ACS Medicinal Chemistry Letters

LETTER

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Bisdionin C—A Rationally Designed, Submicromolar Inhibitor of Family 18 Chitinases

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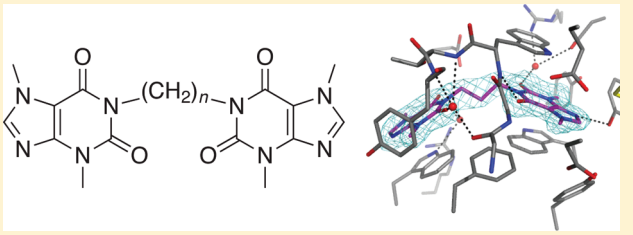
ABSTRACT: Chitinases of the GH18 family play important roles in a variety of pathogenic organisms and have also been shown to be involved in human asthma progression, making these enzymes potential drug targets. While a number of potent GH18 chitinase inhibitors have been described, in general, these compounds suffer from limited synthetic accessibility or unfavorable medicinal-chemical properties, making them poor starting points for the development of chitinase-targeted drugs. Exploiting available structural data, we have rationally designed bisdionin C, a submicromolar inhibitor of GH18 enzymes, that possesses desirable druglike properties and tractable chemical synthesis. A crystallographic structure of a chitinase-bisdionin C complex shows the two aromatic systems of the ligand interacting with two conserved tryptophan residues exposed in the active site cleft of the enzyme, while at the same time forming extensive hydrogen-bonding interactions with the catalytic machinery. The observed mode of binding, together with inhibition data, suggests that bisdionin C presents an attractive starting point for the development of specific inhibitors of bacterial-type, but not plant-type, GH 18 chitinases.



KEYWORDS: GH18 Chitinase, xanthine, ligand design

Chitin, a polymer of β-(1,4)-linked *N*-acetylglucosamine, is an important structural component of the fungal cell wall as well as the exoskeletons of arthropods. Chitinases, the enzymes that hydrolyze chitin, are required for proper morphogenesis of these organisms through their function in cell separation in fungi[1,2] and in molting of arthropods,[3] respectively. Consequently, chitinase inhibitors are thought to have antifungal and insecticidal potential.[3,4] Additionally, humans possess two active chitinases despite our inability to metabolize chitin.[5,6] Human macrophage chitotriosidase (HCHT) has been found to be overexpressed in fungal/bacterial infections and in the lysosomal storage disorder Gaucher's disease,[7,8] while the upregulation of acidic mammalian chitinase (AMCase) in lung tissue is related to asthma progression. Indeed, inhibition of this enzyme has been shown to result in reduced recruitment of inflammatory cells in a mouse model of asthma.[9]

These enzymes, which belong to CAZy family GH18,[10] share an unusual catalytic mechanism involving a nucleophilic attack by the *N*-acetyl group of the −1 sugar itself rather than a protein carboxylate as observed in other retaining glycosidases[11,12] (sugar binding sites are numbered relative to the scissile glycosidic bond between the −1 and +1 sugars[13]). GH18 chitinases can be subdivided at a sequence/structural level into "bacterial-type" and "plant-type" family members, which differ in substrate specificity and active site architecture, with the former possessing a relatively deep active site groove, whereas the latter have a more shallow and accessible open active site.[14−16]

Known inhibitors of these chitinases include cyclic dipeptides,[17,18] cyclic pentapeptides,[19−21] thiazolines,[22] the pseudotrisaccharide allosamidin,[23] and piperazine derivatives.[24] Most of these compounds are natural products or their derivatives, and their use for either in vivo studies or as ligand design leads is significantly impeded by their limited availability and their chemical complexity and subsequent poor synthetic accessibility.

