Structure-based virtual screening and molecular dynamics of potential inhibitors targeting sodium-bile acid co-transporter of carcinogenic liver fluke *Clonorchis sinensis*

**Won Gi Yoo**¹,²,³, Fuhong Dai⁴, Jhang Ho Pak⁵, Sung-Jong Hong¹,⁶, Jin-Ho Song*⁷

1 Department of Basic Science, Chung-Ang University College of Medicine, Seoul, Republic of Korea, 
2 Department of Parasitology and Tropical Medicine, and Institute of Health Sciences, Gyeongsang National University College of Medicine, Jinju, Republic of Korea, 
3 Department of Convergence Medical Science, Gyeongsang National University, Jinju, Republic of Korea, 
4 Department of Parasitology, School of Biology and Basic Medical Sciences, Suzhou Medical College of Soochow University, Suzhou, Jiangsu, PR China, 
5 Department of Convergence Medicine, University of Ulsan College of Medicine and Asan Institute for Life Sciences, Asan Medical Center, Seoul, Republic of Korea, 
6 Convergence Research Center for Insect Vectors, Incheon National University, Incheon, Republic of Korea, 
7 Department of Pharmacology, Chung-Ang University College of Medicine, Seoul, Republic of Korea

* jinhos@cau.ac.kr (JHS)

**Abstract**

**Background**

*Clonorchis sinensis* requires bile acid transporters as this fluke inhabits bile juice-filled biliary ducts, which provide an extreme environment. *Clonorchis sinensis* sodium-bile acid co-transporter (CsSBAT) is indispensable for the fluke’s survival in the final host, as it circulates taurocholate and prevents bile toxicity in the fluke; hence, it is recognized as a useful drug target.

**Methodology and principal findings**

In the present study, using structure-based virtual screening approach, we presented inhibitor candidates targeting a bile acid-binding pocket of CsSBAT. CsSBAT models were built using tertiary structure modeling based on a bile acid transporter template (PDB ID: 3zuy and 4n7x) and were applied into AutoDock Vina for competitive docking simulation. First, potential compounds were identified from PubChem (holding more than 100,000 compounds) by applying three criteria: i) interacting more favorably with CsSBAT than with a human homolog, ii) intimate interaction to the inward- and outward-facing conformational states, iii) binding with CsSBAT preferably to natural bile acids. Second, two compounds were identified following the Lipinski’s rule of five. Third, other two compounds of molecular weight higher than 500 Da (Mr > 500 Da) were presumed to efficiently block the transporter via a feasible rational screening strategy. Of these candidates, compound 9806452 exhibited the least hepatotoxicity that may enhance drug-likeness properties.
Conclusions

It is proposed that compound 9806452 act as a potential inhibitor toward CsSBAT and further studies are warranted for drug development process against clonorchiasis.

Author summary

Clonorchis sinensis, the Chinese liver fluke, is a parasite infecting human. People are infected via ingesting raw freshwater fishes carrying C. sinensis metacercariae, the encysted form of larvae. Clonorchiasis is a disease caused by C. sinensis, which affects primarily the host hepatobiliary organs. World Health Organization has classified C. sinensis as a biological carcinogen inducing cholangiocarcinoma. Praziquantel has been a therapeutic choice for clonorchiasis. However, the flukes resistant to the drug have emerged due to the extensive use of it. Hence, it is necessary to develop new chemotherapeutic agents to outwit the drug-resistant worms. Sodium-dependent bile acid transporter of C. sinensis (CsSBAT) is essential for the survival of C. sinensis in the bile environment. When CsSBAT is inhibited, bile acids accumulate in the worm’s tissues and cells, which are detrimental to the survival of the worm. Structure-based virtual screening is a powerful approach for compound identification and drug development. Here, we prepared CsSBAT structures using tertiary structure modeling and refinement. We identified four putative inhibitory compounds, which competitively targeted the bile acid-binding pocket of CsSBAT. One inhibitor candidate revealed the least oral toxicity and hepatotoxicity that may enhance its druggability.

Introduction

Clonorchis sinensis is a trematode parasite commonly observed in humans and is transmitted by eating raw or undercooked freshwater fish contaminated with its metacercariae [1]. The intensity and duration of the infection determine the disease complications caused by this trematode. World Health Organization has classified C. sinensis as a Group 1 biological carcinogen inducing cholangiocarcinoma in humans [2].

Praziquantel has been strongly recommended for treating trematode infections in humans including clonorchiasis [3]. Due to the extensive use of praziquantel, certain trematodes in the tropical countries have developed low sensitivity to this drug [4,5]. Hence, new chemotherapeutic agents should be developed to circumvent the low sensitivity or drug resistance of these trematodes. To date, several drug candidates for trematodiasis have been tested on animals [6–9]. Some of them are under trials for clonorchiasis patients [10].

Structure-based virtual screening (SBVS) is a powerful approach for compound identification and has recently gained immense attention in drug development [11]. Several studies have reported the effectiveness of computational approaches in discovering novel antiparasitic druggable compounds [12,13]. For C. sinensis, secretory phospholipase A2 enzyme [14] and 20.6-kDa tegumental protein [15] were computationally evaluated as druggable targets. However, more compounds designed by SBVS are necessary for expanding drug candidate repertoire because they can be optionally tested according to the worm burden or developmental stages of C. sinensis.

