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Acetazolamide-based fungal chitinase inhibitors

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ABSTRACT

Chitin is an essential structural component of the fungal cell wall. Chitinases are thought to be important for fungal cell wall remodelling, and inhibition of these enzymes has been proposed as a potential strategy for development of novel anti-fungals. The fungal pathogen *Aspergillus fumigatus* possesses two distinct multi-gene chitinase families. Here we explore acetazolamide as a chemical scaffold for the inhibition of an *A. fumigatus* 'plant-type' chitinase. A co-crystal structure of AfChiA1 with acetazolamide was used to guide synthesis and screening of acetazolamide analogues that yielded SAR in agreement with these structural data. Although acetazolamide and its analogues are weak inhibitors of the enzyme, they have a high ligand efficiency and as such are interesting leads for future inhibitor development.

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1. Introduction

Aspergillus fumigatus is the causative agent of aspergillosis, a life-threatening fungal infection that targets a rising population of immunocompromised patients.¹ Currently available anti-fungal drugs, such as the azoles, amphotericin B and the candins are only partially effective^{2,3} and resistant *Aspergillus* strains have started to appear in hospital settings.^{4,5} Thus there is a need for the identification of novel targets and the development of new anti-fungal agents. Enzymes involved in the biogenesis/turnover of the fungal cell wall are thought to represent possible targets.

Chitin, a polymer of $\beta(1,4)$ -linked *N*-acetylglucosamine (GlcNAc), is an essential structural component of the fungal cell wall, giving it structural rigidity and chemical/biological stability. Because of the inherent rigidity of chitin, fungi need to partially hydrolyse the chitin layer for cell division and morphogenesis, which is carried out by family 18 chitinases.⁶ Two subclasses of family 18 chitinases exist: the 'bacterial-type' chitinases are found in bacteria, fungi and mammals; the 'plant-type' chitinases are found exclusively in plants and fungi. Whereas the 'bacterial-type' enzymes are invariably secreted and mostly possess exochitinase activity,^{7–10} the 'plant-type' chitinases are frequently cell wall associated and possess endochitinase activity. Several studies have shown that these enzymes are involved in yeast mother–daughter

cell separation.^{11,12} Because these enzymes are not intracellular, it is possible to explore a wider area of chemical space for inhibitors, as these would not be required to cross membranes. Whilst humans possess two active chitinases,^{13,14} they are of the 'bacterial-type', and to date the only inhibitors reported are the large, hydrophilic natural products, allosamidin,¹⁵ argifin,¹⁶ argadin¹⁷ and the rationally designed drug-like inhibitor *C*₂-dicaffeine.¹⁸ There are five 'plant-type' chitinases genes in the *A. fumigatus* genome (AfChiA1–5), with a currently unknown transcription profile. Sequence alignments show that they have a high degree of structural similarity in the active site, suggesting that it should be possible to design compounds that inhibit all five enzymes.

Recently we have cloned and over-expressed the 'plant-type' family 18 chitinase Cts1p from *Saccharomyces cerevisiae* (ScCTS1).¹⁹ This enzyme was then screened against the Prestwick chemical library of 880 drug-like molecules. From this, three significant hits were identified, 8-chlorotheophylline, acetazolamide and kinetin (Table 1), all of which were competitive inhibitors of the enzyme and were shown to bind in the active site groove, interacting with the catalytic machinery.¹⁹

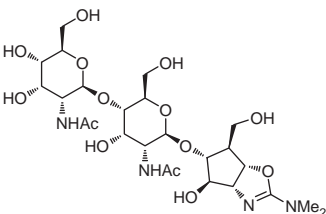
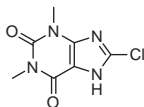
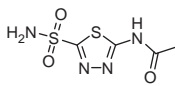
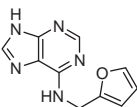
Here we describe a study towards the identification of small inhibitor scaffolds ('fragments') against the 'plant-type' *A. fumigatus* enzyme chitinase A1 (AfChiA1). Two novel ScCTS1 inhibitors, acetazolamide and 8-chlorotheophylline, showed weak inhibition of AfChiA1. We were able to obtain a crystal structure of AfChiA1 in complex with acetazolamide. A number of derivatives of acetazolamide were prepared or purchased and screened against the enzyme AfChiA1; a number were identified with similar activity to acetazolamide.

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Table 1
Inhibitors of ScCTS1 and their activity against AfChiA1

Name	Allosamidin	8-Chloro-theophylline	Acetazolamide	Kinetin
Structure				
ScCTS1 K_i	0.61	340	21	3.2
AfChiA1 IC_{50}	127	410	164	>1000
hCHT IC_{50}	0.04	>2500	>1000	>1000

All data given in μM . hCHT is human chitinase. Data for ScCTS1 have been reported previously.¹⁹ The IC_{50} of allosamidin against hCHT has been reported previously.²²

2. Results and discussion

2.1. Acetazolamide is an efficient inhibitor of *A. fumigatus* chitinase

Previous work has suggested that the plant-type fungal chitinases may be targets for novel anti-fungal strategies.^{11,12,20} So far the only enzyme from this class characterised in some detail is CST1 from *S. cerevisiae* and the plant enzyme hevine.⁶ *A. fumigatus* chitinase A1 (AfChiA1) also belongs to the class of plant-type chitinases family, and has been cloned and characterised recently.²¹