We recently identified xanthine derivatives as promising leads for GH18 inhibitors[25] and subsequently developed a micromolar chitinase inhibitor comprised of two linked caffeine molecules with desirable druglike properties, a crystallographically defined binding mode, and excellent synthetic accessibility.[26] In this work, we present the further development of this class of compounds through in silico and in vitro screens, focusing on modifying the caffeine-caffeine linker and resulting in bisdionin C, the first rationally designed submicromolar inhibitor of GH18 chitinases. The present structural and docking data focus on ChiB1 from the opportunistic fungal pathogen *Aspergillus fumigatus* (*Af*ChiB1) as an experimentally accessible, representative bacterial-type GH18 chitinase, while inhibition data have been determined for a number of chitinases.

We recently described a micromolar inhibitor of family 18 chitinases (Figure 1, n = 2), which was identified in a virtual screen for xanthine derivatives[26] after previous work had shown a

Received: January 11, 2011
Accepted: March 11, 2011
Published: March 23, 2011

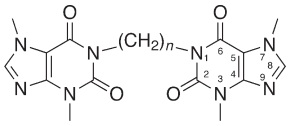


Figure 1. Chemical structure of bisdionin; *n* denotes the number of methylene groups in the linker connecting the two xanthine ring systems, compounds with *n* = 1−5 have been named bisdionin A−E. The ring atom numbering scheme is given for one of the two xanthine rings.

number of these molecules, including caffeine, binding efficiently to the active site of *Af*ChiB1.[25] Upon determining the crystal structure of a complex of *Af*ChiB1 with this compound, it became apparent that the ligand adopts an unexpected strained conformation in the active site. In particular, the "primary" caffeine (which occupies the −1 subsite) is in an energetically less favorable flipped conformation as compared to the previously described crystal structures of complexes with free xanthine derivatives.[25] It was hypothesized that modifying the xanthine-xanthine linker, in particular its length, could alleviate the strain and thus yield a more potent chitinase inhibitor, giving rise to a series of potential chitinase inhibitors, termed the bisdionins here, which are based on the dicaffeine scaffold with variable linker lengths (Figure 1).

To test the effects of linker length on inhibition in vitro, bisdionins C−E were synthesized using established methodology starting from appropriate xanthine and α,ω-dibromoalkane building blocks.[27,28] The inhibitory potency of the bisdionin series was tested against *Af*ChiB1 as well as HCHT and AMCase activity (from mouse lung homogenate) (Table 2). At the expense of introducing a single additional methylene group, in all cases, bisdionin C shows an improvement in inhibition of at least one order of magnitude as compared to bisdionin B. The larger bisdionin variants offer no improvement over bisdionin B.

Like bisdionin B, bisdionin C is a druglike molecule as assessed by Lipinski's rule of five:[29] It has six hydrogen bond acceptors and no hydrogen bond donors, a molecular mass of 400.4 Da, and a predicted logP of approximately zero (calculated using the Molinspiration property calculation service, http://www.molinspiration.com/). The drastic increase in potency from a relatively small increase in compound size also makes it a more efficient chitinase inhibitor (ligand efficiency for *Af*ChiB1 is approximately −0.41 kcal mol^{-1} $atom^{-1}$). This compares favorably with allosamidin, a well-known potent natural product GH18 inhibitor, which violates several of the rule of five criteria by having 10 hydrogen bond donors, 13 acceptors, and a molecular mass of 622.6 Da, as well as having an undesirably low logP of −4.7 and a poorer ligand efficiency for *Af*ChiB1 of approximately −0.25 kcal mol^{-1} $atom^{-1}$.[30]

To investigate the binding mode of bisdionin C, the structure of *Af*ChiB1 in complex with the inhibitor was refined against 2.0 Å synchrotron diffraction data to an R_{work}/R_{free} of 18.3% and 21.6%, respectively (Table 1). The active site reveals clear electron density for the bisdionin C ligand (Figure 2). While the overall binding mode is similar to that of bisdionin B, with one of the caffeines occupying the −1 subsite sandwiched between Trp384 and a flipped-down Trp137 and the second caffeine stacking with Trp52, occupying approximately the −3 subsite, there are a number of striking differences between the two ligands. These differences together explain the significantly improved potency of bisdionin C over bisdionin B.

