Screening-based Discovery and Structural Dissection of a Novel Family 18 Chitinase Inhibitor

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Running Title: Chitinase Inhibitor from Purine Fragments

Family 18 chitinases play key roles in the life cycles of a variety of organisms ranging from bacteria to man. Very recently it has been shown that one of the mammalian chitinases is highly overexpressed in the asthmatic lung and contributes to the pathogenic process through recruitment of inflammatory cells. Although several potent natural product chitinase inhibitors have been identified, their chemotherapeutic potential or their use as cell biological tools is limited due to their size, complex chemistry, and limited availability. We describe a virtual screening-based approach to identification of a novel, purine-based, chitinase inhibitor. This inhibitor acts in the low micromolar ($K_i = 2.8 \pm 0.2 \mu M$) range in a competitive mode. Dissection of the binding mode by X-ray crystallography reveals that the compound, which consists of two linked caffeine moieties, binds in the active site through extensive and not previously observed stacking interactions with conserved, solvent exposed tryptophans. Such exposed aromatics are also present in the structures of many other carbohydrate processing enzymes. The compound exhibits favourable chemical properties and is likely to be useful as a general scaffold for development of pan-family 18 chitinase inhibitors.

Family 18 chitinases (CAZy GH 18 (1)) hydrolyse chitin, a homopolymer of $\beta$-(1,4)-linked N-acetylglucosamine. Chitin is a key component of the fungal cell wall, and chitinases are thought to be required for fungal cell separation and morphogenesis (2–4). Strikingly, while chitin does not occur in humans, it is known that we possess two chitinases (5, 6). Human macrophage chitotriosidase (HCHT) is secreted by activated macrophages and is able to degrade chitin from the cell wall of the fungal pathogen Candida albicans. Although its precise role has not been defined, it has been shown to be upregulated during fungal or bacterial infection (7) and in Gaucher’s disease, a lysosomal storage disorder (8). A second chitinase, termed acidic mammalian chitinase (AMCase) was subsequently discovered and shown to possess an unusually low pH optimum (<3) (6), and its overexpression in the lung has been linked to the progression of asthma.

Most known family 18 chitinase inhibitors are natural products (reviewed in 9), including the cyclic dipeptide CI-4 and related compounds (10, 11), the cyclic pentapeptides argifin and argadin (12–14), and the pseudotrisaccharide allosamidin, a nanomolar inhibitor isolated from Streptomyces sp. (15). Their poor synthetic accessibility and limited availability from natural sources has so far hampered the use of these inhibitors for in vivo studies, while their chemi-
cal complexity makes them unsuitable as starting points for ligand design efforts. Nevertheless, numerous crystallographic and biochemical studies have elucidated the interactions between these compounds and family 18 chitinases (e.g. 11, 14, 16–18).

Recently, a high throughput screen identified three xanthine derivatives, theophylline, caffeine and pentoxifylline, as competitive inhibitors of bacterial, fungal and human family 18 chitinases (19). Although their inhibition was not strong (high micromolar), analysis of their mode of inhibition revealed that these molecules mimic many of the chitinase-substrate hydrogen bonds and in addition display extensive π-π stacking interactions with a conserved tryptophan. Here, we describe a simple, fragment-based approach to virtual screening of commercially available chemical structures, starting from the caffeine scaffold. The virtual screen resulted in the identification of a new xanthine-based chitinase ligand that competitively inhibits several family 18 chitinases in the low micromolar range.