To identify possible inhibitors of AfChiA1, a number of plant-type chitinase inhibitors previously characterised against ScCTS1 were explored as potential scaffolds (Table 1).¹⁹ Allosamidin is an extensively characterised natural product inhibitor of both plant-type and bacterial-type family 18 chitinases,¹⁵ and has recently been reported to competitively inhibit ScCTS1 with a K_i of $0.61 \mu\text{M}$ ¹⁹ and hevamine with a K_i of $3.1 \mu\text{M}$.⁹ Unfortunately, allosamidin is a substrate analogue with poor drug-like properties (high molecular weight, containing glycosidic bonds and an undesirably low $C \log P$ of -5.2) and the total synthesis is costly and complicated.²³ Remarkably, allosamidin only weakly inhibits AfChiA1 ($IC_{50} = 127 \mu\text{M}$, Fig. 1),²¹ which is 30- and 200-fold less potent than values previously reported against hevamine and ScCTS1, respectively. Examination of the purine derivative 8-chlorotheophylline, which had previously been demonstrated to inhibit ScCTS1 with a K_i of $600 \mu\text{M}$,¹⁹ revealed a similar level of inhibition ($IC_{50} = 410 \mu\text{M}$, Fig. 1). Two further compounds, kinetin and acetazolamide, have been identified as ScCTS1 inhibitors by screening the Prestwick Chemical Library, with K_i values of $3.2 \mu\text{M}$ and $21 \mu\text{M}$,

respectively.¹⁹ Kinetin failed to show any discernable effect against AfChiA1 even at concentrations in excess of 1 mM ; acetazolamide on the other hand inhibited AfChiA1 with an IC_{50} of $164 \mu\text{M}$ (Fig. 1), which is an order of magnitude less potent than previously demonstrated against ScCTS1 but not dissimilar to the level of inhibition observed for allosamidin (Fig. 1). It is instructive to compare the ligand efficiencies of the compounds at this stage—that is, the binding energy per non-hydrogen atom.²⁴ Due to their small size acetazolamide and 8-chlorotheophylline are the most efficient of these inhibitors (-0.61 and $-0.57 \text{ kcal mol}^{-1} \text{ atom}^{-1}$, respectively), compared to allosamidin ($-0.19 \text{ kcal mol}^{-1} \text{ atom}^{-1}$). Thus, acetazolamide is a small drug-like molecule that is amenable to preparation of analogues and represents an attractive starting point for further elaboration.

2.2. Crystal structure of the AfChiA1–acetazolamide complex suggests possible derivatives

Of the initial leads investigated, acetazolamide was selected as the most promising starting point for the development of *A. fumigatus* plant-type chitinase inhibitors given its high ligand efficiency. AfChiA1 crystals, reported previously²¹, were soaked with acetazolamide, diffraction data were collected to 2.0 \AA resolution, and the structure of the AfChiA1–acetazolamide complex was solved by molecular replacement and refined to an R_{free} of 0.249 (Table 2) with good stereochemistry. Electron density for the ligand acetazolamide can be seen in both molecules in the asymmetric unit, but it is less clear in chain A, where the active site is partially occluded by a symmetry-related protein molecule. Thus the further discussion of the structure will focus on

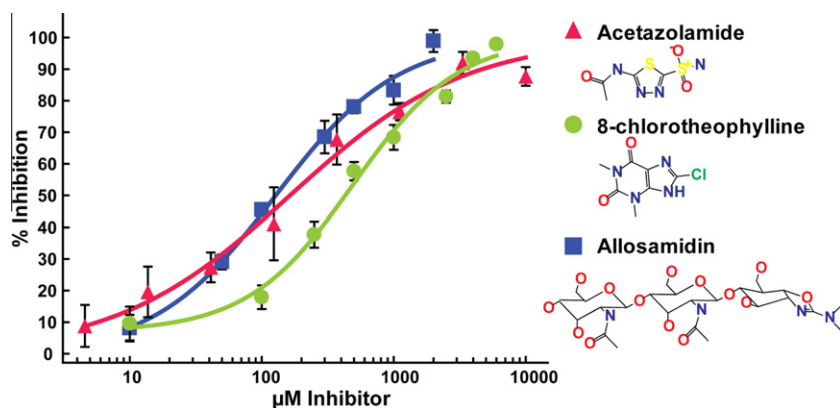


Figure 1. IC_{50} curves determined in triplicate, fitted to a four-parameter logistic dose–response curve (minimum, Hill slope, inflection point and maximum) against AfChiA1 for allosamidin ($IC_{50} = 128 \mu\text{M}$, Hill slope 0.9), acetazolamide ($IC_{50} = 164 \mu\text{M}$, Hill slope 1.1) and 8-chlorotheophylline ($IC_{50} = 410 \mu\text{M}$, Hill slope 1.0) using 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside (4MU-NAG₃) as a substrate.

Table 2
X-ray diffraction/refinement statistics for the AfChiA1–acetazolamide complex

Resolution range (Å)	20.00–2.00 (2.05–2.00)
Number of observed reflections	27,0663
Number of unique reflections	67,879 (4270)
Completeness (%)	98.1 (92.9)
Redundancy	4.0 (3.5)
$I/\sigma(I)$	13.5 (2.5)
R_{merge}	0.085 (0.522)
Wilson B (Å ²)	22.5
$R_{\text{work}}, R_{\text{free}}$	0.216, 0.249
Bond length rmsd from ideality (Å)	0.017
Bond angle rmsd from ideality (°)	1.5
$\langle B \rangle$, overall (Å ²)	27.2
$\langle B \rangle$, protein (Å ²)	26.5
$\langle B \rangle$, solvent (Å ²)	34.1
$\langle B \rangle$, ligand (Å ²)	32.1
Ramachandran plot	
Most favoured (%)	88.6
Additionally allowed (%)	10.8
Generously allowed (%)	00.2

Values in parentheses pertain to the highest resolution shell. Ramachandran plot statistics were calculated with PROCHECK.²⁵

chain B only, which is less impacted and has a more accessible active site.