First, a comparison of intercaffeine distances of bisdionin B (≈8.2 Å) and bisdionin C (≈9.6 Å) suggests that the latter molecule should be a better mimic of a chitooligosaccharide substrate, which, bound to a GH18 active site, has an *n*...*n*+2 sugar ring distance of ≈10 Å (measured in a complex structure of HCgp-39, PDB id 1HJW; Houston et al.[31]). The bisdionin C complex reveals that the longer linker indeed allows the ligand, while maintaining the position of the secondary caffeine, to insert its primary caffeine more deeply into the *Af*ChiB1 active site pocket (Figure 2). In doing so, it displaces the buried water molecule that, in the bisdionin B complex, mediates the hydrogen-bonding interaction between N9 of the primary caffeine and the hydroxyl of Tyr245. In general terms, the tighter fit of the caffeine into the −1 pocket of the protein explains the improved binding of bisdionin C.

Strikingly, the primary caffeine of bisdionin C is flipped by 180° around the N1-linker bond as compared to bisdionin B, reverting it to the binding mode previously observed for smaller xanthine derivatives like caffeine or pentoxifylline.[25] In addition to the deeper insertion into the binding pocket, this orientation also allows the caffeine moiety to make an additional hydrogen bond (a water-mediated H-bond accepted by O2), while the water-mediated hydrogen bond to Tyr245 of the bisdionin B complex is replaced with a direct interaction. These features, together with the fact that smaller xanthine derivatives unrestrained by a second ring system unambiguously adopt this binding mode, suggest that it is energetically more favorable as compared to that observed for bisdionin B. At the same time, it is noteworthy that the active site orientation of the ostensibly less tightly bound secondary caffeine is essentially identical between bisdionin B and C, whereas it is the more buried primary caffeine that undergoes a drastic change in orientation. As has been suggested before,[26] this observation may be explained in part by the fact that

Table 1. Data Collection and Refinement Statistics for the *Af*ChiB1-Bisdionin C Complex[a]

resolution range (Å)	30.0−2.0
total measurements	265212 (24551)
unique reflections	91066 (9004)
completeness	0.998 (0.992)
R_{sym}	0.089 (0.570)
redundancy	2.9 (2.7)
$I/\sigma(I)$	12.3 (2.4)
no. of protein residues	790
no. of ligand residues	2
no. of solvent molecules	691
R_{work}, R_{free}	0.183, 0.216
average *B* factor (Å^2)	
overall	28.8
protein	27.8
ligand	28.4
solvent	36.8
rmsd bonds (Å)	0.010
rmsd angles (°)	1.1
Ramachandran plot statistics (%)	
most favored region	91.6
additional allowed region	8.0
generously allowed region	0.4
disallowed region	0.0

[a] Values in parentheses refer to the highest resolution shell of 0.07 Å width. Ramachandran statistics were determined by PROCHECK.[39]