Notably, bile acids trigger physiological stimuli; however, these metabolites have detrimental effects on C. sinensis survival, as evidence suggests that the accumulated bile acids can cause...
toxicity to the worm’s tissues and cells [16,17]. Recent studies reported that bile components are toxic or detrimental to *C. sinensis* survival [7,18,19]. Under *in vitro* conditions, high concentration (> 0.005%) of bile and lithocholic acid significantly decreased the worm survival [7,18,19]. A defense system against accumulation and toxicity of bile acids is prerequisite for *C. sinensis* survival. In this respect, bile transporters were considered as potential targets against *C. sinensis*.

Apical sodium-dependent bile acid transporter (ASBT) and Na"+-taurocholate co-transporting polypeptide (NTCP), homologs of *C. sinensis* sodium-bile acid cotransporter (CsSBAT), contribute to the enterohepatic circulation of bile salts in human [20]. The SBATs are druggable targets as they are essential for the bile circulation, and thus several inhibitors were developed and evaluated [21–23]. It was found that CsSBAT is essential for the survival of *C. sinensis* in the bile. When CsSBAT was blocked using polyacrylic acid–tetradecoxocholic acid conjugate (PATD) or CsSBAT was knocked down using RNAi, the bile acids were accumulated in *C. sinensis* adults [24]. The bile acid accumulation was detrimental and shorten the survival of *C. sinensis* in the bile. CsSBAT was, therefore, considered as a druggable target. Its taurocholate-binding pocket can be targeted, and further inhibition could impede its transporter function.

The tertiary structures of ASBTs in *Neisseria meningitidis* [25] and *Yersinia frederiksenii* [26] were characterized with inward and outward facing conformations. These findings have contributed greatly to understanding the mechanism of the sodium ion and bile acid transport across the cell membrane. The bacterial ASBTs have α-helices constituting 10 transmembrane domains (TMs) with short loops. In human ASBT (HsASBT), TM2 and TM5 play a major role in transporting and stabilizing sodium ions [27,28]. Both TM3 and TM4 contact with taurocholate on the outward facing (OF) cavity of the transporter, while TM6 and TM7 interact with it on the inward facing (IF) cavity [29–32].

In the present study, CsSBAT structures were reliably prepared using tertiary structure modeling and refinements. For compound screening, the Lipinski’s rule of five and the rational virtual screening strategy were employed. Here, we identified a putative inhibitory compound, which competitively targeted the taurocholate-binding pocket of CsSBAT.

**Methods**

**Tertiary structure modeling and refinement**

The full-length cDNA sequence of CsSBAT (Acc. No. KX756671) was retrieved from GenBank database [33]. To generate three-dimensional (3D) structure of CsSBAT, we compared the 3D structures built using different 3D modeling softwares such as Swiss-Model [34], IntFOLD [35], Phyre2 [36], RaptorX [37], HHpred [38], and I-TASSER [39]. The 3D models were then refined by two steps. First, low free-energy conformations of the 3D structure were refined by full-atomic simulations using either ModRefiner [40] or FG-MD [41]. Thereafter, backbone and side chains of the structure were refined using GalaxyRefine [42] using “both mild and aggressive relaxation” method based on repeated perturbation and overall conformational relaxation with short molecular dynamics simulations. Outward-facing (OF) and inward-facing (IF) conformations of CsSBAT were modeled using the templates *Y. frederiksenii* (YfASBT; PDB ID: 4n7x_A) [26] and *N. meningitidis* ASBT (NmASBT; PDB ID: 3zuy_A) [25], respectively.

**Quality validation and binding pockets of 3D models**

Potential errors in the 3D models were evaluated using PROCHECK [43], ProSA [44], and ERRAT [45]. Residue-by-residue stereochemical quality of 3D models was verified by
Ramachandran plot [46] of PROCHECK. Overall quality score was analyzed by calculating atomic coordinates of the model using ProSA with a Z-score of experimentally determined structures deposited in PDB [47]. Statistics of nonbonded atom–atom interactions were validated in comparison to a database of reliable high-resolution crystallographic structures using ERRAT [45]. All structures and protein–compound interactions were visualized using UCSF Chimera v1.14 [48] and PyMOL v2.4.1 (Schrödinger, LLC, New York, NY, USA). Disordered region was predicted using CSpritz [49]. Substrate-binding sites in CsSBAT were predicted using COACH [50], referring to the analogs with similar binding sites.

**In silico screening of putative inhibitors**

Two strategies were applied to screen the putative inhibitors against IF-/OF-CsSBAT and IF-/OF-*Homo sapiens* ASBT (HsASBT) (Fig 1): i) Compounds (99,288 ea) following the Lipinski’s...
rule of five [51] were screened against “diverse-lib” compounds database using MTiOpenScreen [52] as of June 2018. A list of compounds from each dataset was compared using Venn diagrams generated via jvenn [53]; ii) Compounds (1,255 ea) with high molecular weight (Mr) ranging 500–1,200 Da were collected from PubChem database [54] as of June 2018 and were screened using AutoDock Vina v1.1.2 [55]. Both docking tools were run with following parameters specifying substrate-binding pocket. These were calculated using Autoligand v1.10 [56,57]. SDF and PDBQT formats were converted into MOL2 format using Open Babel [58]. Each protein–ligand interaction map was generated using ViewDock method of UCSF Chimera v1.14 [48] and visualized using LigPlot+ v1.4.5 [59]. Massive data were analyzed iteratively and parsed using in-house Python scripts. For positive controls, bile acids (7 ea) including taurocholic acid were collected from PubChem database [54].

