Gly-Asp in human cathepsin B (green). Enzyme complexes with epoxide inhibitors, SmCB1-CA074 and human cathepsin B-CA030 (Protein Data Bank code 1CSB), were used for the superposition. Loop segment residues (ball-and-stick) and the corresponding inhibitor (sticks) have the same color for carbon atoms, and other atoms are colored by the standard color coding (O, red; N, blue; S, yellow). B, superposition of the complexes SmCB1-K11017 (magenta) and SmCB1-K11777 (cyan). Both vinyl sulfone inhibitors (sticks) contain Hph and phenyl sulfone in P1 and P1/H11032 positions, respectively. The Ile-193 residue (ball-and-stick) interacts with the P1 position of K11017 but with the P1/H11032 position of K11777 (the contacts are highlighted). This is associated with a different orientation of the P1/H11032 moiety that fills the S1/H11032 subsite in K11017 but it is flipped in K11777. Heteroatoms have standard color coding.

FIGURE 3. Binding mode of inhibitors in the SmCB1 active site. A, superposition of vinyl sulfone inhibitors K11777 and K11017 occupying the P3- to P1/H11032-binding positions of SmCB1. B, epoxide inhibitor CA074 occupying the P2'/to P2-binding positions of SmCB1. Left-hand panels, surface representation of SmCB1. Highlighted are the catalytic residues Cys-100 (yellow), His-270 and Asn-290 (salmon), and the occluding loop residues His-180 and His-181 (orange). Inhibitors are in stick representation with differently colored carbon atoms (K11777, green; K11017, magenta; CA074, cyan). Heteroatoms have standard color coding (O, red; N, blue; S, yellow). Middle panels indicate the chemical structures of inhibitors. The C atom forming a covalent bond with the S atom of the catalytic Cys-100 is indicated with an asterisk. Right-hand panels, electron density maps for the inhibitors. The 2Fo/H11002-Fc electron density maps are contoured at 1σ for K11777 and K11017, and 1.5σ for CA074. The covalently bound catalytic Cys-100 is depicted.
occluding loop at the S2’ subsite leading to cathepsin B specific inhibition (18, 55, 56). An additional stabilization is conferred by nonpolar interaction between Ile-193 and the P2’ proline ring. The S1’ subsite is a hydrophobic pocket (Val-247, Leu-252, His-270, and Trp-292) in the R domain and stably holds the P1’ Ile of CA074 through hydrogen bonding (Trp-292 and Gln-94). CA074 does not protrude deeply into the S1 subsite composed of Gln-94, Gly-98, Gly-144, and Gly-269. The inhibitor binding in this region is stabilized by the following interactions: C6 atom covalently bound to Cys-100 and two carbonyl oxygen atoms interacting with Gln-94 (in the “oxyanion hole”) and Gly-144 (Fig. 4). The terminal propyl of CA074 occupies part of the S2 subsite, where it is directed toward Glu-316. This P2 group is bound through hydrophobic interactions with the backbone of Leu-146 and Ala-271.

The chemical structures of K11777 and K11017 are identical at the P1 position (Hph) and the P1’ position (VSPh) but differ at the P2 position (Phe and Leu, respectively) and P3 position (N-Mpip and Mu, respectively) (Fig. 3). Contrary to CA074, the vinyl sulfone inhibitors do not occupy the S2’ subsite of SmCB1. When comparing the binding mode of K11777 and K11017, a striking difference was observed for the conserved P1’ substituent; the phenyl sulfone moiety fills the S1’ subsite in K11017 but is flipped ~90° out of the active site in K11777 (for all three molecules in the asymmetric unit) (Fig. 3 and supplemental Fig. S4). Both conformations of phenyl sulfone are stabilized by polar contacts, mainly with Leu-252 and Trp-292 in K11017, and with Cys-97 and Gly-98 in K11777 (supplemental Table S2). The S1 subsite of SmCB1 binds the Hph residue of both K11777 and K11017; however, a net of polar contacts in this subsite is influenced by the situation in S1’. The residue Ile-193 located on the Lys-Ile-Tyr-Lys segment of the occluding loop has different orientation in the K11777 and K11017 complexes as it interacts with different positions in the inhibitors (Fig. 2B). In the SmCB1-K11017 complex, Ile-193 makes contacts with Hph in the S1 subsite and, in the SmCB1-K11777 complex, with phenyl sulfone that is flipped out from the S1’ subsite. SmCB1 contains an acidic residue (Glu-316) at the bottom of the S2 subsite similarly to mammalian cathepsins B (57). Leu in the P2 position of K11017 is able to establish a polar contact with Glu-316, whereas for the bulkier Phe in K11777, Glu-316 points out of the pocket to avoid a steric clash (supplemental Fig. S5). Similar conformational changes of the acidic residue in S2 were reported for vinyl sulfone complexes of cathepsin L-type peptidases of Trypanosoma and Plasmodium (58). The flexibility of Glu-316 in SmCB1 is further demonstrated by its dual conformation in the SmCB1-K11017 complex (supplemental Fig. S5). The S3 subsite located at the entrance of the SmCB1 active site cleft is generally hydrophobic. This wide subsite accommodated loosely the terminal groups Mu and N-Mpip as the P3 substituents of vinyl sulfone inhibitors.

