Glycoside hydrolase family 18 and 20 enzymes are novel targets of the traditional medicine berberine

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Berberine is a traditional medicine that has multiple medicinal and agricultural applications. However, little is known about whether berberine can be a bioactive molecule toward carbohydrate-active enzymes, which play numerous vital roles in the life process. In this study, berberine and its analogs were discovered to be competitive inhibitors of glycoside hydrolase family 20 β-N-acetyl-d-hexosaminidase (GH20 Hex) and GH18 chitinase from both humans and the insect pest Ostrinia furnacalis. Berberine and its analog SYU-1 inhibit insect GH20 Hex from O. furnacalis (OfHex1), with Ki values of 12 and 8.5 μM, respectively. Co-crystallization of berberine and its analog SYU-1 in complex with OfHex1 revealed that the positively charged conjugate plane of berberine forms π-π stacking interactions with Trp490, which are vital to its inhibitory activity. Moreover, the 1,3-dioxole group of berberine binds an unexplored pocket formed by Trp322, Trp483, and Val164, which also contributes to its inhibitory activity. Berberine was also found to be an inhibitor of human GH20 Hex (HsHexB), human GH18 chitinase (HsCht and acidic mammalian chitinase), and insect GH18 chitinase (OjCht1). Besides GH18 and GH20 enzymes, berberine was shown to weakly inhibit human GH84 O-GlcNAcase (HsOGA) and Saccharomyces cerevisiae GH63 α-glucosidase 1 (ScGlu1). By analyzing the published crystal structures, berberine was revealed to bind with its targets in an identical mechanism, namely via π-π stacking and electrostatic interactions with the aromatic and acidic residues in the binding pockets. This paper reports new molecular targets of berberine and may provide a berberine-based scaffold for developing multitarget drugs.

Berberine is an isoquinoline quaternary alkaloid widely distributed in the root, stem, and bark of plants from the Berberis and Coptis families, such as Berberis aristata, Berberis aquifolium, Berberis vulgaris, Coptis chinensis, Coptis japonica, and Coptis rhizome (1–3). Berberine has been used for more than 3,000 years in Ayurvedic, Chinese, and Middle-Eastern folk medicine for its antimicrobial, antiprotozoal, antiidiarrheal, and antitrichomonal activities (1–3). With the development of modern biomedicine, berberine has been revealed to have a very wide range of pharmacological properties, including anticancer, antidiabetic, antidepressant, antihyperlipidemic, and antihypertensive activities (4–8). Moreover, berberine has also been revealed to have potential applications in agriculture for its antifungal, insecticidal, and herbicidal activities (9–11). Corresponding to its multispectral activities, several molecular targets of berberine, such as glycogen synthase kinase, calmodulin kinase, matrix metalloproteinase, acetylcholinesterase, butyrylcholinesterase, monoamine oxidase, DNA topoisomerase, cyclin, and transcriptional factor p53 (12–18), have been discovered.

Glycoside hydrolase family 20 β-N-acetyl-d-hexosaminidase (GH20 Hex) catalyzes the removal of N-acetyl-d-glucosamine (GlcNAc) or N-acetyl-d-galactosamine (GalNAc) from various glycans, glycolipids, and glycoproteins (19, 20). Insect Hex has been proven to be vital for the survival of agricultural pests (21–26). Human Hex is also important for health. Dysfunction of human Hex results in lysosomal storage diseases and osteoarthritis (27, 28). Glycoside hydrolase family 18 (GH18) chitinase not only catalyzes chitin degradation in bacteria, fungi, and insects but also plays different roles in other organisms (29). For example, human chitinases (HsCht and AMCase) have been reported to be involved in asthma (30) and other immunological disorders (31–33). Chitinases from parasites causing nematodosis (34) and malaria (35) are also important for the development and pathogenesis of these organisms. In view of the abovementioned roles of GH20 Hex and chitinases, inhibitors targeting these enzymes are potential therapeutic agents and agrochemicals (36–39). Glycoside hydrolase family 84 O-GlcNAcase (HsOGA) removes O-linked GlcNAc (O-GlcNAc) from both humans and the insect pest Ostrinia furnacalis. Berberine and its analog SYSU-1 inhibit insect GH20 Hex from O. furnacalis (OfHex1) revealed that the positively charged conjugate plane of berberine forms π-π stacking interactions with Trp490, which are vital to its inhibitory activity. Moreover, the 1,3-dioxole group of berberine binds an unexplored pocket formed by Trp322, Trp483, and Val164, which also contributes to its inhibitory activity. Berberine was also found to be an inhibitor of human GH20 Hex (HsHexB), human GH18 chitinase (HsCht and acidic mammalian chitinase), and insect GH18 chitinase (OjCht1). Besides GH18 and GH20 enzymes, berberine was shown to weakly inhibit human GH84 O-GlcNAcase (HsOGA) and Saccharomyces cerevisiae GH63 α-glucosidase 1 (ScGlu1). By analyzing the published crystal structures, berberine was revealed to bind with its targets in an identical mechanism, namely via π-π stacking and electrostatic interactions with the aromatic and acidic residues in the binding pockets. This paper reports new molecular targets of berberine and may provide a berberine-based scaffold for developing multitarget drugs.