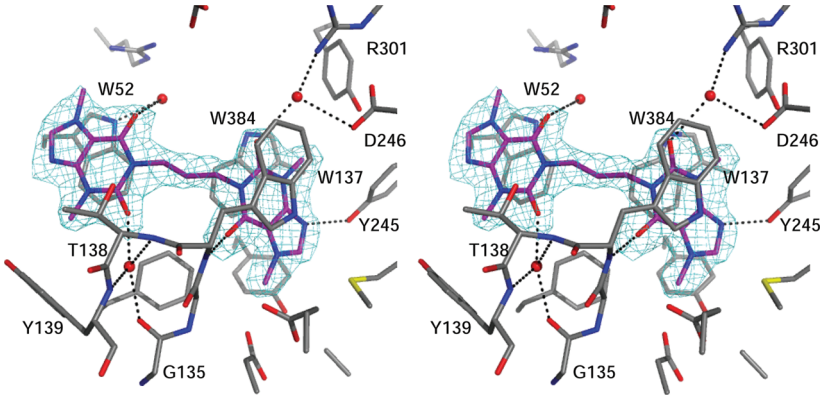


Figure 2. Stereoview of bisdionin C bound to *Af*ChiB1. The ligand is shown in purple, protein residues are colored gray, and selected water molecules are shown as red spheres. Potential hydrogen bonds are indicated by black dotted lines. Residues interacting with the ligands are labeled. The unbiased $|F_o| - |F_c|$, φ_{calc} electron density map covering the ligand is shown contoured at 2.75σ.

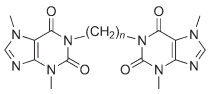
the bisdionin B-like binding mode likely allows for improved π-π stacking between the primary xanthine ring and its sandwiching tryptophan residues, which could alleviate the energetic penalty imposed by the flipped xanthine.

The two xanthine rings of bisdionin C are almost perfectly coplanar with their tryptophan stacking partners. This is in marked contrast to bisdionin B, where an unfavorable xanthine-xanthine angle precludes coplanarity with the tryptophans and instead adopts two conformations in the active site, each of which approximates coplanarity on one side of the molecule, resulting in nonideal π-π stacking interactions and in fact slight clashes with the protein. The longer linker of bisdionin C increases flexibility, and its odd number of methylene groups is more compatible with the two xanthines being at an angle suitable for optimal stacking in the chitinase active site. This is further supported by the fact that the aromatic rings of bisdionin C are essentially coplanar with the "unrestrained" ring systems found in the *Af*ChiB1-caffeine complex structure,[25] which shows two caffeine molecules bound in the −1 and the −3 subsites.

The three tryptophan residues (52, 137, and 384 in *Af*ChiB1) that are the hallmark features of the *Af*ChiB1-bisdionin C interaction are conserved in the majority of bacterial-type GH18 chitinases, suggesting that bisdionin C might be a potent inhibitor of other members of this subfamily, including the two human chitinases. Indeed, these two enzymes are inhibited by bisdionin C, albeit with an approximately 1.5 orders of magnitude higher IC_{50} (Table 2). Considering the expected degree of similarity between the active sites of these three enzymes,[26] the source of this significant reduction in affinity is unclear. At the same time, two of these tryptophan residues are missing from plant type GH18 enzymes, which would suggest that bisdionin C should be a poor inhibitor of this GH18 subfamily. To test this, bisdionin IC_{50} values for *Af*ChiA1, a representative plant type chitinase, were determined (Table 2). As expected, bisdionins B−D show no significant inhibition of *Af*ChiA1. Surprisingly, bisdionin E was found to inhibit *Af*ChiA1 weakly; it is possible that due to the longer tether its secondary caffeine is able to interact with an unknown new *Af*ChiA1-specific binding site. Being specific for the bacterial-type chitinases, bisdionin C could thus provide a chemical tool to dissect the different functions of plant type and bacterial type enzymes in biological systems.

Through in vitro screening of bisdionin compounds with varying linker lengths, we have identified bisdionin C as a potent

Table 2. Experimental IC_{50} Values for Inhibition of Family 18 Chitinases by the Bisdionins



	IC_{50} (μM)			
bisdionin (n)	*Af*ChiB1	HCHT	AMCase	*Af*ChiA1
B (2)	4.8 ± 1.4	110 ± 10	90 ± 4	>1000
C (3)	0.20 ± 0.01	8.3 ± 0.7	3.4 ± 0.2	>1000
D (4)	9.0 ± 2.0	91 ± 17	ND	>1000
E (5)	5.7 ± 0.8	260 ± 70	ND	820 ± 50