**Computational Analysis of the Inhibitor Binding Mode**—The quantum chemical calculations on the crystallographic complexes were employed to determine the noncovalent interaction energy of the K11017, K11777, and CA074 inhibitors in the subsites of SmCB1. Fig. 5 shows the interaction energies of the individual side- and main-chain segments in P3 to P2’ positions.

For CA074, by far the largest favorable contribution comes from the P2’ position containing C-terminal Pro residue (~32.8 kcal/mol). The other contributions vary in range from −4.5 to 5.3 kcal/mol with favorable interactions formed by the P2/P1 and P1 segments and unfavorable interactions by the side...
Structural Analysis of SmCB1 Inhibition

Inhibitor position

![Interaction energy (kcal/mol)]

FIGURE 5. Subsite interaction energies between inhibitors and SmCB1. The noncovalent interaction energy was determined using quantum chemical calculations on the crystallographic complexes of SmCB1 with K11017, K11777, and CA074. The inhibitor structures were fragmented into the side-chain segments (P3 to P2’) and main-chain segments (Pi/P(i-1) connecting Pi and P(i-1)). The P1/P1’ segment forming a covalent bond with the catalytic Cys-100 was not calculated. Positions absent in inhibitor structures are marked with a ×.

chains of P2 and P1’ (Fig. 5). This is contrasted with the interaction energies of the vinyl sulfone inhibitors that represent rather smaller favorable contributions. The comparison of K11017 and K11777 revealed that there are large differences in the interaction energy between both inhibitors at the P2 and P1’ positions (Fig. 5). The side chain of the P2 residue, Leu of K11017 does not bring any favorable interaction (0.1 kcal/mol) in contrast to Phe of K11777 (−6.9 kcal/mol). The phenyl sulfone moiety at P1’ adopts a distinct conformation in each of the both vinyl sulfone-SmCB1 complexes, contributing −13.8 kcal/mol in K11017 and −4.3 kcal/mol in K11777 to binding. To evaluate the effect of the two orientations of the P1’ residue in the respective inhibitor complexes, we calculated the interaction energies for artificial complexes of K11017 and K11777, in which the side chains were built with interchanged conformations (supplemental Table S3). The calculated interaction energy of P1’ is reduced substantially to less negative values, which strongly indicates that only the crystallographic conformation is favorable for binding of the respective inhibitor to SmCB1.

In the crystallographic complexes of SmCB1 with CA074 and K11017, we found dual conformations of the side chain of Glu-316 in S2 interacting with the P2 residues. Subsite interaction energies in both alternative conformations were calculated (supplemental Table S3). The energy differences were 1.3 and 0.1 kcal/mol for K11017 and CA074, respectively. We conclude that two conformations of Glu-316 in the S2 subsite are nearly isoenergetic and might be favorable for the complex formation in terms of the conformational entropy.

Inhibitor Specificity of the SmCB1-binding Subsites—A set of 20 vinyl sulfone inhibitors was screened in vitro against SmCB1 to explore the structural requirements of the inhibitor-binding subsites in the SmCB1 active site cleft. These compounds are listed in Table 1 (Fig. 6) together with their IC_{50} values determined using a kinetic assay with the fluorogenic substrate Cbz-Phe-Arg-AMC. The substitutions at the positions P3 to P1’ were introduced on the inhibitor scaffold derived from the K11777 molecule.