The overall binding mode of the ligand to AfChiA1 is essentially identical to that observed for ScCTS1 (Fig. 2B).¹⁹ The thiadiazole ring stacks with the conserved Trp312, while its ring nitrogens accept hydrogen bonds from the backbone amides of Ala124 and Tyr125, in the latter case indirectly via an active site-bound water molecule. The acetamido group enters, and essentially fills, the small AfChiA1 active site pocket formed by Tyr238, Gln230, Met310, Ala205, Tyr34 and Asp172. It is oriented by two hydrogen bonds, one from its amide to the side chain of Asp172 and one from the Tyr232 side chain hydroxyl to its carbonyl oxygen. The sulfonamide group on the other hand forms few direct interactions with the protein: it accepts a poor hydrogen bond from the Trp312 side chain and otherwise points away from the protein and into the bulk solvent.

The unexpectedly poor inhibition of AfChiA1 by kinetin can be explained by the presence of methionine 310 in the AfChiA1 active site, which replaces an alanine in the corresponding position in ScCTS1 (Fig. 2A), a substitution found in all *A. fumigatus* plant-type chitinases. These residues define the bottom of the active site pocket that accepts the furanyl group of kinetin.¹⁹ While the pocket is still present in AfChiA1, it is shallower due to the larger Met310 side chain (Fig. 2), rendering it unable to accommodate bulky ligands like kinetin.

A. fumigatus is predicted to possess five plant-type GH18 chitinases (AfChiA1–5) that may have overlapping, if not interchangeable, functions. Thus, for an inhibitor to be useful in vivo, it would have to bind effectively to all five AfChiA active sites. The AfChiA1 sequence in Fig. 2A is shaded based on a sequence alignment of AfChiA1–5, indicating residues identical among all five proteins in purple, residues conserved among four or fewer proteins in shades of blue and completely non-conserved residues in white. The same colouring has also been applied to the AfChiA1 active site surface shown in Fig. 2C, demonstrating that, with the exception of the non-conserved but flexible Tyr125,²¹ the part of the active site cleft interacting with acetazolamide is completely conserved among all five *A. fumigatus* plant-type chitinases. This suggests that acetazolamide could bind similarly, both in orientation and in affinity, to these five enzymes. Fig. 2C also highlights additional conserved active site areas that could be used for the further elaboration of the ligand.

To investigate in silico the potential for such elaboration, we used the docking program LIGTOR¹⁸ to screen for beneficial substitutions/modifications of either the acetamido or the sulfonamide group, while keeping the rest of the molecule constant. Not surprisingly, the scope for modification at the acetamido group is limited.

Docking runs predict that a slight increase in size of this group, for example, by substituting a trifluoroacetamido moiety, could improve overall binding affinity, and even an additional methyl group, yielding a propionamido group, may be tolerated with slight changes to the overall binding mode, but anything larger (including, e.g., isobutyramido groups) cannot be accommodated in the active site pocket and would most likely abolish binding. Modifications/substitutions of the sulfonamide group on the other hand face the opposite problem: as the ligand is essentially pointing away from the active site, most small modifications are tolerated but do not yield additional interactions between ligand and protein. Larger additions to the existing scaffold may be able to interact with additional parts of the AfChiA1 active site, but the required flexibility of such ligands and the corresponding entropy cost associated with orienting the flexible parts on binding to the protein could negate any positive effects on the predicted ligand affinity. To test these computational predictions, a number of acetazolamide derivatives were either synthesised or obtained from commercial suppliers and their binding to AfChiA1 was investigated.

2.3. Synthesis and screening of acetazolamide derivatives

As acetazolamide provided an attractive small molecule starting point for a rational focused inhibitor screen, including a structurally defined binding mode, a number of analogues were screened against AfChiA1 (Table 3). The acetazolamide analogues were either synthesised (Scheme 1) or acquired from commercial sources. The synthesis of compounds carried out to is shown in the scheme.

Compounds were screened against AfChiA1 in duplicate. The assay performance statistics generated from screening plates were well within acceptable screening parameters (Z' 0.72 ± 0.04) and the replicate potency determinations correlated well, yielding errors below 45% for all but one compound (Table 3).

The structures of the compounds allowed determination of the effects of changing the both the sulphonamide (R_1 in Table 3) and acetamide (R_2) portions of the molecule. The screen gave a number of compounds with potencies in the 100–500 μM range, that is, similar to the parent compound. A few trends in the SAR can be deduced. Increasing the size of the acetamide moiety by adding an extra methyl (**2**) or a chloro (**20** and **21**) substituent leads to a reduction in activity, while substitution with a trifluoroacetamide group (compare **11** and **15**) is energetically neutral or slightly favourable; this is in accordance with the structural and docking data, as the methyl of the acetamide group essentially fills the active site pocket as described above. At the same time 'deacetylating' R_2 to a free amine also abolishes inhibitory activity (cf. **6**). Replacement of the sulphonamide is generally tolerated: $-\text{SH}$, $-\text{Ph}$, $-\text{CF}_3$ and $-\text{Br}$ substituents as R_1 (**9–12**) produce compounds with similar activity to acetazolamide (**1**). This is perhaps not surprising as the sulphonamide group does not appear to make significant interactions with the protein. Nonetheless the R_1 substituent does affect affinity as its removal ($R_1 = -\text{H}$, **7**) or replacement with a methyl ($R_1 = -\text{CH}_3$, **8**) again abrogate activity.