### Toxicity assessment

Toxicity risk and oral toxicity (LD$_{50}$) were predicted using ProTox [60]. Higher LD$_{50}$ dose led to less toxic compound. Toxicity class ranging 4–6 indicates that the compound is safe. Human hepatotoxicity (H-HT) was predicted using ADMETLab v2.0 [61]. H-HT value close to 1 indicates that a compound may cause liver injury.

### Molecular dynamics (MD) simulation and binding free energies

All MD simulations were performed using YASARA Structure v22.8.22 [62]. The AMBER14 force field was applied under periodic boundary conditions. The simulation cell was defined to include 20 Å surrounding the protein and filled with water, as a solvent, at a density of 0.997 g/mL. The initial energy minimization was carried out under relaxed constraints using steepest descent minimization in vacuo. All simulated systems were maintained at pH 7 by adding counter ions to replace the water containing 0.9% NaCl. Simulations were performed in water at a temperature of 298 K and constant pressure. The snapshots were saved every 250 ps for 50 ns.

Each snapshot was subjected to built-in "md_analyzebindenergy.mcr" script of YASARA Structure for the binding free energy ($\Delta E_{\text{bind}}$) calculation through Molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) using the following formula:

$$\Delta E_{\text{bind}} = \left( (E_{\text{potProt}} + E_{\text{solvProt}}) + (E_{\text{potLigand}} + E_{\text{solvLigand}}) \right) - \left( E_{\text{potComplex}} + E_{\text{solvComplex}} \right)$$

where $E_{\text{potProt}}$, $E_{\text{solvProt}}$, $E_{\text{potLigand}}$, and $E_{\text{solvLigand}}$ are the total energies of protein, ligand, and the protein-ligand complex in solvent, respectively, whereas $E_{\text{potProt}}$, $E_{\text{potLigand}}$, and $E_{\text{potComplex}}$ are the total potential energies of those molecules in vacuum. The total energy is calculated as expressed below:

$$E_{\text{total}} = E_{\text{coul}} + E_{\text{vdw}} + (\text{molsurf} \times \text{surfcost})$$

where $E_{\text{coul}}$ is the Coulombic energy, also called electrostatic energy, $E_{\text{vdw}}$ is the Van der Waals energy, molsurf parameter is the Van der Waals surface from the viewpoint of a solvent molecule, surfcost parameter is set to 0.65 to estimate the entropic cost of exposing an Å$^2$ to the solvent. More positive $\Delta E_{\text{bind}}$ values indicate better binding. The final $\Delta E_{\text{bind}}$ values were obtained by averaging the $\Delta E_{\text{bind}}$ calculated for each snapshot of the MD simulation.

### Results and discussion

#### Generation and validation of CsSBAT homology model

Both N- and C-terminal regions of CsSBAT were predicted to be hypothetical as well as disordered. In particular, disordered regions can result in long simulation time and may lead to
errors in the structural clustering process [63]. Therefore, these regions were excluded from building 3D models (S1 Fig). Functional region (residues 185–492) of CsSBAT matched well with experimentally characterized ASBTs; 34.4% identity with IF-NmASBT (PDB ID: 3zuy_A) [25] and 21.4% identity with OF-YfASBT (PDB ID: 4n7x_A) [26]. Selected templates were suitable for building homology models for CsSBAT since homology model of human NTCP having 27.5% identity with NmASBT was successfully applied for analyzing new inhibitory candidates [64].

To obtain reliable homology models of the parasite protein, combined approach of 3D modeling methods and refinement were employed [15–17,65,66]. All predicted 3D models of IF-CsSBAT were evaluated using homology modeling programs such as Swiss-Model [34], IntFOLD [35], Phyre2 [36], RaptorX [37], and HHpred [38], and threading-based modeling program such as I-TASSER [39] (Table 1). Swiss-Model, IntFOLD, and HHpred revealed values greater than 91.0% in the most favored region of Ramachandran plot [46]. Except for IntFOLD presenting erroneous ERRAT value, Swiss-Model, I-TASSER, and HHpred were evaluated further in terms of refinement because I-TASSER is suitable in building structure of unaligned regions by employing ab initio modeling [67].