EXPERIMENTAL PROCEDURES

LIGTOR: a simple and efficient virtual screening algorithm— Several examples of successful fragment-based drug discovery have recently been reported (reviewed in 20, 21). The approach is to identify binding modes of very small, but highly efficient, chemical entities, which are then further elaborated or connected together through small linkers to yield dramatic increases in inhibition. Unfortunately, no freely available, non-commercial programs for such elaboration of fragments have so far been reported. Here we describe LIGTOR, a program for rapidly evaluating compounds starting from a 2D library of chemical molecules and a structurally characterised starting fragment. As a first step, a virtual compound library is searched for molecules containing the fragment as a substructure; depending on the choice of fragment, this step will leave only a small number of compounds for consideration. Using the fragment structure as a template, chemically sensible three-dimensional coordinates are generated for the selected compounds, so that orientation and conformation of the fragment-equivalent substructure matches that of the template as closely as possible, even if the molecules differ in atom or bond types due to degeneracy in the search pattern. If a library compound contains more than one instance of the fragment substructure, several coordinate sets are produced. These initial binding modes are then optimised by evaluation of torsion angles (outside the fragment substructure) and, optionally, the overall position/orientation in the context of the protein receptor. Subsequently, compounds with poor predicted binding or excessive coordinate shifts of their fragment substructure are discarded. The docked complexes of the remaining compounds are inspected visually, promising candidates are chosen for in vitro testing and cocrystallisation.

The substructure search is carried out with SDFSCAN, a command-line tool for selecting compounds from a library by (sub-)structure; SDFSCAN accepts input either in SDFile format or in an enriched SDFSCAN-specific format which can be generated using PRODRG (22). Template-based coordinate generation is also performed with PRODRG. Ligands are docked using LIGTOR, a PRODRG extension for evaluating and optimising ligand poses against a receptor described by affinity grid maps. Currently, LIGTOR uses the AutoDock 3.0 force field (23) with a modified ligand-internal energy term to accelerate computation. Another difference to AutoDock is that LIGTOR minimises the predicted ΔG, not ΔH. LIGTOR obtains ligand descriptions including a list of optimisable torsion angles from PRODRG. Depending on the application, LIGTOR can also optimise the overall position/orientation of the ligand or, alternatively, keep a part of the ligand (in this case the fragment substructure) fixed. In the former case harmonic restraints may be applied to either the coordinates of a substructure or to the center of mass of parts/all of the ligand.
Instructions for obtaining SDFSCAN, LIGTOR and PRODRG are available from http://davapc1.bioch.dundee.ac.uk.

Protein purification and enzyme assays— Chitinase B1 from Aspergillus fumigatus (Af/ChiB1) was overexpressed in Escherichia coli and purified as described previously (19). Selected hits from the virtual screen against Af/ChiB1 were purchased from suppliers indicated in Table I. C2-dicafeine was later re-synthesised according to the method of Cavallaro et al. (24). The compound was characterised by 1H and 13C NMR and electrospray mass spectrometry, and its purity confirmed by analytical RP-HPLC.

Enzyme activity was determined using 4-methylumbelliferonyl-β-D-N,N'-diacetylchitobiose (4MU-GlcNAc2; Sigma) or 4-methylumbelliferonyl-β-D-N,N',N''-triacetylchitobiose (4MU-GlcNAc3; Sigma) as substrates in a standard assay as described previously (5, 18, 19). In a final volume of 50 µl, 5 nM of enzyme was incubated with 20 µM substrate in McIlvain buffer (100 mM citric acid, 250 mM sodium phosphate pH 5.5) containing 0.25 mg/ml bovine serum albumin, for 10 min at 37 °C in the presence of different concentrations of inhibitor. 5-30 µM 4MU-GlcNAc2 was used for determination of K_i for C2-dicafeine against Af/ChiB1. Experiments were performed in triplicate. Production of 4MU was linear for the incubation period used, and less than 10% of available substrate was hydrolysed. Kinetic data were fitted with GRAFIT 5.0 (25) against standard equations for dose response curves and competitive inhibition. A similar approach was employed to evaluate inhibition against the human chitotriosidase (22 µM 4MU-GlcNAc3, 0.4 nM enzyme) and chitinase A from Serratia marcescens (60 µM 4MU-GlcNAc2, ≈ 7.4 nM enzyme; Sigma).