3. Conclusion

A. fumigatus contains five plant-type GH18 chitinases; based on the structural information for AfChiA1, it is predicted that the acetazolamide binding sites of AfChiA1–5 are identical, suggesting it may be possible to develop compounds that inhibit all of these enzymes. We have previously reported various inhibitors of ScCTS1; these showed different inhibition profiles against AfChiA1; in particular the binding pocket which accommodated the acetamide group is much smaller in the case of AfChiA1 compared to ScCTS1. The most promising inhibitor was acetazolamide. Although acetazolamide

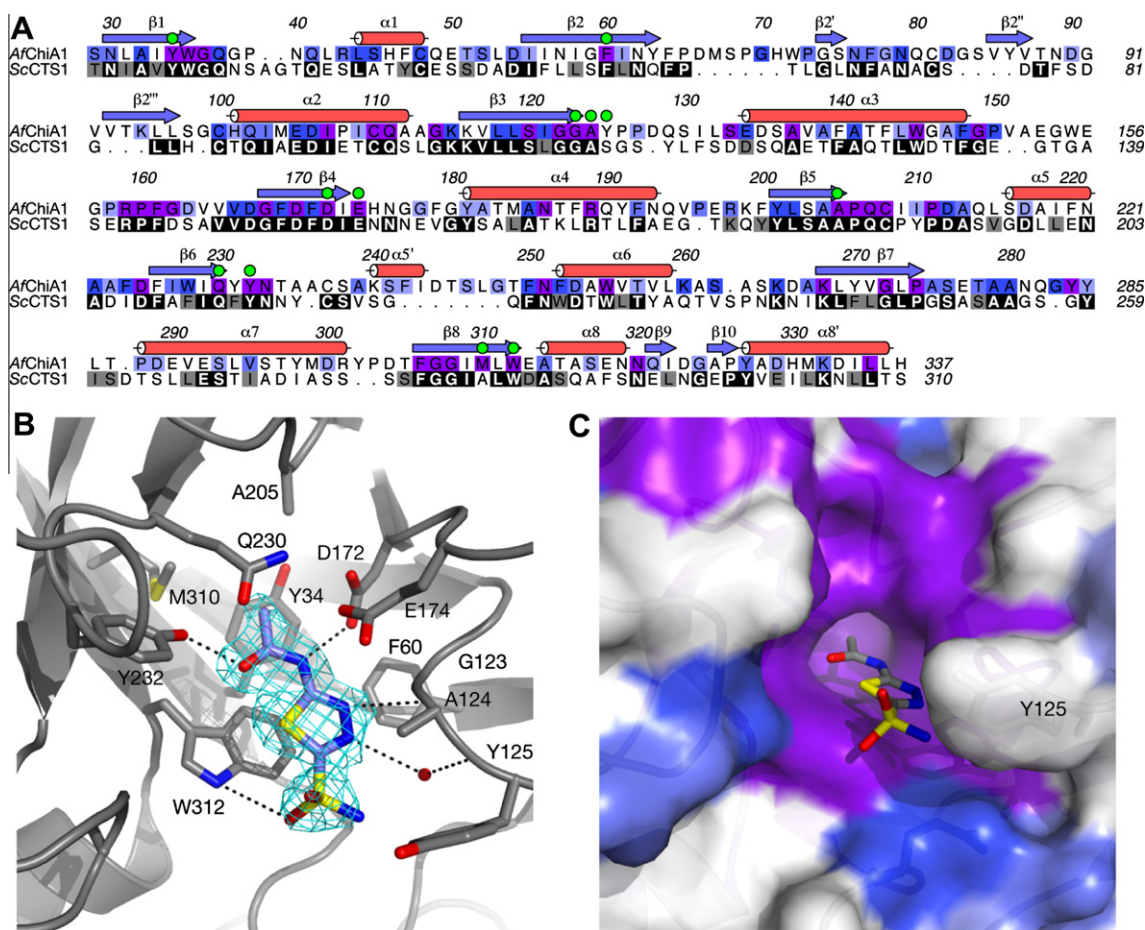


Figure 2. (A) Structure-based sequence alignment of *A. fumigatus* ChiA1 and *S. cerevisiae* CTS1. AfChiA1 secondary structure elements are indicated above the sequence and labelled. Residue numbers are given for AfChiA1. The ScCTS1 sequence is shaded by sequence similarity between the two enzymes shown (black = identical, grey = chemically similar residues), while AfChiA1 is shaded by sequence conservation among *A. fumigatus* ChiA enzymes (purple = 100% identity, then a gradient from blue (mode identical) to white (less identical)). Residues lining the AfChiA1 active site are highlighted by green filled circles. (B) Acetazolamide (slate) binding to the active site of AfChiA1. The protein is shown as a grey cartoon with the side chains of active site residues shown as sticks and labelled. Unbiased (i.e., calculated before the addition of the ligand to the model) σ_A -weighted $F_o - F_c$ density for acetazolamide contoured at 3.0σ is shown in cyan. Possible hydrogen bonds are indicated as black dotted lines, a water participating in indirect hydrogen bonding between ligand and protein is shown as a red sphere. (C) The active site cavity of AfChiA1 (with bound acetazolamide) coloured by similarity among AfChiA proteins as described for panel A. Y125, the only non-conserved residue of the acetazolamide-binding site, is labelled.