Homology modeling of membrane proteins is challenging when the target protein shares low sequence identity (approximately 20%) with a template [68]. This issue was circumvented by refining the initial models with poor quality [15,16]. Therefore, the initial models of

### Table 1. Quality verifications of initial models of IF-CsSBAT according to different programs.

| 3D modeling program | Verification value/score | Ramachandran\(^a\) | ERRAT | ProSA | Cov\(^b\) |
|---------------------|--------------------------|-------------------|-------|-------|-------|
| Swiss-Model         | 91.0% (0.8%)             | 46.0              | –3.2  | 0.6   |
| I-TASSER            | 75.8% (1.9%)             | 92.0              | –1.1  | 1.0   |
| IntFOLD             | 93.8% (0.2%)             | n.a.\(^c\)       | –1.8  | 1.0   |
| Phyre2              | 73.2% (5.1%)             | 26.9              | –3.3  | 1.0   |
| RaptorX             | 89.3% (0.4%)             | 41.9              | –2.9  | 1.0   |
| HHpred              | 91.7% (0.0%)             | 50.2              | –1.4  | 1.0   |

\(^a\)Ramachandran plot, % of most favored region (disallowed region).

\(^b\)Cov: sequence coverage, ratio of predicted region relative to whole region.

\(^c\)n.a.: not available.

### Table 2. Comparison on refined structural evaluation.

| 3D modeling program | Refinement program | Verified score/value produced by | Ramachandran\(^a\) | ERRAT | ProSA | Cov\(^b\) |
|---------------------|---------------------|---------------------------------|-------------------|-------|-------|-------|
|                     | ModRefiner | FG-MD | GalaxyRefine | Swiss-Model | 93.3% (0.8%) | 89.7 | –3.8 | 0.6 |
|                     | I-TASSER    | n.a.\(^d\) | n.a.         | 92.5% (1.2%) | 97.7 | –3.7 | 0.6 |
|                     | HHpred      | n.a. | n.a.         | 86.8% (1.9%) | 89.0 | –1.1 | 1.0 |
|                     |            | n.a. | n.a.         | 74.7% (1.9%) | 92.9 | –1.2 | 1.0 |
|                     |            | n.a. | n.a.         | 94.1% (0.0%) | 69.2 | –1.9 | 1.0 |
|                     |            | n.a. | n.a.         | 76.0% (1.3%) | 81.5 | –1.9 | 1.0 |

\(^a\)Ramachandran plot, % of most favored region (disallowed region).

\(^b\)Cov: sequence coverage, ratio of predicted region relative to whole region.

\(^c\)Symbol “○”: corresponding software was applied.

\(^d\)n.a.: not available.
IF-CsSBAT were refined with ModRefiner, FG-MD, and GalaxyRefine (Table 2). After comprehensive evaluation based on the good quality of the most favored region (> 90%) and ERRAT value (> 95%), we applied Swiss-Model for reliable 3D modeling, and thereafter, FG-MD and GalaxyRefine were used for effective refinement. In fact, Swiss-Model is a powerful tool for transporter modeling [69,70]. Refining enhanced the structural quality of the final model compared to that of the initial model, particularly in terms of ERRAT values, which increased from 46.0% to 97.7% (Tables 1 and 2). However, I-TASSER and HHpred could not overcome poor values either in most favored regions of Ramachandran plot or in unacceptable ERRAT plot. Moreover, 3D models of OF-CsSBAT, OF-HsASBT, and IF-HsASBT were prepared as aforementioned.

**Bile acid-binding cavity and sodium-binding sites**

For the translocation of bile acids across the cell membrane, alternative conformational changes in IF and OF conformations of secondary active transporters were proposed [26,71]. One of the two conformational states was observed only in a particular state because it is difficult to crystallize the structure under other states. Structural information of one state was applied to predict another conformation of transporters [72]. Here, we predicted the OF-CsSBAT and IF-CsSBAT models based on OF-YfASBT [26] and IF-NmAASBT [25], respectively.

In CsSBAT, the bile acid-binding pocket was presumed to be formed with 9 residues in an extracellular cavity with a volume of 908 Å³ in OF-CsSBAT (Fig 2A) and with 11 residues in an intracellular cavity with a volume of 986 Å³ in IF-CsSBAT (Fig 2B). Among them, five residues (Phe₁₉₆, Phe₂₂₂, Ala₂₈₈, Ala₂₉₁, and Met₂₉₅) participated in pocket forming in both conformations.

ASBT is a symporter dependent on Na⁺ gradient that drives bile acid transport, which carries one bile acid with two Na⁺ ions [73]. Two Na⁺-binding sites of CsSBAT, Na1 and Na2, were predicted based on NmAASBT (S2A Fig). Na1 site comprised Ser₂₈₉, Asn₂₉₀, Ser₃₀₃, Thr₃₀₇, and Glu₄₄₁ residues, whereas Na2 site comprised Gln₂₅₂, Glu₄₄₁, Thr₄₄₂, Ile₄₄₄, and Gln₄₄₅ residues.

---

**Fig 2. Conformation changes of CsSBAT accompanied with transport of Na⁺ and taurocholate (TCH).** Both OF-CsSBAT (A) and IF-CsSBAT (B) are colored in tan. OF-YfASBT (A) is colored in pink and IF-NmAASBT (B) in blue. ASBTs presenting identical conformation are superposed. Tunnel leading to the TCH-binding site is visualized in yellow eclipse. Residues forming TCH-binding cavity are depicted as green spheres in CsSBAT. Other residues participating in TCH-binding site are provided at each side. Na⁺ ions are in purple circle (Na1 and Na2). Taurocholate (TCH) is depicted as maroon pentagon.

https://doi.org/10.1371/journal.pntd.0010909.g002
residues (S2B and S2C Fig). These two sites were highly conserved with ASBTs and NTCPs [26]. Furthermore, Glu441 was predicted to participate in Na\(^{+}\)-binding of the two sites, implying that it might have a functionally significant role (S2B and S2C Fig). In NmASBT, mutation at Glu260 (corresponding to Glu441 of CsSBAT) markedly alters the taurocholate transporting activity [25].