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The atomic coordinates and structure factors (codes SY10 and SY18) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The abbreviations used are: GH20 Hex, glycoside hydrolase family 20 β-N-acetyl-d-hexosaminidase; AMCase, acidic mammalian chitinase; GlcNAc, N-acetyl-d-glucosamine; Hex, HsCht, human chitotriosidase; HsHexB, human β-N-acetyl-d-glucosaminidase B; MU-β-GlcNAc, 4-methylumbelliferyl-β-N-acetyl-d-glucosaminide; MU-α-glucose, 4-methylumbelliferyl α-d-glucopyranoside; OCht1, group I chitinase from O. furnacalis; OfHex1, group I β-N-acetyl-d-hexosaminidase from O. furnacalis; HsOGA, O-GlcNAcase from human; ScGlu1, α-glucosidase I from S. cerevisiae; PPA, porcine pancreatic α-amylase; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
Novel targets of berberine

![Figure 1. Structure of berberine and its analogs.]

from nucleocytoplasmic proteins that are involved in transcriptional regulation and stress response (40). Glycoside hydrolase family 63 α-glucosidase I (GluI) is a key member of the eukaryotic N-glycosylation-processing pathway. Inhibition of GluI activity decreases infectivity of several enveloped viruses, including hepatitis B and C (41).

In the previous work, we noticed that compounds with a large conjugated plane were highly potent inhibitors of GH20 Hex (21, 42) and GH18 chitinase (39). Berberine is a typical compound with a large conjugated plane. Here, we report that berberine and its analogs (Fig. 1) act as inhibitors of GH20, GH18, GH84, and GH63 enzymes. The inhibition mechanism of berberine for these enzymes was revealed by crystallography and molecular docking. By comparison with published structures, berberine was revealed to have a similar inhibition mechanism for these structurally and functionally diverse proteins. This work provides the first report of berberine targeting glycoside hydrolases.

Results

Inhibition of GH20, GH18, GH84, GH63, and GH13 enzymes by berberine and its analogs

SYSU-1 is a berberine derivative originally reported as a telomeric G-quadruplex DNA-stabilizing ligand (43). In our preliminary screening, SYSU-1 was found to display an inhibition rate of 58.7% against OfHex1, an insect GH20 member, at a concentration of 10 μM. In this study, the $K_i$ value of SYSU-1 for OfHex1 was determined to be 8.5 μM (Fig. 2). Then, the inhibitory activities of berberine itself against OfHex1 as well as HsHexB, a human GH20 member, were studied. Inhibition kinetics demonstrated that berberine inhibits both OfHex1 and HsHexB in a competitive mode, but the $K_i$ value of berberine for HsHexB was 20-fold higher than that of berberine against OfHex1 (Fig. 2 and Table 1). Berberine analogs, including thalifendine and palmatine, were also found to be inhibitors of OfHex1 and HsHexB, and they all showed ~5-fold higher $K_i$ values for HsHexB than for OfHex1 (Fig. 2 and Table 1). However, tetrahydroberberine did not inhibit OfHex1 and HsHexB at the concentration of 100 μM (its solubility limit in 2% DMSO).