and various analogues did not show very potent inhibition, they have relatively low molecular weights. Ligand efficiency is a good way to characterise how efficiently these core scaffolds bind and the potential for them to be optimised to low nanomolar compounds.²⁴ Some of the compounds (Table 3) have ligand efficiencies of better than $-0.3 \text{ kcal mol}^{-1} \text{ atom}^{-1}$. Therefore these possess the potential to be elaborated to compounds with IC_{50} of $<10 \text{ nM}$ and molecular weight of <500 , provided good binding interactions are retained. Most of the interactions with the protein are focused around the amide bond and thiadiazole ring. There is not much scope for further substitution of the acetyl group as the methyl nearly completely occupies a small pocket. However the sulphonamide does not appear to make strong interactions and it is possible to replace this. Therefore optimisation will have to focus on substitution or replacement of this sulphonamide and enhancement of the interactions of the thiadiazole core with the protein.

4. Experimental

4.1. Expression and purification

AfChiA1 Ser29-Leu335 was expressed and purified as described previously.²¹ Briefly, the enzyme was expressed in *Pichia pastoris* as

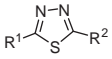
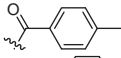
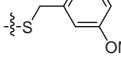
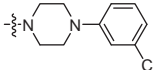
a secreted protein. The culture supernatant was subjected to dialysis and concentration, then AfChiA1 was purified using anion exchange chromatography followed by gel filtration. The resulting pure AfChiA1 protein was used for both kinetic analysis and crystallization trials.

4.2. Crystallisation and structure solution

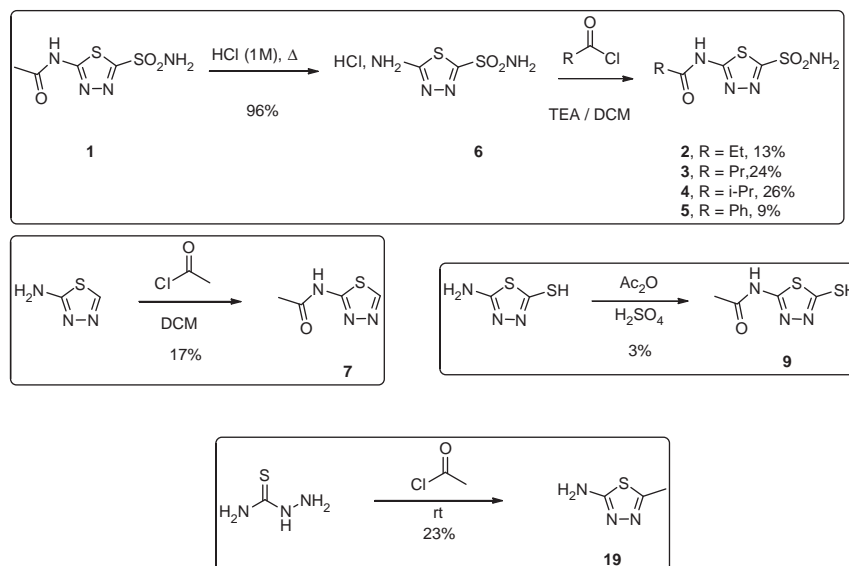
The protein was concentrated to 36 mg mL^{-1} and crystallized by hanging drop vapour diffusion as described previously.²¹ Acetazolamide was incorporated by adding the solid ligand to a crystal-containing drop and incubating for 30 min at room temperature. After cryoprotection by short immersion in $2.5 \text{ M Li}_2\text{SO}_4$, data were collected at 100 K on beamline ID14-EH3 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).

Data were processed and scaled to 2.0 \AA using HKL software²⁶ and the structure was solved by molecular replacement with AMoRe²⁷ using the AfChiA1 apo-structure²¹ as a search model. Refinement of the AfChiA1–acetazolamide complex structure proceeded through rounds of minimisation with REFMAC5²⁸ and model building with Coot.²⁹ Ligand coordinates and topologies were generated with PRODRG.³⁰ PyMol³¹ and ALINE³² were used in the preparation of Fig. 2.