The P1’ position of K11777 (IC_{50} = 2.09 nM) contains a phenyl group directly attached to the vinyl sulfone moiety. A modified linkage of the P1’ phenyl through a one-atom spacer (-NH-Ph, -O-Ph, -CH_{2}-Ph) led to inhibitors with similar or slightly weaker potency (IC_{50} in the range of 2.02–7.80 nM for WRR-282, WRR-284, and WRR-285); however, a three-atom linker (-NH-0-CH_{2}-Ph) provided the best inhibitor WRR-286 (IC_{50} = 0.61 nM) of the whole set. On the other hand, the inhibition dramatically decreased by 2 orders of magnitude when the P1’ phenyl was modified to give -O-Ph-O-CH_{3} (WRR-347) or was replaced with -O-CH_{2}-CH_{3} (WRR-283).

At the P1 position, Hph is the favored residue that is present in all tested inhibitors with IC_{50} of <10 nM. Its substitution with Lys (K11006) or Tyr (WRR-453) led to 1 and 4 orders of magnitude higher IC_{50} respectively, as compared with K11002. Also, a change of configuration at P1 Hph (and adjacent P2 Phe) from S to R substantially decreased the inhibitory potency as shown for WRR-359 derived from WRR-284 (IC_{50} ~114 and 7.8 nM, respectively). Unfavorable substitutions at P1 (containing (R)-Ala) and P1’ resulted in low inhibition of WRR-185 and WRR-200 (compare with WRR-145).

At the P2 position, both Phe and Leu are highly effective as demonstrated with K11002 and K11017 (IC_{50} around 1.7 nM). In the K11777 scaffold, replacement of the P2 Phe by Phe-4-CH_{3} (AR-198049) and Phe-4-CF_{3} (AR-198048) resulted in 3- and 5-fold weaker inhibition, and His (WRR-499) and Arg (WRR-483) afforded 14- and 24-fold weaker inhibition, respectively. The N-terminal modification of inhibitors corresponding to the P3 position was by N-Mpip, Mu, and Cbz capping groups. The heterocycles are present in the best inhibitors and do not differ importantly in their contribution to the inhibitory effect, as shown with K11777 and K11002 (IC_{50} of 2.09 and 1.73 nM, respectively).

Cleavage Mode and Substrate Specificity of SmCB1—Hydrolisis by SmCB1 was analyzed with the physiological protein substrate, hemoglobin, and with a series of synthetic peptide substrates. SmCB1 degraded hemoglobin at acidic pH between 4 and 6 as measured by a fluorescamine assay that directly quantifies hemoglobin fragments (Fig. 7A). SDS-PAGE visualization of the hemoglobin fragmentation showed that the disappearance of the substrate band is not associated with a corresponding accumulation of large hemoglobin fragments of >3.5 kDa (Fig. 7B). A detailed pattern of hemoglobin-derived products was obtained by RP-HPLC separation (Fig. 7C). Like those after SDS-PAGE, the RP-HPLC profiles indicated that hemoglobin is gradually converted into a pool of dipeptides with little accumulation of peptides of intermediate size. The detected intermediate fragments ranging in size from 10 to 41 amino acids are mostly derived from the interior of the hemoglobin sequence (supplemental Table S4); this demonstrates the involvement of endopeptidase activity. The combined data suggest that upon endopeptidolytic cleavage of hemoglobin by SmCB1, the substrate is rapidly processed via carboxypeptidase activity.

SmCB1 was then tested with various fluorogenic peptide substrates that allowed for the discrimination of endo- and exopeptidase activities. The supplemental Table S5 compares...
TABLE 1
Inhibition of SmCB1 by vinyl sulfone inhibitors and their anti-schistosomal activity

The IC50 values for 20 vinyl sulfone inhibitors were determined in a kinetic activity assay with SmCB1 and the fluorogenic peptide substrate, Cbz-Phe-Arg-AMC, at pH 5.5. The epoxide inhibitor CA074 was assayed for comparison. The vinyl sulfone structures are defined by the compound core (see scheme below) and substituents R3 to R1'. Inhibitors are ranked according to their IC50 values. Mean values ± S.E. are given for triplicate measurements. Induction of phenotypic alterations by the inhibitors was determined with NTS of *S. mansoni*. The inhibitors were tested at 1 and 10 μM concentrations, and the resulting phenotypes, arising as a function of time and concentration, were graded I to III, with I being the most severe (see Fig. 6).