To evaluate whether berberine can act as a scaffold for developing an inhibitor for a broad spectrum of glycosyl hydrolases, the inhibitory activities of berberine and its analogs toward GH18, GH84, GH63, and GH13 enzymes were assayed, and the $K_i$ values were determined. Berberine, thalifendine, and palmatine showed inhibitory activities against GH18, GH84, and GH63 enzymes in a competitive mode, but it did not inhibit GH13 porcine pancreatic α-amylase (PPA) even at a concentration of 400 μM. Tetrahydroberberine did not inhibit all these enzymes at the concentration of 100 μM (Figs. 3 and 4 and Table 1). In addition, berberine and its analogs showed moderate selectivity between two human chitinases. They had 3–5-fold higher $K_i$ values for AMCase than for HsCht.

Crystal structure of OfHex1 in complex with berberine

To reveal the inhibition mechanism of berberine against OfHex1, the complexed structure of OfHex1 and berberine was prepared by soaking and was resolved to a solution of 2.4 Å. The statistics of data collection and structure refinement are shown in Table 2. The coordinates of the OfHex1–berberine complex and OfHex1–SYSU-1 complex have been deposited in the Protein Data Bank under accession numbers 5Y0V and 5Y1B.

The electron-density map supports the location of berberine in the active pocket of OfHex1 (Fig. 5A). Berberine binds OfHex1 across the −1 and +1 subsites mainly via a π–π stacking interaction with Trp490 and van der Waals interactions with the surrounding residues (Fig. 5B and C). The positive charge of berberine can be neutralized by the negative electrostatic potential in the active pocket (Fig. 5B). Trp490 appears to be important for berberine’s binding because it forms a π–π stacking interaction with the berberine ring and a water-mediated hydrogen bond with the O1 of berberine. The mutant OfHex1–W490A was not inhibited by berberine at 100 μM (data not shown). Moreover, a hydrophobic recess composed of Trp432, Trp483, and Val484 also contributed to the binding of berberine by accommodating its 1,3-dioxole group (Fig. 5). Palmatine without the 1,3-dioxide group showed a $K_i$ value for OfHex1 that was more than 4-fold higher than that of berberine (Table 1). Notably, this hydrophobic recess has not been occupied by other OfHex1 inhibitors, such as N,N,N-trimethyl-β-glucosaminyl-chitotriomycin (TMG-chitotriomycin) (44), N-acetylglucosaminino-1,5-lactone O-(phenylcarbamoyl)-oxime (PUGNAc) (45), 3aR,5R,6S,7R,7aR)-5-(hydroxymethyl)-2-methyl-5,6,7,7a-tetrahydro-3aH-pyran[3,2-d]thiazole-6,7-diol (NAG-thiazoline) (46), 2-(2-(((5-methyl-1,3,4-thiadiazol-2-yl)methyl)amino)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (Q1), and 6-(dimethylamino)-2-(2-(((5-methyl-1,3,4-thiadiazol-2-yl)methyl)amino)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (Q2) (42).

Crystal structure of OfHex1 in complex with SYSU-1

The complexed structure of OfHex1 and SYSU-1 was also prepared by soaking and was resolved to a solution of 2.2 Å. The statistics of data collection and structure refinement are shown
in Table 2. The coordinates and the OfHex1-SYSU-1 complex have been deposited into the Protein Data Bank under accession number 5Y1B.

The electron-density map of the berberine ring of SYSU-1 is clear and supports the binding of SYSU-1 in the active pocket of OfHex1 (Fig. 6A). Superimposition of the complex structures of OfHex1–SYSU-1 and OfHex1–berberine revealed that the binding mode of the berberine moiety of SYSU-1 in OfHex1 is identical to that of berberine (Fig. 6B). Most of the residues in the active pockets that interacted with the ligand were in the same conformation except Trp448, which was rotated $\sim10^\circ$ outward from the center of the active pocket. The electron density for the 1-butylquinolin-1-ium group of SYSU-1 is not clear, indicating disorder in this region. Nevertheless, the 1-butylquinolin-1-ium group as well as the linker region may enhance the binding affinity of SYSU-1 via van der Waals interactions or stacking interactions with the surrounding aromatic residues, such as Trp$^{448}$ and Tyr$^{471}$ (Fig. 6B).