Recently, a putative third Na\(^{+}\)-binding site was proposed, albeit rather speculative without experimental evidence [71]. The third Na\(^{+}\)-binding site was reported from similar transporters such as glutamate [74] and leucine [75] transporters. In CsSBAT, residues Ile280, Gly281, Ser283, and Gln445 were predicted to act as binding sites of the third Na\(^{+}\) ion, which were superposed with the corresponding residues on NmASBT (S2D Fig). Residue Gln445 could be a key residue carrying Na\(^{+}\) ion from Na2 to Na3 site (S2 Fig). Mutation of Gln258 in YfASBT (corresponding to Gln445 in CsSBAT) was reported to significantly reduce the Na\(^{+}\)-binding capacity of Na2 and Na3 sites [26]. Therefore, Glu441 and Gln445 in CsSBAT might act as molecular arms and transport Na\(^{+}\) ion from one site to the next.

**Putative inhibitors targeting at bile acid-binding pocket**

For a successful large-scale virtual screening for potential compounds interacting with CsSBAT, our virtual screening protocol was validated through the re-docking of taurocholate within the binding pocket of NmASBT as suggested by Hernandez Alvarez et al. [13]. The root-mean-square deviation (RMSD) for the backbones of the re-docked pose having the best binding energies was only 2.0 Å with respect to the experimental binding mode (Fig 3). Moreover, two hydrogen bonds between taurocholate and residues, Thr112 and His294, were reproduced in the predicted NmASBT-taurocholate complex without two Na\(^{+}\) ions (Fig 3). This result provided a reasonable prediction of experimental binding mode of taurocholate which validates our docking protocol.

For accurate molecular docking of CsSBAT against compounds in the library, grid center and size were precisely specified in the extracellular and intracellular bile acid-binding pockets of OF-CsSBAT (Fig 2A) and IF-CsSBAT (Fig 2B), respectively, rather than sodium-binding sites (S2B–S2D Fig), as described in “In silico screening of putative inhibitors” section of Methods.

SBVS was carried out to select putative inhibitors of CsSBAT, which satisfied the following criteria (Fig 1): i) A compound should interact more favorably with CsSBAT than with HsASBT to ensure accurate targeting. ii) OF-ASBT conformation should be considered as a target although IF-ASBT binding with taurocholate was used as a template for virtual docking [23], because the ASBTs transfer bile acid inward via conformational change from OF- to IF-conformation [76]. The Cα RMSD values between OF- and IF-conformations are 2.4 Å for CsSBAT and 1.9 Å for HsASBT. iii) The compound to be identified as a competitive inhibitor of taurocholate should reveal higher affinity than natural bile acids, ranging from −6.2 to −9.0 kcal/mol [23]. Theoretically, the binding energies of several bile acids against IF-CsSBAT and OF-CsSBAT conformations ranged from −6.1 to −8.7 kcal/mol (Table 3 and S3 Fig) [17]; however, those of HsASBT (CsSBAT homolog) were −9.0 and −9.2 kcal/mol against natural bile acids and PATD (a lead compound blocking HsASBT), respectively [23]. Thus, a cut-off value was set at −9.2 kcal/mol since the present study aimed to explore the most probable inhibitor candidates binding to CsSBAT.

**Compounds satisfying the Lipinski’s rule of five**

Compounds following the Lipinski’s rule of five [51] were screened using MTiOpenScreen [52]. Of the top 1,000 scoring compounds under docking simulation against OF- and IF-
**Fig 3.** Taurocholate re-docking into the substrate-binding pocket of NmASBT. Superposition of taurocholate in the best-re-docking pose (yellow) and crystal structure (blue) (RMSD = 2.0 Å). Hydrogen bonds between the residues of NmASBT binding pocket and taurocholate in both the crystal (blue; Lys\textsubscript{13} and Asn\textsubscript{295}) and the best-re-docked (yellow; Thr\textsubscript{112} and His\textsubscript{294}) complex structures are shown as yellow dotted lines, and NmASBT interacting residues (green) are depicted as sticks. The known hydrogen bonds are marked in red asterisks.

https://doi.org/10.1371/journal.pntd.0010909.g003

**Table 3.** Binding energy for bile acids to OF-/IF-SBAT of *Clonorchis sinensis* and OF-/IF-ASBT of *Homo sapiens* using AutoDock Vina.

| Bile acids                  | PubChem ID    | *C. sinensis* (kcal/mol) | *H. sapiens* (kcal/mol) |
|-----------------------------|---------------|--------------------------|-------------------------|
|                             |               | OF | IF  | OF  | IF  |
| Chenodeoxycholic acid       | 24875071      | −6.7 | −7.6 | −7.7 | −7.0 |
| Taurochenodeoxycholic acid  | 312642451     | −6.4 | −7.7 | −8.0 | −8.0 |
| Glycochenodeoxycholic acid  | 177011774     | −6.4 | −7.6 | −7.7 | −7.3 |
| Glycodeoxycholic acid       | 57309861      | n.a.* | n.a. | n.a. | n.a. |
| Deoxycholic acid            | 222528\textsuperscript{b} | −6.1 | −7.7 | −7.3 | −6.9 |
| Taurocholic acid            | 828139        | −6.6 | −8.7 | −7.1 | −7.0 |
| Glycocholic acid            | 177011773     | −6.8 | −8.5 | −8.0 | −6.8 |

\*n.a.: not available.