Crystallisation and structure determination— Crystals of Af/ChiB1 were grown by vapour diffusion from 0.1 M Tris/HCl, pH 9.5 and 1.4 M Li2SO4. Crystals were washed three times in 0.1 M sodium citrate, pH 5.5 and 1.4 M Li2SO4, then soaked for 25 min in mother liquor containing ≈ 125-fold molar excess of C2-dicafeine before cryoprotection in 3 M Li2SO4. Data were collected at beamline BM14, European Synchrotron Radiation Facility (ESRF), and processed with the HKL suite (26). Refinement was started from the native Af/ChiB1 structure (PDB id 1W9P) and proceeded through cycles of model building with O (27) and positional/B-factor refinement using REFMAC (28). A model for C2 dicafeine was not included until completely defined by the unbiased |F_o| – |F_c|, ϕ, e calculations electron-density maps (Fig. 1C). Ligand starting structure and topology were generated with PRODRG (22). Data and refinement statistics are given in Table II. Fig. 1 was rendered with PyMOL (www.pymol.org).

RESULTS

Identification of elaborated 3-methylxanthishines by virtual screening— Recently, three 1,3-dimethylxanthine derivatives, theophylline, caffeine and pentoxifylline (Fig. 1A), were identified as inhibitors of Aspergillus fumigatus chitinase B1 (Af/ChiB1) in a high-throughput screen (19). Despite their relatively poor affinity (K_i = 37 µM for pentoxifylline), these compounds present interesting starting points for ligand design, as they displayed a common binding mode, not previously observed in family 18 chitinase inhibitors, involving extensive π–π stacking interactions with a fully conserved tryptophan and mimicry of chitinase-substrate hydrogen bonds (Fig. 1). Furthermore, these compounds show good ligand efficiency (expressed as ΔG per non-hydrogen atom (29), Δg = –0.43 kcal·mol⁻¹·atom⁻¹ for pentoxifylline) and are easy to synthesise or derivatise. The rigid xanthine moiety of the three compounds binds in a virtually identical orientation to the Af/ChiB1 active site (19), making it an ideal starting point for further optimisation.

From a chemical point of view, further functionalisation of the xanthine scaffold is mainly possible at four positions: at N1, N3, N7 and
C8 (Fig. 1A). However, the binding mode of the xanthine suggests that of these, modification at N7 and C8 is restricted as these both point towards small cavities in the structure. In comparison, both N1 and, to a lesser extent N3, should be able to accept larger modifications (Fig. 1A). The oxohexyl group of pentoxifylline is an example for such a modification on N1 (Fig. 1A) and its presence improves binding to AfChiB1 almost four-fold (19).

Purine scaffolds have been extensively used in inhibitor design (e.g. 30–33). We searched a database of 5.1 million commercially available compounds for molecules containing a 3-methylxanthine substructure, identifying 50193 candidate ligands. We then evaluated all these ligands using the LIGTOR program developed as part of this study (see Experimental procedures). The coordinates of the 3-methylxanthine substructure were kept fixed, while performing a torsional evaluation of the substitutions, in the context of the protein component of the published AfChiB1-pentoxifylline complex (19) as the receptor. After docking with PRODRG/LIGTOR, compounds were selected by predicted free enthalpy of binding (∆G < −10.0 kcal · mol⁻¹), predicted ligand efficiency (∆g < −0.35 kcal · mol⁻¹ · atom⁻¹) and coordinate shift of the 3-methylxanthine substructure atoms (RMSD between starting and docked conformations < 0.6 Å). The predicted conformations of the 263 unique compounds fulfilling all three criteria were inspected visually and finally twelve compounds listed in Table I were purchased for in vitro testing. The docked conformations for these compounds are shown together in Fig. 1B.