**Modeled structures of other GH20, GH18, GH84, GH63, and GH13 enzymes in complex with berberine**

The binding mode of berberine to HsHexB was studied by the molecular docking of berberine to the crystal structure of HsHexB (47). As shown in Figs. 6B and 7A, berberine could be only partially inserted into the active pocket and formed a $\pi-\pi$ stacking interaction with Trp$^{489}$. Compared with berberine binding to OfHex1, berberine bound to HsHexB is more solvent-exposed, which may weaken the hydrophobic interaction with the active-site residues. Moreover, the positive charge of berberine may be repulsed by the positive electrostatic potential in the active pocket (Fig. 7A).

The binding modes of berberine to HsCht, OfChtI, and AMCase were studied by molecular docking (Fig. 7, C–F). The results demonstrated that berberine was placed into an identical position in the substrate-binding clefts of these chitinases by forming $\pi-\pi$ stacking interactions with a conserved tryptophan residue (Fig. 7F). Although the electrostatic potentials in the active pockets of these chitinases are from negative to neutral (Fig. 7, C–E), the positive charge of berberine might be neutralized by a conserved aspartate residue (Fig. 7F).

The binding modes of berberine to HsOGA, ScGluI, and PPA were also studied by molecular docking (Fig. 8). As the electrostatic potentials in the active pockets of HsOGA and ScGluI are negative, the positive charge of berberine might be neutralized by the surrounding negatively charged residues (Fig. 8, A and B). As for PPA, although berberine could be docked into the wide active pocket of PPA, it could not form any $\pi-\pi$ stacking interactions or electrostatic interactions with PPA (Fig. 8C).

![Figure 2. Inhibition kinetics of berberine and its analogs toward GH20 Hexs.](image-url)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>GH20 OfHex1</th>
<th>OfHexB</th>
<th>GH18 OfChtI</th>
<th>OfCht</th>
<th>AMCase</th>
<th>GH84, HsOGA</th>
<th>GH63, ScGluI</th>
<th>GH13, PPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td>12</td>
<td>240</td>
<td>23</td>
<td>19</td>
<td>65</td>
<td>118</td>
<td>130</td>
<td>NI</td>
</tr>
<tr>
<td>Thalifendine</td>
<td>11</td>
<td>65</td>
<td>15</td>
<td>15</td>
<td>55</td>
<td>72</td>
<td>74</td>
<td>NI</td>
</tr>
<tr>
<td>Palmatine</td>
<td>53</td>
<td>300</td>
<td>38</td>
<td>15</td>
<td>70</td>
<td>194</td>
<td>600</td>
<td>NI</td>
</tr>
<tr>
<td>Tetrahydroberberine</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

*NI indicates not inhibited at a concentration of 400 $\mu$M.

*NI indicates not inhibited at a concentration of 100 $\mu$M.
To test their bioactivity, the artificial diet that contained berberine and SYSU-1, respectively, was used to feed 4th-instar day 1 *Ostrinia furnacalis* larvae. Compared with the control group, compounds fed larvae grew slowly, and some of them died after 6 days (Fig. 9).