\textsuperscript{b}PubChem compound ID but other bile acids are PubChem substance ID.

https://doi.org/10.1371/journal.pntd.0010909.t003
conformations of CsSBAT and HsASBT, 19 compounds that could interact with only OF-CsSBAT or IF-CsSBAT were selected (Fig 4 and S1 Table). Of these, two compounds met our strict criteria. Compound 49734421 formed a hydrogen bond with Ala<sub>291</sub> of IF-CsSBAT and Asn<sub>446</sub> of OF-CsSBAT. Compound 124948115 formed two hydrogen bonds with OF-CsSBAT but not with IF-CsSBAT (Table 4 and Fig 5). Majority of the residues of these two compounds were

![Figure 4](https://doi.org/10.1371/journal.pntd.0010909.g004)

Table 4. Inhibitory compounds subjected to virtual screening using MTIOpenScreen and selected by applying the Lipinski’s rule of five.

| PubChem ID | Binding energy (kcal/mol) | nRot | HBA | HBD | LogP | Mr  | TPSA | Hydrogen bond | Toxicity class (LD<sub>50</sub> mg/kg) | H-HT |
|------------|--------------------------|------|-----|-----|------|-----|------|---------------|-------------------------------------|------|
|            | OF          | IF    |     |     |      |     |      |               |                                     |      |
| 49734421   | −10.9       | −9.3  | 4   | 7   | 1    | 2.3 | 396.5| 1             | 1                                   | 4    |
|            |             |       |     |     |      |     |      |               |                                     | ++++ |
| 124948115  | −10.3       | −9.3  | 5   | 6   | 1    | 2.8 | 397.5| 2             | 0                                   | 4    |
|            |             |       |     |     |      |     |      |               |                                     | ++++ |

Abbreviations: nRot, number of rotatable bonds; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; H-HT, human hepatotoxicity; LogP, lipophilicity; Mr, molecular weight; TPSA, topological polar surface area.

https://doi.org/10.1371/journal.pntd.0010909.t004
involved in hydrophobic interaction with residues on CsSBAT, implying that these interactions might play a crucial role in compound–protein interactivity. It has been reported that aromatic moieties with high hydrophobicity enable beneficial interactions with nonpolar residues in the binding pocket [77]. However, both compounds were predicted to have adverse hepatic effects, and thus their hepatotoxicity should be experimentally confirmed using human tissue and samples.

Since the Lipinski’s rule of five was introduced in 1997 [51], drug absorption or permeability has been presumed to be more likely when it has less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, a molecular weight less than 500, and a calculated LogP smaller than 5. Recently, it was suggested that antiparasitic drugs should be exempted from this rule because several drug leads for infectious diseases do not follow the Lipinski’s rule of five [51,78]. Less stringent criteria may allow to identify more lead compounds for further assays. The suggestion motivated us to find out more effective strategy for antiparasitic drugs.

Fig 5. Docked pose of two compounds with IF-CsSBAT and OF-CsSBAT following the Lipinski’s rule of five. Binding modes of compounds were obtained from the MTiOpenScreen [52]. In each panel, left box ribbon and stick indicate a compound placed in the active site of OF-CsSBAT (A and C) and IF-CsSBAT (B and D). Right panel generated by LigPlot+ v1.4.3 [59] indicates the schematic interactions of CsSBAT with 124948115 (A and B) and 49734421 (C and D). The compound and residues with hydrophobic interactions are visualized with 2D diagrams. Amino acid residues involved in hydrophobic interactions are presented as red spoked arcs. Residues contributing to hydrogen bonds are depicted in green and atomic distance (Å) is given as green number. Nitrogen, oxygen, carbon, and sulfur atoms are shown in blue, red, black, and yellow, respectively. Black arrow indicates compound movement.

https://doi.org/10.1371/journal.pntd.0010909.g005
**Large compounds with high affinities**

PATD was recently synthesized and evaluated as a potent inhibitor against ASBT [23]. Molecular weight of PATD is larger than 500 Da because it has several polyacrylic acids and tetra-deoxycholic acids. Surprisingly, PATD is a hydrophobic substance, which violates ideal molecular weight of the Lipinski’s rule of five [51]. Nonetheless, it successfully inhibits ASBT by filling up the bile acid-binding cavity [23]. This finding motivated us to screen compounds with molecular weight larger than 500 Da, which are assumed to tightly dock CsSBAT.