Desirable chemical features are enriched in the virtual screening hits—A question in assessing the validity of the virtual screening approach is to what extent the docking-based compound selection leads to an enrichment of the discussed features (small groups on N7/C8, larger groups on N1/N3), especially as the set of 50193 xanthine derivatives that serve as the starting point show an unfavourable distribution of functionalisations: less than 9% of the compounds carry more than one non-hydrogen atom on N1, while 90% and 88% of all compounds have undesirably large groups attached to N7 and C8, respectively (Fig. 2B). However, analysis of the group size distributions for the set of 263 compounds selected after docking shows, as expected, a clear preference for large modifications on N1 (97% of the set carry two or more attached non-hydrogen atoms) and at slight preference for large modifications on N3 (Fig. 2B). Similarly, the expected restrictions on substitutions on N7 (over 95% of the selected compounds are modified with a single non-hydrogen atom) and C8 (a relative increase of unmodified compounds, although more than half of the selected molecules still carry a ‘heavy’ modification). In conclusion, the set of functionalisations selected by the virtual screen are generally in agreement with the distribution expected based on experimental data and visual inspection of the active site.

The virtual screen identifies a low micromolar inhibitor—Twelve compounds from the selected set were chosen for in vitro evaluation (Fig. 2). They were picked to approximately represent the chemical features found in the entire set. Most of them have functional groups attached at N1 which may interact with Trp⁵², as observed in the pentoxifylline complex (Fig. 1A). One compound has a free N7, the others present a methyl group at N7. One compound each carries a large group on N3 and C8, otherwise these atoms are methylated or unmodified, respectively. The compounds are listed, with their predicted free enthalpy of binding and efficiency, in Table I, as is pentoxifylline, which was also identified as a candidate ligand by the virtual screen.

Of the twelve compounds, three were not delivered by the suppliers, for the remaining compounds IC₅₀ values for the inhibition of AfChiB1 were determined. Only two of them, compounds 2 and 5 (Fig. 2A), showed significant inhibition. Compound 5 performs significantly worse than its parent compound caffeine and was not considered any further. Only compound 2, (1-
(2-((theobromine-1-yl)ethyl)-theobromine, from here on referred to as C$_2$-dicaffeine), showed a significantly increased affinity to AfChiB1. With an IC$_{50}$ of (4.8±1.4) μM it improves inhibition by two orders of magnitude compared to its parent compound (caffeine) and even when compared to pentoxifylline, so far the highest-affinity xanthine-based chitinase inhibitor, it improves inhibition $\approx$ 25-fold.

C$_2$-dicaffeine is a competitive inhibitor with an extensive $\pi$-$\pi$ stacking binding mode—
The inhibition of AfChiB1 by C$_2$-dicaffeine was further investigated using kinetic measurements. Lineweaver-Burk analysis of steady-state kinetic experiments with varying substrate and inhibitor concentrations showed that C$_2$-dicaffeine is a competitive inhibitor of the chitinase with a $K_i$ of (2.8 ± 0.2) μM (Fig. 2C). This makes it more than one order of magnitude more potent than pentoxifylline ($K_i = (37 ± 2) \mu M$ (19)).

We next investigated the binding mode of C$_2$-dicaffeine by solving a high resolution crystal structure of the AfChiB1-C$_2$-dicaffeine complex (Table II, Fig. 1C). The active site Asp$^{175}$ is pointing down into the catalytic core similarly to the other xanthine derivative complexes (RMSD = 0.244 Å for 185 atoms of 19 active site residues comparing to the pentoxifylline complex). Defined electron density for C$_2$-dicaffeine shows the ligand bound to the protein in an overall orientation similar to that predicted by docking: the ‘primary’ caffeine moiety is sandwiched between Trp$^{384}$ and Trp$^{52}$ (Fig. 1C). The crystal structure also reveals two major differences from the known xanthine binding mode and the docked conformation: first, C$_2$-dicaffeine is shifted out of the active site by about 1.5 Å and second, both purine rings are flipped by 180° around their N1-linker bond. This binding mode significantly improves the $\pi$-$\pi$-stacking between the secondary caffeine and Trp$^{52}$ compared to the docked orientation.