**Discussion**

In this study, berberine was discovered to be a competitive inhibitor of GH20 and GH18 enzymes. Similar to the amylase inhibitor montobretin A (48), berberine binds with the target enzymes by a noncanonical mode that is not the binding mode of the transition state analog inhibitor or a substrate.
As revealed by X-ray crystallography as well as molecular docking, berberine inhibits these enzymes via an identical mechanism. As a positively charged conjugate plane, berberine usually binds in a narrow pocket with negative electrostatic potential and forms π–π stacking interactions with a conserved tryptophan residue (Trp⁴⁹⁰ in OfHex1, Trp⁴⁸⁹ in HsHexB, Trp⁹⁹ in HsCht, Trp⁹⁹ in AMCase, and Trp¹⁰⁷ in OfChtI) (Figs. 5B and 7, A and B) and electrostatic interactions with a conserved negatively charged residue (Glu³²⁸ in OfHex1, Glu⁴⁹¹ in HsHexB, Asp²¹3 in AMCase, Asp²¹⁸ in OfChtI, Asp¹⁷⁵ in HsOGA, and Glu⁷⁷¹ in ScGluI) (Figs. 5B, 7, A and B, and 8). To determine whether berberine binds other known target proteins by the same mechanism, the reported complexed structures of berberine with other protein targets were analyzed. These protein targets included the multidrug binding protein QacR from Staphylococcus aureus (49), the multidrug resistance regulator BmrR from Bacillus subtilis (50), and RamR from Salmonella typhimurium (51). Although the structures and functions of these proteins vary greatly, we observed that berberine binds these proteins in a similar mode to that observed with GH20 and GH18 enzymes (Fig. 10). The conjugate plane of berberine formed π–π stacking interactions with aromatic residues (Trp⁶¹, Tyr⁷³, Tyr¹²³ in QacR; Phe²²₄, Tyr²₂₉, and Tyr₂₆₈ in BmrR; Phe¹₅₅ in RamR). Moreover, the positive charge of berberine could be neutralized by the surrounding
be retained in the further design of berberine-based inhibitors. Additionally, berberine is a good starting point to pursue better affinity or specificity because it can be readily modified at the C8, C13, and O9 sites (3).

**Conclusion**

In this study, we discovered berberine and its analogs to be inhibitors of GH20, GH18, GH84, and GH63 glycoside hydrolases. By steady inhibition kinetics, X-ray crystallography, and molecular docking, we revealed berberine and its analogs interacted with these enzymes through π–π stacking or electrostatic interactions. This work not only expands the molecular target library of berberine but also provides a scaffold for developing inhibitors of carbohydrate hydrolases.

**Experimental procedures**

**Materials**

4-Methylumbelliferyl-β-D-GlcNAc (MU-β-D-GlcNAc), 4-methylumbelliferyl-β-D-β-N,N'-diacetylchitobiose (MU-β-D-GlcNAc), 4-methylumbelliferyl α-D-glucoyanoside (MU–α-glucose), Saccharomyces cerevisiae α-glucosidase I (ScGlul), PPA, amylase activity assay kit, berberine, and palmatine were purchased from Sigma (Shanghai, China). The compound SYSU-1 was synthesized by Ma et al. (43) and was kindly provided by Associate Prof. Min Li (Sun Yat-Sen University, China). Thalilfendine and tetrahydroberberine were kindly provided by Prof. Xuhong Qian (East China University of Science and Technology, China). The yeast strain Pichia pastoris GS115 and the expression vectors pPIC9 and pPIC9K were purchased from Invitrogen (Beijing, China). The chromatographic columns for protein purification were purchased from GE Healthcare. The BCA protein assay kit was purchased from TaKaRa (Dalian, China).

**Enzyme preparation**

QfHex1 and the mutant QfHex1−W490A were expressed in P. pastoris GS115 and purified as described previously with some modifications (52). Briefly, the positive clones were cultured in BMGY broth at 30 °C for 72 h, and methanol (1% of the total volume) was added every 12 h. WT and mutant QfHex1 were purified from the culture supernatant by ammonium sulfate precipitation (65% saturation), followed by affinity chromatography on a HisTrap™ crude column (5 ml).