Table 3
Activity of compounds investigated

Compd			IC ₅₀ (μM)	Hill slope	L.E.	% inhibition at 1 mM
	R ¹	R ²				
1	–SO ₂ NH ₂	–NHCOCH ₃	164 ± 75	1.1	–0.40	88
2	–SO ₂ NH ₂	–NHCOCH ₂ CH ₃	315 ± 65	0.71	–0.34	76
3	–SO ₂ NH ₂	–NHCO(CH ₂) ₂ CH ₃	>1000			33
4	–SO ₂ NH ₂	–NHCOCH(CH ₃) ₂	>1000	N/A		N/A
5	–SO ₂ NH ₂	–NHCOPh	850 ± 74	0.5	–0.23	51
6	–SO ₂ NH ₂	–NH ₂	>1000	N/A		N/A
7	–H	–NHCOCH ₃	>1000	N/A		25
8	–Me	–NHCOCH ₃	>1000	N/A		N/A
9	–SH	–NHCOCH ₃	730 ± 120	1.1	–0.43	64
10	–Ph	–NHCOCH ₃	479 ± 210	0.5	–0.30	60
11	–CF ₃	–NHCOCH ₃	141 ± 210	1.1	–0.44	91
12	–Br	–NHCOCH ₃	243 ± 98	0.7	–0.65	76
13		–NHCOCH ₃	>1000	N/A		22
14		–NHCOCH ₃	>1000			38
15	–Ph	–NHCOCF ₃	320 ± 60	1.2	–0.26	85
16	Morpholino	–NHCOCF ₃	>1000	N/A		22
17		–NHCOCF ₃	>1000	N/A		30
18	–Et	–NHCOCH ₂ CH ₃	>1000	N/A		18
19	–Me	–NH ₂	>1000	N/A		21
20	–CH ₂ CH(CH ₃) ₂	–NHCOCH ₂ Cl	>1000	N/A		N/A
21	–CF ₃	–NHCOCH ₂ Cl	>1000	N/A		N/A
22	–CF ₃	–CO(CH ₂) ₂ CO ₂ H	>1000	N/A		N/A
Allosamidin			127		–0.12	
8-Chloro-theophylline			410		–0.33	
Kinetin			>1000			

L.E. = ligand efficiency in kcal mol^{–1} atom^{–1}.²⁴ This was calculated from the equation: $\Delta G = -RT \ln(1/IC_{50})$. IC₅₀ standard deviations calculated with 95% confidence limit.

**Scheme 1.** Synthesis of acetazolamide analogues.

4.3. AfChiA1 inhibition assays

AfChiA1 activity was assayed in McIlvain's buffer (pH 5.5).³³ The final reaction mixture consisted of AfChiA1 (10 nM), 0.05 mg/mL BSA (Thermo) and 4-methylumbelliferyl β-D-N,N,N'-triacetylchitotrioxide (Sigma) (100 μM). Final assay volume was 42 μl in 384-

well black polystyrene plates with a final dimethyl sulphoxide (DMSO) concentration of 1% in all samples, including controls. Test and standard compound concentrations ranged from 1000 to 0.15 μM and 10,000 to 1.5 μM, respectively.

Test and standard compounds were placed into columns 1 and 13 of a 384-well polypropylene plate and then serially diluted in

100% DMSO through half log increments using a JANUS 8-channel Varispan automated workstation (PerkinElmer). This produced a compound source plate containing 30 test and 2 standard compounds curves ($100 \times$ final assay concentration). From this source plate, 0.42 μ L of each compound concentration was then stamped into replicate black 384-well polystyrene assay plates using a Hummingbird (Genomic Solutions).

To the assay plates, 20.8 μ L AfChIA1 (20 nM) was added to all wells with the exception the negative controls. The reaction was initiated by the addition of 20.8 μ L of (200 μ M) 4-methylumbelliferyl β -D-N,N',N''-triacylchitotrioside (stock concentration 200 μ M), both previous additions were executed using a FlexDrop reagent dispenser (PerkinElmer).

Assay plates were then incubated on a microtitre plate shaker (Heidolph) at room temperature for 70 min. Fluorescence generated from the release of 4-methylumbelliferone was quantified using an Envision 2102 Multilabel Reader (PerkinElmer) equipped with 340 nm excitation (band width 60 nm) and 460 nm emission (band width 25 nm) filters.

ActivityBase (Abase) version 5.4 from IDBS was used for the data processing and analysis. All curve fitting was undertaken using a 4 Parameter Logistic dose–response curve using XLFit 4.2 Model 205.

4.4. Compound Synthesis

4.4.1. Synthesis of 5-amino-2-sulfamoyl-1,3,4-thiadiazole monohydrochloride (6)

Hydrochloric acid (70 mL, 70.00 mmol, 5.2 equiv) was added to acetazolamide (2.995 g, 13.34 mmol, 1.0 equiv) and the mixture stirred for 3 h at reflux. The crude material was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 70/30) to yield the product (2.768 g, 96%); mp 179–180 °C; R_f = 0.26 ($\text{CHCl}_3/\text{MeOH}$: 80/20); δ_{H} (500 MHz, DMSO) 7.91 (2H, br s, NH_2 -6), 8.09 (3H, s, NH_3 -7); δ_{C} (125 MHz, DMSO) 157.8 (C-2), 171.6 (C-5); m/z (ES^+): 181.1 ($[\text{M}+\text{H}-\text{Cl}]^+$, 100%); HRMS (ES^+) 180.9849. ($[\text{M}+\text{H}-\text{Cl}]^+$ $\text{C}_2\text{H}_5\text{N}_4\text{O}_2\text{S}_2$ requires 180.9848).