Similar to the other xanthine inhibitors the primary caffeine moiety of C$_2$-dicaffeine accepts two hydrogen bonds, one from the backbone amide of Trp$^{137}$ and one from the hydroxyl of Tyr$^{245}$. Due to the flip of the ring system the former H-bond is formed by O2 instead of O6, and the direct interaction between Tyr$^{245}$ and N9 is replaced with a water-mediated hydrogen bond involving the same groups (Fig. 1C). O2 of the secondary caffeine occupies a position similar to that of the pentoxifylline side chain carbonyl oxygen and, like it, accepts a hydrogen bond from a water molecule which in turn is held in place through interactions with the 136-139 loop forming one wall of the active site (Fig. 1A,C). The secondary O6 also hydrogen bonds to a water which interacts with the indole NH of Trp$^{52}$ and the guanidino group of Arg$^{57}$.

Further inspection of the ligand electron density revealed it to be most compatible with the C$_2$-dicaffeine binding in a dual conformation. Refinement supported a major conformation with occupancy 0.75 and a minor conformation with occupancy 0.25. The two conformations are approximate mirror images of each other with the mirror plane being the average plane through all ligand atoms. This means that the two conformations occupy essentially the same space and form the same hydrogen bonds with the active site; the main differences between them are the tilt of the purine rings relative to their stacking partners and the torsion angles of the ethylene linker. The linker adopts a staggered conformation, i.e. the central torsion angle is approximately 180°, while the torsion angles with the purine rings are $\approx 90°$ and $-90°$ on either side. This linker conformation prevents the two caffeine rings from both achieving ideal stacking - the major ligand conformation has the primary caffeine significantly tilted against its tryptophan stacking partners (angle $\approx 19°$), whereas the minor conformer has an unfavourable angle of over 23° between the Trp$^{52}$ indole and its secondary caffeine. Together, the ligand shift/flip, the disorder and tilting of the aromatic systems suggest that C$_2$-dicaffeine is in a strained conformation in the observed complex, which is non-ideal for tight binding.

C$_2$-dicaffeine inhibits several family 18
chitinases— Comparing the sequence of AfChiB1 with that of other ‘bacterial-like’ family 18 chitinases shows a high degree of conservation of the active site residues (Fig. 1C). With the exception of Thr138 and Tyr139, all residues directly interacting with the C2-dicaffeine ligand in the complex structure are conserved in > 70% of known bacterial-like chitinases. In particular, the three tryptophan residues stacking with the aromatic systems of the ligand, Trp52, Trp137 and Trp384, are conserved in > 80% of sequences, suggesting that C2-dicaffeine may be useful as a ligand for other bacterial-like family 18 chitinases. To investigate this possibility, three additional family 18 chitinases were assayed for inhibition by C2-dicaffeine: human chitotriosidase (HCHT), the acidic mammalian chitinase activity from mouse lung samples (mAMCase) and the bacterial chitinase A from Serratia marcescens (SmChiA).

The HCHT active site contains two amino acid substitutions compared to AfChiB1: Thr138 is replaced by an asparagine and Tyr139 is replaced by a phenylalanine. Despite the relatively conservative nature of these substitutions C2-dicaffeine inhibits HCHT with an IC50 of 107 ± 5 µM, which is 1.3 orders of magnitude weaker compared to its inhibition of AfChiB1. Thr138 and Tyr139 form one wall of the subsite binding the secondary caffeine and while their side chains do not form hydrogen bonds with the ligand, they make extensive van der Waals interactions which are likely to contribute significantly to ligand binding and stabilisation. Crystal structures of HCHT (17, 34) show that the asparagine replacing Thr138 adopts a conformation similar to that of the threonine and thus is unlikely to significantly alter binding. On the other hand, the side chain of the phenylalanine replacing Tyr139 points away from the active site, into a pocket created by a glycine in the place of AfChiB1 Asn78. This leaves the binding site for the secondary caffeine more open, explaining the reduced affinity of HCHT for C2-dicaffeine, especially considering that ‘monocaffeine’ ligands like theophylline or pentoxifylline bind more tightly to HCHT compared to AfChiB1.