![Scheme of vinyl sulfone inhibitors](image)

| Compound name | Substituent positiona | Inhibition | Severity of phenotype against parasite |
|---------------|-----------------------|------------|----------------------------------------|
| Vinyl sulfone | R3 | R2 | R1 | R1' | IC50 (nM) | Gradeb |
| WRR-286 | N-Mpip | Phe | Hph | NH-O-CH2-Ph | 0.61 ± 0.05 | I |
| K11017b | Mu | Leu | Hph | Ph | 1.71 ± 0.13 | I |
| K11002 | Mu | Phe | Hph | Ph | 1.73 ± 0.21 | I |
| WRR-282 | N-Mpip | Phe | Hph | CH2-Ph | 2.02 ± 0.10 | I |
| K11777b | N-Mpip | Phe | Hph | Ph | 2.09 ± 0.08 | II |
| WRR-285 | N-Mpip | Phe | Hph | NH-Ph | 2.13 ± 0.03 | I |
| K11747 | N-Mpip | Phe | Hph | Naphthyl | 2.50 ± 0.12 | I |
| AR-198048 | N-Mpip | Phe-4-CH3 | Hph | Ph | 6.00 ± 0.21 | II |
| WRR-284 | N-Mpip | Phe | Hph | O-Ph | 7.80 ± 0.23 | II |
| AR-198049 | N-Mpip | Phe-3-CF3 | Hph | Ph | 10.5 ± 0.5 | II |
| WRR-145 | Cbz | Phe | Ala | Ph | 22.9 ± 0.5 | III |
| WRR-499 | N-Mpip | His | Hph | Ph | 29.5 ± 0.4 | III |
| K11006 | Mu | Phe | Lys | Ph | 33.2 ± 0.3 | III |
| WRR-483 | N-Mpip | Arg | Hph | Ph | 48.9 ± 0.4 | III |
| WRR-359 | N-Mpip | Phe | Hph | O-Ph | 114 ± 2 | III |
| WRR-185 | Cbz | Phe | Ala | NH-CH(Bz)-COO-CH3 | 126 ± 2 | II |
| WRR-283 | N-Mpip | Phe | Hph | O-COCH2-CH3 | 192 ± 3 | III |
| WRR-200 | Cbz | Phe | Ala | NH-COCH2-Ph-4-CF3 | 247 ± 4 | II |
| WRR-347 | N-Mpip | Phe | Hph | O-Ph-4-O-CH3 | 747 ± 20 | III |
| WRR-453 | Mu | Phe | Tyr | Ph | 11849 ± 101 | III |

Epoxide

CA074b 63.9 ± 2.8 II

a The following abbreviations used are as follows: N-Mpip, N-methylpiperazinylcarbonyl; Mu, morpholinylcarbonyl; Cbz, benzyloxycarbonyl; Ph, phenyl; Bz, benzyl.

b Compounds were analyzed by x-ray crystallography in complex with SmCB1.

c Residues are in R configuration; all other residues are in S configuration.

d Grade I was the most severe; see text for details.

FIGURE 6. Phenotypes induced in vitro in *S. mansoni* NTS by cysteine peptidase inhibitors (listed in Table 1). Examples of three inhibitor-induced phenotypes in the parasite versus untreated controls. Phenotypes arise as a function of time and inhibitor concentration and were graded as follows: Grade I, dead NTS by 2 days of culture at 10 μM and dying/dead NTS by 3 days at 1 μM; Grade II, dead NTS by 3 days at 10 μM and round/dark/dying by 3 days at 1 μM; Grade III, round/dark/dying phenotype in 3 days at 1 and 10 μM concentrations. Scale bar, 0.2 mm.

kinetic parameters of the mode-selective substrates. The endopeptidase substrates of cathepsins B (59), Abz-Gln-Val-Val-Ala-Gly-Ala-EDDnp, and Abz-Ala-Phe-Arg-Phe-Ser-Gln-EDDnp, displayed 2 orders of magnitude lower $k_{cat}/K_m$ values than the carboxydipeptidase substrates Abz-Phe-Arg-ValNph and Abz-Phe-Arg-Nph-Val (60, 61). With the minimized endopeptidase substrate, Cbz-Phe-Arg-AMC (62), $k_{cat}/K_m$ for SmCB1 was 1 order of magnitude lower than for the carboxydipeptidase substrates. This analysis indicates that the exopeptidase catalysis is more efficient than endopeptidase catalysis.