**HSHexB was also expressed in P. pastoris** GS115. The selected region of the gene encoding HsHexB (GenBank™ accession number NM_000521.3) was synthesized, and a C-terminal His6 tag was introduced. The DNA fragment was ligated into pPIC9K, and the expression plasmid pPIC9K–HsHexB was transformed into P. pastoris GS115 by electroporation. The cells expressing HsHexB were grown in 200 ml of BMGY medium at 30 °C for 24 h and then collected and resuspended in 1 liter of fresh BMGY medium. Methanol was added to a final concentration of 1% (v/v) at 24-h intervals as an inducer. After incubation for an additional 72 h, the supernatant was harvested via centrifugation. HsHexB was purified using immobilized metal ion affinity chromatography with a HisTrap™ crude column (5 ml).
The catalytic domains of OjChtI from *O. furnacalis*, human HsCht, and human AMCase were expressed in *P. pastoris* GS115 and purified as described previously (39, 53, 54). HsOGA was expressed in *Escherichia coli* and purified as described previously (40). All of the purified proteins were desalted using a HiTrap desalting column (5 ml) with 20 mM bis-tris at pH 6.5 and quantitated using a commercial BCA protein assay kit. The purities of the target proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining.

**Inhibitory activity assay**

The activities of GH20 Hex and GH84 HsOGA were determined using MU-β-GlcNAc as a substrate. The reaction mixtures used for inhibitor screening consisted of 100 μl of 0.4 nM enzyme, 10 μM MU-β-GlcNAc, 10 μM inhibitors, and 2% DMSO in the buffer (20 mM sodium phosphate, pH 6.5). The reaction in the absence of inhibitors was used as a positive control. After incubating at 30 °C for OjHex1 and 37 °C for HsHexB and HsOGA for an appropriate time, 0.5 mM sodium carbonate was added to the reaction mixture, and the fluorescence produced by the released MU was quantified as described above. Experiments were performed in triplicate. For *K*<sub>i</sub> value determination, three substrate concentrations (1, 2, and 4 μM) for OjChtI and 5, 10, and 20 μM for HsCht and AMCase) and varied inhibitor concentrations were used. The *K*<sub>i</sub> values and types of inhibition were also determined by linear fitting of the data in Dixon plots.

The activity of GH18 chitinase was determined using MU-β-(GlcNAc)<sub>2</sub> as a substrate. The reaction mixtures used for inhibitor screening consisted of 100 μl of 0.4 nM enzyme, 10 μM MU-β-(GlcNAc)<sub>2</sub>, 10 μM inhibitors, and 2% DMSO in the buffer (20 mM sodium phosphate, pH 6.5, for OjChtI and HsCht; 20 mM sodium citrate, pH 5.2, for AMCase). The reaction in the absence of inhibitors was used as a positive control. After incubating at 30 °C for OjChtI and 37 °C for HsCht and AMCase for an appropriate time, 0.5 M sodium carbonate was added to the reaction mixture, and the fluorescence produced by the released MU was quantified as described above. Experiments were performed in triplicate. For *K*<sub>i</sub> value determination, three substrate concentrations (1, 2, and 4 μM for OjChtI and 5, 10, and 20 μM for HsCht and AMCase) and varied inhibitor concentrations were used. The *K*<sub>i</sub> values and types of inhibition were also determined by linear fitting of the data in Dixon plots.

The activity of GH63 ScGlu1 was determined using MU–α-glucose as a substrate. The reaction mixtures used for inhibitor screening consisted of 100 μl of 0.4 nM enzyme, 10 μM MU–α-glucose, 10 μM inhibitors, and 2% DMSO in the buffer (20 mM sodium phosphate, pH 6.5). The reaction in the absence of inhibitors was used as a positive control. After incubating at 30 °C for an appropriate time, 0.5 M sodium carbonate was added to the reaction mixture, and the fluorescence produced by the released MU was quantified as described above. Experiments were performed in triplicate. For *K*<sub>i</sub> value determination, three substrate concentrations (10, 20, and 40 μM) and varied inhibitor concentrations were used. The *K*<sub>i</sub> values and types of inhibition were also determined by linear fitting of the data in Dixon plots.