4.4.2. Synthesis of 5-propylamido-2-sulfamoyl-1,3,4-thiadiazole (2)

5-Amino-2-sulfamoyl-1,3,4-thiadiazole monohydrochloride (218 mg, 1.01 mmol, 1.0 equiv) was dissolved in DCM (6 mL). Triethylamine (0.30 mL, 2.16 mmol, 2.1 equiv) was added and the solution stirred for 1.5 h at rt. Then, propionyl chloride (0.20 mL, 2.26 mmol, 2.2 equiv) was slowly added and the mixture left stirring for 1.5 h at rt. Water (1 mL) was added and the precipitate filtered and dried under vacuum. The solid (158 mg) was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 78/22) to yield the product (32 mg, 13%); mp 253–255 °C; R_f = 0.58 ($\text{CHCl}_3/\text{MeOH}$: 80/20); δ_{H} (500 MHz, DMSO) 1.12 (3H, t, H-10, J = 7.5), 2.55 (2H, q, H-9, J = 7.5), 8.34 (2H, br s, NH_2 -6), 13.00 (1H, br s, NH -7); δ_{C} (125 MHz, DMSO) 8.8 (C-10), 28.2 (C-9), 161.2 and 164.1 (C-2 and C-5), 173.0 (C-8); m/z (ES^+): 237.0 ($[\text{M}+\text{H}]^+$, 100%), 495.0 ($[\text{2M}+\text{H}]^+$, 71%); HRMS (ES^+) 237.0101. ($[\text{M}+\text{H}]^+$ $\text{C}_5\text{H}_9\text{N}_4\text{O}_3\text{S}_2$ requires 237.0111).

4.4.3. Synthesis of 5-butylamido-2-sulfamoyl-1,3,4-thiadiazole (3)

5-Amino-2-sulfamoyl-1,3,4-thiadiazole monohydrochloride (286 mg, 1.32 mmol, 1.0 equiv) was dissolved in DCM (7 mL). Triethylamine (0.35 mL, 2.51 mmol, 1.9 equiv) was added and the solution stirred for 1.5 h at rt. Then, butyryl chloride (0.25 mL, 2.36 mmol, 1.8 equiv) was slowly added and the mixture left stirring for 4 h at rt. Water (1 mL) was added and the precipitate filtered and dried under vacuum. The solid (90 mg) was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 78/22) to yield the product (79 mg, 24%); mp 244–246 °C; R_f = 0.54 ($\text{CHCl}_3/\text{MeOH}$:

80/20); δ_{H} (500 MHz, DMSO) 0.91 (3H, t, H-11, J = 7.4), 1.65 (2H, sext, H-10, J = 7.4), 2.52 (2H, m, H-9), 8.33 (2H, br s, NH_2 -6), 12.99 (1H, br s, NH -7); δ_{C} (125 MHz, DMSO) 13.4 (C-11), 17.9 (C-10), 36.7 (C-9), 161.1 and 164.2 (C-2 and C-5), 172.2 (C-8); m/z (ES^+): 251.0 ($[\text{M}+\text{H}]^+$, 73%); 523.0 ($[\text{2M}+\text{H}]^+$, 100%); HRMS (ES^+) 251.0257. ($[\text{M}+\text{H}]^+$ $\text{C}_6\text{H}_{11}\text{N}_4\text{O}_3\text{S}_2$ requires 251.0267).

4.4.4. Synthesis of 5-(2-methyl-propylamido)-2-sulfamoyl-1,3,4-thiadiazole (4)

5-Amino-2-sulfamoyl-1,3,4-thiadiazole monohydrochloride (274 mg, 1.26 mmol, 1.0 equiv) was dissolved in DCM (7 mL). Triethylamine (0.35 mL, 2.51 mmol, 2.0 equiv) was added and the solution stirred for 1.5 h at rt. Then, isobutyryl chloride (0.25 mL, 2.34 mmol, 1.9 equiv) was slowly added and the mixture left stirring for 2 h at rt. Water (1 mL) was added and the precipitate filtered and dried under vacuum. The solid was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 80/20) to yield the product (90 mg, 26%); mp 254–255 °C; R_f = 0.65 ($\text{CHCl}_3/\text{MeOH}$: 80/20); δ_{H} (500 MHz, DMSO) 1.16 (6H, d, H-10, J = 6.9), 2.82 (1H, Sept, H-9, J = 6.8), 8.34 (2H, br s, NH_2 -6), 13.01 (2H, br s, NH -7); δ_{C} (125 MHz, DMSO) 18.9 (C-10), 33.9 (C-9), 161.3 and 164.3 (C-2 and C-5), 176.1 (C-8); m/z (ES^+): 523.0 ($[\text{2M}+\text{Na}]^+$, 100%), 251.0 ($[\text{M}+\text{H}]^+$, 33%); HRMS (ES^+) 251.0272. ($[\text{M}+\text{H}]^+$ $\text{C}_6\text{H}_{11}\text{N}_4\text{O}_3\text{S}_2$ requires 251.0267).