Mouse (and human) AMCase carry the same two substitutions as HCHT; additionally Arg301 is replaced by a histidine and a glutamine takes the place of Glu322. The IC50 for C2-dicaffeine against mouse lung AMCase activity is 90 ± 4 µM, which is similar to the value obtained for HCHT, suggesting that neither the arginine-to-histidine substitution nor the replacement of Glu322, which does not directly interact with the dicaffeine ligand, significantly influence ligand binding.

SmChiA differs from AfChiB1 in only one active site residue — Tyr139 is replaced by a leucine. Still, this substitution increases the IC50 for C2-dicaffeine against SmChiA to 71 ± 3 µM. Inspection of SmChiA crystal structures (e.g. PDB id 1CTN (35)) shows that the smaller leucine side chain could not reach a C2-dicaffeine ligand bound in the conformation observed for AfChiB. This again results in a loss of van der Waals interactions, thus explaining the reduced affinity of the dicaffeine for SmChiA compared to AfChiB1.

While the wide conservation of the three ‘essential’ tryptophans among bacterial-like family 18 chitinases suggests that the dicaffeine molecule provides a useful general scaffold for the generation of inhibitors against this group of enzymes, comparisons of several family 18 members reveal that relatively subtle changes in active site construction can significantly affect affinity. This makes it likely that dicaffeine derivatives selective for only a small number or even individual family 18 targets can be designed. It should also be noted that the conservation of active site features amongst bacterial-like family 18 chitinases does not extend to their ‘plant-like’ siblings: most AfChiB1 active site residues, including the three tryptophans, are conserved in less than 15% of plant-like family 18 chitinase sequences.
DISCUSSION

This work presents an approach to performing rapid virtual screening experiments based on the known conformation of a ligand fragment in a target binding site, as well as the application of this method to elaborate a methylxanthine fragment common to several recently identified inhibitors of A/fChIB1 (19). While the method identifies both known and novel A/fChIB1 inhibitors, a large number of predicted hits turn out to be false positives, which is unexpected, in particular as the assumptions made to speed up the virtual screening should, if anything, give rise to false negative results. To re-evaluate the results, the compounds in Table I were re-docked using AutoDock 3.0, which uses essentially the same force field as LIGTOR, but, while slower than LIGTOR, is better suited to ab initio docking and produces a list of favourable conformations instead of a single ‘best’ conformation. Table I identifies the AutoDock cluster (re-ranked by $\Delta G$) most similar to the LIGTOR-generated pose and the RMSD between the two conformations. The results suggest that the large number of false positives is not due to a shortcoming of the employed docking protocol, but instead most likely stems from problems of the force field employed. At the same time the data indicate that the assumption that the fragment substructure binds to the active site in a fixed conformation may not hold in all cases, in particular for compounds with large extensions like 3, 4 or 6. In addition, the rapid evaluation with LIGTOR may not find the desired energy minimum in cases of highly flexible attachments, as seen in the case of compound 6, leading to an expected increase of false negatives. To improve the predictions from the virtual screen, the use of a separate scoring function is being investigated. Alternatively, a small number of candidate ligands could be evaluated using free-energy perturbation calculations (36).

LIGTOR together with PRODRG provides a simple affinity grid-based docking implementation. It can be used as a building block for a variety of docking protocols, including the fragment-based approach described here, but also for evaluating series of synthesisable compounds, in ligand growing protocols or for fitting ligands to electron density maps (A.W.S., unpublished data). It should also be noted that the grid-based docking as implemented by LIGTOR is relatively force field-agnostic and more elaborate force fields could be adopted with little effort.