**Protein crystallization and structure determination**

Crystallization experiments were performed by the hanging drop–vapor diffusion method at 4 °C. OjHex1 was desalted in 20 mM bis-tris with 20 mM NaCl, pH 6.5, and concentrated to 15.0 mg/ml by ultracentrifugation. The reservoir solution used for crystallization consisted of 100 mM HEPES, pH 6.6–7.5, 100 mM MgCl<sub>2</sub>, and 26–35% PEG400. Berberine and SYSU-1 were dissolved in the mother liquor with 5% DMSO at 1 mM and soaked into the crystals 1 h before they were transferred to a glycerol solution and flash-cooled in liquid nitrogen.
Diffraction data were collected at the National Center for Protein Science, Shanghai (BL19U1, Pilatus3–6M detector), and processed using HKL2000 (55). The structures of berberine- and SYSU-1-complexed OfHex1 were solved by molecular replacement with PHASER (56) using the structure of unliganded OfHex1 (PDB code 3NSM) as the search model. PHENIX (57) was used for structure refinement. The molecular models were manually built and extended using Coot (58). The stereochemistry of the models was checked by PROCHECK (59). The coordinates of berberine- and SYSU-1-complexed OfHex1 have been deposited under accession codes 5Y0V and 5Y1B. All structural figures were generated using PyMOL (DeLano Scientific LLC, San Carlos, CA). The electrostatic surfaces were calculated using APBS and PDB 2PQR (60–62).

**Molecular docking**

The PRODRG2 server was used to generate and optimize the initial structure of the compound before docking (63). The molecular docking methodology, performed using AutoDock4.2 software (64, 65), consisted of two steps. First, the protein–ligand complex was obtained by rigid docking and then by flexible docking via setting the active pocket outside ligand-binding residues as flexible. Polar hydrogen atoms and Gasteiger charges were added using AutoDockTools. The center of the grid box was placed at the center of the active pocket of HsHexB (PDB code 1O7A), HsCht (PDB code 1HKK), OfChtI (PDB code 3WQW), AMCase (PDB code 2YBT), HsOGA (PDB code 5UN9; β-subunit), ScGluI (PDB code 4J5T), and PPA (PDB code 1DHK), and the dimensions of the active site box were set at 50 × 50 × 50 Å, 70 × 70 × 70 Å, 60 × 80 × 60 Å, 90 × 46 × 54 Å, and 50 × 80 × 50 Å. All maps were calculated with a 0.375 Å spacing between the grid points. Docking calculations were carried out using the Lamarckian genetic algorithm, and all parameters were the same for each docking. A population of random individuals (population size: 150), a maximum number of 25,000,000 energy evaluations, and a maximum number of generations of 27,000 were used.

**In vivo activity of berberine and SYSU-1**

*O. furnacalis* larvae were fed an artificial diet and reared at 26 ± 1 °C under a 16:8 light/dark photoperiod and 70% relative humidity. Day 1 4th-instar larvae were selected for the feeding experiment. In the experimental groups, an artificial diet containing 0.5 mM compounds (dissolved in DMSO) was used. In the control groups, an artificial diet containing DMSO was

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**Figure 10. Aromatic residues and negatively charged residues involved in the binding of berberine to OfHex1 (PDB code 5Y0V) (A), QacR (PDB code 3BTI) (B), BmrR (PDB code 3D6Y) (C), and RamR (PDB code 3VW2) (D). Berberine is shown in green. Aromatic and negatively charged residues are shown in yellow and white, respectively.**

**Figure 11. Analysis of the structural characteristics of berberine and its analogs.** The energy-minimized structures of berberine and its analogs were generated with MM2 on ChemBio3D (PerkinElmer Life Sciences). The electrostatic potential surfaces for berberine and its analogs were generated with DelPhi on Accelrys Discovery Studio 2016 (Dassault Systèmes). Red and cyan represent the electronegative and electropositive potentials, respectively, and green represents a potential halfway point between the two extremes. The pK_a of tetrahydroberberine was predicted by Marvin Beans (ChemAxon).
used. Each group contained 10 individual larvae and was continuously fed for 7 days. Mortality and developmental defects were recorded every day.

Author contributions—Y. D. and T. D. data curation; Y. D. and Y. Z. software; Y. D., T. L., Y. Z., and T. D. formal analysis; Y. D. and Q. Y. methodology; T. L. and Q. Y. conceptualization; T. L. writing-original draft; T. L. and Q. Y. writing-review and editing; Q. Y. resources; Q. Y. validation; Q. Y. project administration.

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Novel targets of berberine


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