Compounds of high molecular weight (500–1,200 Da) were retrieved from PubChem compound library [54] and screened using AutoDock Vina v1.1.2 [55]. Of the 1,255 compounds, 49 compounds satisfied the three given criteria. By the third criterion (higher affinity than natural bile acids), we selected 25 compounds with high affinity for CsSBAT but low affinity for HsASBT (S2 Table).

![Docked poses of large compounds with OF-CsSBAT and IF-CsSBAT.](https://doi.org/10.1371/journal.pntd.0010909.g006)

**Table 5.** Inhibitory compounds with high molecular weight (Mr > 500 Da) selected using AutoDock Vina v1.1.2.

| PubChem ID | Mr<sup>a</sup> | MF<sup>b</sup> | Binding energy (kcal/mol) | Hydrogen bond | Toxicity class (LD<sub>50</sub> mg/kg) | H-HT<sup>c</sup> |
|------------|----------------|----------------|--------------------------|---------------|-----------------------------------|---------------|
|            |                |                | C. sinensis            | H. sapiens    | OF | IF | OF | IF | OF | IF |     |           |
| 441243     | 670.8          | C<sub>39</sub>H<sub>40</sub>N<sub>8</sub>O<sub>5</sub> | −12.3 | −10.0 | −8.9 | −7.9 | 5 | 1 |    |    | 4  | (500) | +++  | (0.95) |
| 4701       | 508.6          | C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub> | −11.2 | −10.9 | −8.9 | −8.7 | 4 | 0 |    |    | 4  | (800) | ++   | (0.82) |
| 92727      | 628.8          | C<sub>37</sub>H<sub>40</sub>N<sub>8</sub>O<sub>4</sub> | −10.0 | −10.0 | −9.0 | −8.7 | 4 | 0 |    |    | 5  | (5,000) | +    | (0.69) |
| 45375808   | 529.5          | C<sub>22</sub>H<sub>13</sub>F<sub>3</sub>N<sub>9</sub>O<sub>4</sub>P | −9.7 | −9.7 | −8.4 | −8.4 | 2 | 4 |    |    | 6  | (12,000) | +++  | (0.95) |
| 9806452    | 512.7          | C<sub>30</sub>H<sub>41</sub>F<sub>3</sub>N<sub>9</sub>O<sub>4</sub> | −9.3 | −9.3 | −7.6 | −8.3 | 4 | 2 |    |    | 5  | (3,990) | +    | (0.67) |

<sup>a</sup>Mr, molecular weight.

<sup>b</sup>MF, molecular formula.

<sup>c</sup>H-HT, human hepatotoxicity.

https://doi.org/10.1371/journal.pntd.0010909.t005
Fig 7. Stability assessment of two SBATs and two ASBTs docking with compound 9806452. (A) OF-CsSBAT, (B) IF-CsSBAT, (C) OF-HsASBT, and (D) IF-HsASBT. (Left panel) RMSD values of Cα (RMSDc in blue), backbone (RMSDb in red), and all heavy atoms (RMSDAll in green) from each initial structure during 50 ns simulation time. (Right panel) The RMSD pattern of the compound within each complex.

https://doi.org/10.1371/journal.pntd.0010909.g007
complexes (Fig 6A–6F). Compounds 441243 and 3693566 presented 5 and 6 hydrogen bonds, respectively (S2 Table); however, these compounds were excluded owing to less hydrogen bonds in compound–IF-CsSBAT interactions, which could result in off-target binding with adverse drug reactions [79].

Two compounds 45375808 (Fig 6A and 6B) and 9806452 (Fig 6C and 6D) could form more than two hydrogen bonds each with OF-CsSBAT and IF-CsSBAT without predicted toxicity (Table 5). Compound 45375808 was predicted to have drug-induced liver injury, and thus its hepatotoxicity should be validated through experimental settings.

Compound 45375808, known as sofosbuvir, was proposed to inhibit nonstructural protein 5B polymerase in hepatitis C virus (HCV) [80]. Analogs of this compound exhibited inhibitory effect on HCV [81]. Compound 9806452 was reported as an inhibitor of matrix metalloprotei-nases [82] such as gelatinase A associated with tumor metastasis [83] and stromelysin-1 found in osteoarthritis [84]. Carboxyalkyl peptides containing a biphenylylethyl group inhibits adult Schistosoma mansoni [85]. Considering these reports, it is suggested that compound 9806452 could be an anthelminthic candidate for C. sinensis.

**Table 6. Binding free energy and its components during MD simulation.**

| Systems | CsSBAT | HsASBT |
|---------|--------|--------|
|         | OF     | IF     | OF    | IF |
| ΔE<sub>bind</sub> (kcal/mol)<sup>b</sup> | 42.3   | 88.7   | 38.5  | 22.5 |
| E<sub>potProt</sub> (kcal/mol) | −1657.1 | −1678.6 | −566.8 | −535.7 |
| E<sub>solvProt</sub> (kcal/mol) | −2009.6 | −2163.0 | −2194.9 | −2218.9 |
| E<sub>potLigand</sub> (kcal/mol) | 38.7   | 21.6   | 18.9  | −0.5 |
| E<sub>solvLigand</sub> (kcal/mol) | −45.6  | −35.6  | −33.4 | −36.2 |
| E<sub>potComplex</sub> (kcal/mol) | −1684.8 | −1727.0 | −561.2 | −583.8 |
| E<sub>solvComplex</sub> (kcal/mol) | −2031.1 | −2217.4 | −2253.5 | −2230.1 |

<sup>a</sup>In all systems, 12–50 ns simulation was used for calculation of MM/PBSA except for OF-CsSBAT for 24–50 ns.
<sup>b</sup>More positive ΔE<sub>bind</sub> values indicate better binding.