family 18 chitinase inhibitor. The crystal structure of a *Af*ChiB1-bisdionin C complex elucidates how the addition of a single CH_2 group can improve ligand affinity by at least 10-fold for all tested bacterial-type chitinases: The longer linker not only allows the primary caffeine to penetrate deeper into the active site but fortuitously also enables the ligand to engage in concurrent and close-to-ideal $\pi-\pi$ stacking interactions with both of its aromatic systems. At the same time, bisdionin C retains its desirable druglike properties and good synthetic accessibility, making it an excellent lead for the development of drugs targeted at the bacterial-type chitinases involved in a variety of disease processes. In particular, the inhibition of AMCase, while decreased as compared to that of *Af*ChiB1, suggests that bisdionin C will provide a good starting point for the development of AMCase inhibitors, which could be useful in the treatment of allergic asthma.[9] In addition, the specificity of bisdionin C for bacterial-type over plant-type GH18 chitinases will enable its use as a chemical tool to dissect the different roles these subclasses of enzymes play in, for example, fungal organisms.

EXPERIMENTAL PROCEDURES

A. fumigatus ChiB1 (*Af*ChiB1) and human chitotriosidase were expressed and purified as described previously.[25,26] AMCase activity was determined from mouse lung homogenate. Lung homogenates were prepared from female BALB/C mice. Tissue (100 mg) was taken from isolated lung lobes, homogenized in 2 mL of HBSS (Invitrogen), and

centrifuged (800*g*, 10 min), and the resulting supernatant was collected. *Af*ChiA1 was expressed and purified as described by Rush et al.[32].

The bisdionins B−E (Itaharo and Imamura;[27] Figure 1) with alkyl linkers ranging from two ($n = 2$, bisdionin B) to five methylene units ($n = 5$, bisdionin E) were synthesized according to the method of Cavallaro et al.,[28] whereby a suspension of theobromine (2 equiv) and potassium carbonate (2 equiv) in dry DMF was heated to 120 °C under argon for 1 h, followed by treatment with 1 equiv of the appropriate α,ω-dibromoalkane. The reaction mixture was poured into water and neutralized with 0.1 M HCl. The precipitate formed was then collected by filtration and purified by precipitation from a chloroform solution with ether. The compounds were characterized by ^{1}H and ^{13}C NMR and electrospray mass spectrometry, and their purity was confirmed by analytical RP-HPLC.

The enzyme activity was determined as described before,[26,32] using 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobiose (4MU-GlcNAc$_2$; for *Af*ChiB1 and lung homogenate) and 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose (4MU-GlcNAc$_3$; for HCHT and *Af*ChiA1), both purchased from Sigma, as substrates. All IC_{50} determinations were carried out with triplicate measurements for each inhibitor concentration. Data analysis was performed in GRAFIT 5.0.[33]

Crystals of *Af*ChiB1 were grown as described before.[25] After they were washed in 0.1 M sodium citrate, pH 5.5, and 1.4 M Li_2SO_4 to remove bound Tris, they were soaked in mother liquor containing ≈125-fold molar excess of bisdionin C for up to 30 min. After cryoprotection in 3 M Li_2SO_4, data were collected at BM14, European Synchrotron Radiation Facility (ESRF) (Grenoble, France). Data were processed using the HKL suite[34] and truncated using CCP4 software.[35] After phasing by manual molecular placement of the apo-*Af*ChiB1 structure (PDB id 1W9P), refinement was performed using REFMAC[36] and model building in O.[37] Starting coordinates and topologies for bisdionin C were generated by PRODRG.[38] Data and model statistics are given in Table 1. Figures were generated using PyMOL (www.pymol.org). Atomic coordinates and structure factors have been deposited with the Protein Data Bank (www.rcsb.org).

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Funding Sources

This work was supported by a Wellcome Trust Senior Research Fellowship and an MRC Programme Grant (D.vA.), a Wellcome Trust Project Grant (I.M.E. and D.vA.), a Wellcome Trust Ph.D. Studentship (M.A.), and a BBSRC CASE Studentship (F.V.R.). We thank the staff at ESRF BM14 for their support.

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