We next designed FRET-based substrate libraries for mapping the carboxydipeptidase activity and residue preferences in
Structural Analysis of SmCB1 Inhibition

FIGURE 7. Hydrolysis of hemoglobin by SmCB1. A, human hemoglobin (Hb) was digested with SmCB1 at various pH values. The degradation rate was determined with the fluorescamine derivatization assay quantifying the liberated fragments. The mean values ± S.E. are expressed relatively to the maximum value. B and C, Hb digest at pH 4.5 was performed at two time points; the reaction mixture was electrophoretically and chromatographically separated and compared with the undigested control. B, Tricine-SDS-PAGE of the Hb digest visualized by protein staining. C, RP-HPLC of the Hb digest resolved on a C4 column using a TFA/acetonitrile system. Elution positions are indicated for the intact Hb substrate (α and β subunits) and for Hb-derived fragments, which form the pools of dipeptides and large peptides (ranging in size from 10 to 41 amino acids, see supplemental Table S4). The flow-through peak (see profile at 0 h) contains nonpeptide components of the reaction mixture.

FIGURE 8. Carboxydipeptidase specificity of SmCB1. The libraries of FRET-based peptide substrates Abz-Phe-Arg-Xaa-Nph-OH and Abz-Phe-Arg-Nph-Xaa-OH were designed to span the P2 to P2’ positions of SmCB1 and introduce substitutions (X) at the P1’ and P2’ positions, respectively. The amino acids at the X position are grouped according to their chemical characteristics (aliphatic, aromatic, polar, acidic, and basic). The substrate hydrolysis was measured in a kinetic activity assay with SmCB1 at pH 5.5. Mean values ± S.E. for triplicate measurements are given normalized to the maximum value in each series (100%).

DISCUSSION

SmCB1 is one of a number of digestive peptidases in the gut of the flatworm parasite *S. mansoni* (8, 12). Both reverse genetics and chemical experiments suggest that it is a critical for parasite growth and a valuable target for the development of novel anti-schistosomal drugs (14, 15). In this study, we provide a comprehensive structure-activity analysis of SmCB1 that includes a series of crystal structure determinations. We also describe the SmCB1 interaction with inhibitors and characterize its specificity with both peptidyl and protein substrates.

Structure of SmCB1—The three-dimensional structure of SmCB1 was solved for three inhibitor complexes; the best resolution achieved was 1.3 Å. SmCB1 possesses an occluding loop that is characteristic of cathepsin B-type peptidases (37). It is known to regulate access to the active site, where it partially blocks the primed substrate-binding subsites (at S3’ and beyond), and thus confer carboxydipeptidase activity to cathepsins B (59, 61, 64). The occluding loop of SmCB1 presents local structural rearrangements compared with mammalian homologs; however, these changes retained the overall loop fold suggesting its functional competence. This was probed by determining the mode of SmCB1 action using specific peptide substrate; carboxydipeptidase activity was clearly manifested. In addition, SmCB1 displayed endopeptidase activity indicating that steric hindrance by the SmCB1 occluding loop is flexible such that the loop can move to accommodate endopeptidase substrate in the active site cleft as has been reported for human cathepsin B (59).
SmCB1, an Efficient Endo- and Exopeptidolytic Machine—

The carboxydipeptidase catalytic efficiency of SmCB1 as measured with peptide substrates was greater than its endopeptidase efficiency. The screening of carboxydipeptidase substrate libraries showed that a broad range of residues is tolerated in the primed positions P1’ and especially P2’. This suggests that SmCB1 is able to effectively the C termini of peptides. SmCB1 has more promiscuous substrate specificity in P2’ than human cathepsin B (60). Furthermore, we identified a combined carboxydipeptidase/endopeptidase action of SmCB1 on the physiological substrate hemoglobin. Analysis of the reaction products indicates that endopeptidolytic fragments are rapidly converted into dipeptides. Thus, oligopolypeptide fragments do not accumulate to the extent observed for hemoglobin digestion by helminth cathepsin L-type endopeptidases (65–67). With regard to hemoglobinolytic capability, SmCB1 resembles cathepsins B from the Southeast Asian liver fluke Opisthorchis viverrini and the hookworm Ancylostoma caninum (66, 68) but differs from cathepsins B of the avian fluke Trichobilharzia regenti and the hookworm Necator americanus that cannot initiate hemoglobinolysis (69, 70). We conclude that SmCB1 operates as an effective proteolytic machine to degrade the major protein in the parasite’s blood meal.