The newly identified dicaffeine compound represents a promising starting point for the design of family 18 chitinase inhibitors. With a molecular weight of 386 Da, a cLogP of -0.77, six hydrogen bond acceptors and no hydrogen bond donors, it conforms with Lipinski’s rule of five (37). It also is reasonably rigid, possessing only three free torsions involving non-hydrogen atoms. C$_2$-dicaffeine can be derivatised in numerous locations, both on the xanthine ring systems and on the linker, and many derivatives are easily synthetically accessible. Sequence comparisons and assays of additional enzymes suggest that the dicaffeine scaffold can form a basis for the design of specific inhibitors of bacterial-like, but not plant-like, family 18 chitinases.

CONCLUSIONS

Family 18 chitinases comprise drug targets from fungal and protozoan pathogens, and also have been implicated as effectors in allergic asthma. Using a new docking program, LIGTOR, a purine fragment common to several previously characterised chitinase inhibitors is elaborated into a new inhibitor, C$_2$-dicaffeine, which has low micromolar affinity for a model chitinase and desirable drug-like properties. The crystal structure of a C$_2$-dicaffeine-chitinase complex shows the inhibitor interacting with two separate subsites in the active site cleft and suggests that its scaffold should be widely applicable to the development of specific inhibitors of family 18 chitinases.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. The active site of AfChiB1 in complex with xanthine derivatives. Active site residues are shown as grey stick models, possible hydrogen bonds are indicated by dotted black lines, crystallographic waters interacting with ligands are shown as orange spheres. A, The crystallographic complex with pentoxifylline (purple) (19), which provided the starting point for virtual screening. The 3-methylxanthine moiety used as the rigid fragment is shown darker, while the 7-methyl group and the oxohexyl tail are paled. B, The docked conformations (pale green) of the compounds listed in Table II. Possible hydrogen bonds have been omitted. C, Crystal structure of the AfChiB1-C2-dicaffeine complex (stereo). The major conformation of the ligand is shown in purple, the minor \((q = 0.25)\) conformation is shown semitransparently in orange. The unbiased \(|F_o| - |F_c|, \varphi_c\) electron density map is shown contoured at 2.5 \(\sigma\). The label colour indicates conservation of the corresponding residue among bacterial-like family 18 chitinases using a colour gradient from red (not conserved) to green (completely conserved): to evaluate sequence conservation, a list of family 18 glycoside hydrolases was obtained from CAZy (1). After removing entries not marked as chitinases (EC 3.2.1.14) and entries lacking a full-length UniProt entry sequences were downloaded from UniProt and aligned with ClustalW (38). Elimination of sequences lacking the active site motif DxxDxDxE yielded 165 putative chitinase sequences, which were separated by sequence length into 129 ‘bacterial-like’ chitinases (proteins longer than 370 amino acids) and 36 ‘plant-like’ chitinases (proteins shorter than 370 amino acids).

Figure 2. A, The chemical structures of the compounds discussed. Standard purine/xanthine atom numbering is shown for compound 1. B, Enrichment of particular fragment structure modifications after docking. The bars show the size distributions for groups attached to N1, N3, N7 and C8 of the xanthine fragment structure for the set of 50193 fragment search matches (labeled ‘db’) and the set of 263 docked compounds selected as possible ligands (labeled ‘docked’), distinguishing no attachment (light colour; not for N3), a single non-hydrogen atom (medium colour) and more than one non-hydrogen atoms (dark colour). C, Lineweaver-Burk plot of C2-dicaffeine inhibition of AfChiB1 at different concentrations. The data is compatible with a competitive inhibition model, giving a \(K_i\) of \((2.8 \pm 0.2)\ \mu\text{M}\).
TABLE I