4.4.5. Synthesis of 5-benzylamido-2-sulfamoyl-1,3,4-thiadiazole (5)

5-Amino-2-sulfamoyl-1,3,4-thiadiazole monohydrochloride (315 mg, 1.45 mmol, 1.0 equiv) was dissolved in DCM (7 mL). Triethylamine (0.40 mL, 2.87 mmol, 2.0 equiv) was added and the solution stirred for 1.5 h at rt. Then, benzoyl chloride (0.30 mL, 2.56 mmol, 1.8 equiv) was slowly added and the mixture left stirring for 2.5 h at rt. Water (1 mL) was added and the precipitate filtered and dried under vacuum. The solid (317 mg) was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 78/22) to yield the product (36 mg, 09%); mp 260–261 °C; R_f = 0.66 ($\text{CHCl}_3/\text{MeOH}$: 80/20); (found: C, 38.3; H, 3.2; N, 17.9; S, 21.2. $\text{C}_9\text{H}_8\text{N}_4\text{O}_3\text{S}_2 \cdot 0.7\text{MeOH}$ requires C, 38.0; H, 3.5; N, 18.3; S, 20.9); δ_{H} (500 MHz, DMSO) 7.60 (2H, dt, H-11, J = 7.8, 1.8), 7.71 (1H, tt, H-12, J = 7.4, 1.2), 8.16 (2H, dd, H-10, J = 8.3, 1.1), 8.38 (2H, br s, NH_2 -6), 13.55 (1H, br s, NH -7); δ_{C} (125 MHz, DMSO) 128.6 and 128 (C-10 and C-11), 130.9 (C-9), 133.4 (C-12), 162.2 and 164.7 (C-2 and C-5), 165.7 (C-8); m/z (ES^+): 285.0 ($[\text{M}+\text{H}]^+$, 100%), 569.0 ($[\text{2M}+\text{H}]^+$, 20%); HRMS (ES^+) 285.0102. ($[\text{M}+\text{H}]^+$ $\text{C}_9\text{H}_9\text{N}_4\text{O}_3\text{S}$ requires 285.0111).

4.4.6. Synthesis of 5-acetamido-1,3,4-thiadiazole (7)

2-Amino-1,3,4-thiadiazole (161 mg, 1.54 mmol, 1.0 equiv) was dissolved in DCM (2 mL). Acid chloride (0.15 mL, 2.11 mmol, 1.4 equiv) was slowly added (exothermic), and the mixture stirred for 5 h at rt. The solution was concentrated after addition of H_2O (1 mL). The crude product was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$: 100/0 to 90/10) to yield the product (38 mg, 17%); mp 276–277 °C; R_f = 0.83 ($\text{CHCl}_3/\text{MeOH}$: 80/20); (found: C, 33.9; H, 3.4; N, 27.6; Cl, 21.8. $\text{C}_4\text{H}_5\text{N}_3\text{OS} \cdot 0.03\text{HCl} \cdot 0.18\text{MeOH}$ requires C, 33.5; H, 3.8; N, 28.0; S, 21.4); δ_{H} (500 MHz, DMSO) 2.20 (3H, s, H-8), 9.15 (1H, s, H-2), 12.55 (1H, br s, NH -6); δ_{C} (125 MHz, DMSO) 22.4 (C-8), 148.5 (C-5), 158.5 (C-2), 168.6 (C-7); m/z (ES^+): 144.0 ($[\text{M}+\text{H}]^+$, 60%), 166.0 ($[\text{M}+\text{Na}]^+$, 100%); HRMS (ES^+) 144.0226. ($[\text{M}+\text{H}]^+$ $\text{C}_4\text{H}_6\text{N}_3\text{OS}$ requires 144.0226).

4.4.7. 5-Acetamido-2-thiol-1,3,4-thiadiazole (9)

5-Amino-1,3,4-thiadiazole-2-thiol (554 mg, 4.08 mmol, 1.0 equiv), acetic anhydride (1.8 mL, 19.08 mmol, 4.7 equiv) and concd sulphuric acid (20 mL, 0.37 mmol, 0.09 equiv) were stirred for 30 min on a steam bath. After cooling, the mixture was

concentrated under vacuum, and then purified by column chromatography (CHCl₃/MeOH: 100/0 to 70/30) to yield the product (22 mg, 03%); mp 293–295 °C; *R*_f 0.24 (CHCl₃/MeOH: 90/10); (found: C, 28.1; H, 3.0; N, 22.5; S, 35.1. C₄H₅N₃OS₂·0.3MeOH requires C, 27.9; H, 3.4; N, 22.7; S, 34.7); δ_H (500 MHz, DMSO) 2.14 (3H, s, H-9), 12.45 (1H, br s, NH-7), 14.06 (1H, br s, SH-6); δ_C (125 MHz, DMSO) 22.3 (C-9), 152.2 (C-5), 169.4 (C-8), 183.5 (C-2); *m/z* (ES⁺): 176.0 ([M+H]⁺, 100%); HRMS 175.9946. ([M+H]⁺ C₄H₆N₃OS₂ requires 175.9947).

4.4.8. 5-Amino-2-methyl-1,3,4-thiadiazole (19)

Acetyl chloride (0.50 mL, 7.04 mmol, 2.2 equiv) was slowly added to thiosemicarbazide (291 mg, 3.16 mmol, 1.0 equiv) and the mixture stirred for 4 h at rt. A solution of NaOH 50% was added till pH 12–14, and the mixture concentrated under vacuum. The crude material was purified by column chromatography (CHCl₃/MeOH: 100/0 to 91/09) to yield the product (85 mg, 23%); mp 273–274 °C; *R*_f = 0.79 (CHCl₃/MeOH: 80/20); (found: C, 31.9; H, 4.4; N, 35.4; S, 27.4. C₃H₅N₃S·0.05AcOH requires C, 31.5; H, 4.4; N, 35.6; S, 27.1); δ_H 500 MHz, DMSO 2.17 (3H, s, H-7), 13.15 (2H, br s, NH₂-6); δ_C (125 MHz, DMSO) 10.8 (C-7), 148.8 (C-5), 165.8 (C-2); *m/z* (ES⁺): 115.8 ([M+H]⁺, 100%); 253.2 ([2M+Na]⁺, 100%); HRMS (ES⁺) 116.0275. ([M+H]⁺ C₃H₆N₃S requires 116.0277).

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