Structure-based Insights for Drug Design—The interaction of SmCB1 with peptidomimetic inhibitors was investigated using the following: (i) crystal structures of three SmCB1-inhibitor complexes, (ii) computational analysis of interaction energies, and (iii) inhibition profiling with a panel of vinyl sulfones. These enabled us to evaluate the critical interactions of inhibitors in the binding subsites and provide a basic SAR for improving inhibitory potency and selectivity.

The S2’ subsite was efficiently occupied in the complex of CA074, a specific inhibitor of cathepsin B-type peptidases. The hydrogen bonding of the inhibitor’s C-terminal P2’ residue with the occluding loop (especially the two His residues) was the largest favorable subsite interaction among the three crystallographic complexes (Fig. 5). The vinyl sulfone inhibitors, by contrast, do not contain a P2’ residue, and therefore, we would consider re-designing the scaffold to extend into the S2’ position, which may improve both potency and selectivity to SmCB1. For the vinyl sulfone P1’, aromatic sulfone moieties were preferred over the Ile residue in CA074 (in accordance with the low substrate specificity for Ile in P1’ (Fig. 8)). An important discovery is the strikingly different conformation between the P1’ phenyl sulfone moieties in the K11777 and K11017 complexes, which suggests a cooperativity between S1’ and other subsite(s) of SmCB1. This conformational switch should be taken into account in future docking experiments to optimize P1’ substituents, e.g. by aromatic groups with a longer linker (Table 1). Interestingly, two types of orientation of the P1’ phenyl sulfone were also observed in the crystal structures of several vinyl sulfone inhibitors with cathepsin L-type peptidases from protozoan parasites, as shown in supplemental Fig. S4 (58, 71, 72). In these complexes, the particular phenyl sulfone orientation was regulated by the structural environment of S1’; however, a dual (transient) conformation of this substituent was also documented (71).

In the S1 subsite of SmCB1, Hph of the vinyl sulfone inhibitors was energetically favored (Fig. 5). A basic residue at this position reduced inhibitory potency (Table 1), although basic P1 residues are preferred in SmCB1 substrates (63). This may reflect the effect of the overall scaffold of the active-site ligand that has been reported to change the P1 specificity of human cathepsin B (60, 61). Based on the structural difference, the S1 pocket can be exploited to engineer selective inhibition of SmCB1 over human cathepsin B. For this purpose, the interaction can be optimized between the P1 residue and Ile-193 located on an nonconserved sequence segment of the occluding loop of SmCB1 (Fig. 2). At P2, bulky hydrophobic residues such as Phe and Leu on the vinyl sulfone scaffold afforded highly potent inhibitors, which agrees with the known P2 substrate preferences of SmCB1 (63). The bottom of the S2 pocket of SmCB1 and other cathepsins B contains Glu, which facilitates the recognition of positively charged residues at P2 (57, 60, 63). Further focus can be placed on this P2-S2 interaction by introducing basic substituents of a suitable size to make contact without displacing the flexible side chain of Glu-316 (supplemental Fig. S5). Finally, for S3, occupation by monocylic heterocycles of the vinyl sulfones generated favorable interaction energies. More bulky substituents can be tested at P3 to improve inhibition, as reported for human cathepsin B inhibitors (73).

SmCB1 as Priority Drug Target—Although SmCB1 is one of a number of peptidases expressed in the gut (7–9) and elsewhere in the parasite (74), the correlation between the severity of phenotypes induced by vinyl sulfone inhibitors and the potency of inhibition of SmCB1 encourage the view that SmCB1 is a valuable drug target. This is congruent with the identification of SmCB1 as a major target for infection by K11777 during experimental therapy in a murine model of S. mansoni infection (15). Given the catalytic efficiency of SmCB1 against hemoglobin described here and being the major cysteine peptidase activity in the parasite (13, 75), it might be anticipated that inhibition of this enzyme would impact the parasite’s ability to thrive. Indeed, RNA interference of SmCB1 slowed the growth of the parasite both in culture and in an animal model of infection (14). To conclude, the SmCB1 crystal structures described herein provide the necessary first step in a structure-based drug development program to improve inhibitor specificity and potency, and possibly, generate new lead anti-schistosomal compounds.

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