Compounds selected for testing against *Af*ChiB1, sorted by predicted affinity. Compound numbers refer to Figure 2A. The IC$_{50}$ for pentoxifylline is taken from (19). $\Delta G_{\text{pred}}$ = predicted free enthalpy of binding in kcal·mol$^{-1}$; $\Delta g_{\text{pred}}$ = ligand efficiency in kcal·mol$^{-1}$·atom$^{-1}$; IC$_{50,\text{exp}}$ = experimental IC$_{50}$ in µM; n/a = not available. Rank and RMSD (in Å) pertain to the *ab initio* docking and are explained in the text.

| Compound | Supplier | $\Delta G_{\text{pred}}$ | $\Delta g_{\text{pred}}$ | IC$_{50,\text{exp}}$ | Rank | RMSD |
|----------|----------|--------------------------|--------------------------|----------------------|------|------|
| 2        | Chembridge | -13.42 | -0.48 | 4.8 ± 1.4 | 1 | 0.256 |
| 3        | Specs     | -13.07 | -0.45 | n/a       | 7 | 2.051 |
| 4        | Chembridge | -12.84 | -0.46 | > 1000   | 2 | 1.056 |
| 5        | Specs     | -12.73 | -0.58 | 520 ± 80 | 1 | 1.050 |
| 6        | Specs     | -12.72 | -0.49 | > 1000   | 2 | 0.515 |
| 7        | Specs     | -11.73 | -0.43 | > 1000   | 1 | 0.591 |
| 1, R$_1$=-F, R$_2$=-Cl | Chembridge | -11.45 | -0.52 | > 1000   | 1 | 0.371 |
| 8        | Sigma     | -10.91 | -0.55 | 126 ± 7  | 1 | 0.211 |
| 9        | IBS       | -10.86 | -0.52 | n/a       | 1 | 0.843 |
| 1, R$_1$=-H, R$_2$=-CH$_3$ | Chembridge | -10.84 | -0.52 | > 1000   | 1 | 0.174 |
| 1, R$_1$=-H, R$_2$=-F | Chembridge | -10.77 | -0.51 | > 1000   | 1 | 0.142 |
| 1, R$_1$=R$_2$=-H | Chembridge | -10.68 | -0.53 | > 1000   | 1 | 0.176 |
| 10       | Specs     | -10.48 | -0.52 | n/a       | 2 | 0.279 |
### TABLE II

**Data collection and structure refinement statistics for the \(Af\)ChiB1-C\(_2\)-dicaffeine complex.** Values in parentheses pertain to the highest resolution shell.

| Description                             | Value                                      |
|-----------------------------------------|--------------------------------------------|
| Resolution (Å)                          | 20–1.95 (2.02–1.95)                       |
| No. of observed reflections             | 283642 (25771)                             |
| No. of unique reflections               | 96150 (9510)                              |
| Redundancy                              | 2.9 (2.7)                                 |
| \(I/\sigma I\)                          | 21.0 (3.4)                                |
| Completeness (%)                        | 99.2 (99.2)                               |
| \(R_{merge}\)                           | 0.061 (0.452)                             |
| \(R_{work}\), \(R_{free}\)             | 0.187, 0.223                             |
| RMSD from ideal geometry:              |                                            |
| bonds (Å)                               | 0.010                                     |
| angles (°)                              | 1.5                                       |
| \(B\)-factor RMSD:                      |                                            |
| bonds (main chain, Å\(^2\))            | 1.6                                       |
| angles (main chain, Å\(^2\))           | 2.1                                       |
| \(\langle B_{protein} \rangle\) (Å\(^2\)) | 24.2 ± 7.5                               |
| \(\langle B_{ligand} \rangle\) (Å\(^2\))    | 29.2 ± 2.6                                |
| \(\langle B_{solvent} \rangle\) (Å\(^2\))    | 35.6 ± 10.2                               |
Figure 2
Screening-based discovery and structural dissection of a novel family 18 chitinase inhibitor
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