All abbreviations are defined in “Molecular dynamics (MD) simulation and binding free energies” section of Methods.

https://doi.org/10.1371/journal.pntd.0010909.t006

Binding free energies

Prior to binding free energy estimations, the stability of MD simulations was investigated through the inspection of structural properties. All heavy atoms of both OF-CsSBAT and IF-CsSBAT showed relatively stable RMSD values at 3.5 Å and 4.5 Å on average (Fig 7A and 7B), respectively, indicating a delay on their stabilization into binding site, whereas OF-HsASBT displayed structural fluctuations during the first 12 ns and IF-HsASBT remained unstable (Fig 7C and 7D). Overall, these results suggest that 24 ns for OF-CsSBAT and 12 ns for the remainder are suitable equilibration time and, therefore, the last 26 ns and 38 ns MD simulation trajectories were used to calculate mean ΔE<sub>bind</sub> values using the MM/PBSA method.

To better understand the binding free energies of compound 9806452–protein complex, we analyzed the MM/PBSA energy components (Table 6). MM/PBSA results show that OF-CsSBAT and IF-CsSBAT have better ΔE<sub>bind</sub> values (42.3 kcal/mol and 88.7 kcal/mol) than OF-HsASBT and IF-HsASBT (38.5 kcal/mol and 22.5 kcal/mol). In this regard, movement of compound 9806452 revealed that OF-CsSBAT and IF-CsSBAT was stabilized around at RMSD values of 4.5 and 4.0 Å (Fig 7A and 7B), whereas OF-HsASBT and IF-HsASBT reached RMSD values of 70 Å and 10 Å, respectively (Fig 7C and 7D).
Limitations and countermeasures of CsSBAT-targeted virtual screening

It should be noted that experimental validation is necessary to derive any meaning from this computational study. Protein-ligand docking could result in a high false positive prediction due to several limitations, such as unreliability of scoring methods, and lack of protein flexibility and water molecules during the simulation.

We attempted to address these limitations. First, we used the experimentally-characterized ligand-bound structures (4n7x and 3zuy) to find the geometries of more accurate binding pockets. Then, we validated our virtual docking protocol by re-docking of the substrate between experimental binding mode and predicted mode. Second, docking values were not used as a measure for compound activity, but rather we applied the relative affinities between OF- and IF-conformations, and between parasite and human. Then, we applied MM/PBSA method that accounts for the influence of solvents on the stability of the protein-ligand complex. Third, we did not incorporate water molecules in our docking simulations due to the computational complexity involved. Instead, MD simulation was applied because it enables protein-ligand complex to be simulated in water.

Conclusions

Clonorchis sinensis sodium-bile acid cotransporter (CsSBAT) is indispensable for the worm’s survival in the bile duct. Inhibition of the bile transporters may perturb bile acid transport and is proven to be detrimental to C. sinensis. We identified the inhibitory compounds targeting the bile acid-binding pockets in CsSBAT, based on the physiological essence of crucial importance using structure-based drug discovery approach. First, two PubChem compounds were selected by applying the Lipinski’s rule of five. Furthermore, we devised a feasible rational screening strategy to search inhibitor candidates with molecular weight greater than 500 Da. Two large inhibitory compounds were selected with expectation of tight binding to CsSBAT. Of these candidates, compound 9806452 revealed the least oral toxicity and hepatotoxicity that may enhance its druggability. Collectively, compound 9806452 was proposed as a putative inhibitor of CsSBAT, deserving further in vitro and in vivo evaluation toward anthelminthic development.

Supporting information

S1 Fig. Homologous template searches for building tertiary structure of CsSBAT. Termini of CsSBAT were both hypothetical polypeptides. CsSBAT model was built on residues 185–492, based on two reliable templates (PDB ID: 3zuy_A and 4n7x_A).

S2 Fig. Potential Na⁺- and taurocholate-binding sites of CsSBAT. All ligands and interaction were predicted using COACH [50] except for Na3 site (A). Na⁺-binding sites (B, C), Na1 site (B) and Na2 site (C). Putative Na3 site (D) as suggested by Alhadeff et al. [71]. The consensus residues forming the binding site are presented in stick mode and labeled. Side-chain oxygen, nitrogen, and sulfur atoms are indicated in red, blue, and yellow, respectively. Na⁺ ion is depicted as a purple ball and taurocholate substrate as stick mode.

S3 Fig. Docked pose of taurocholate with OF-CsSBAT and IF-CsSBAT. Schematic interactions of taurocholate with OF-CsSBAT (A) and IF-CsSBAT (B) were plotted using LigPlot + v1.4.3. Residues in close contact with a compound are visualized with 2D diagrams. Amino acid residues involved in hydrophobic interactions are presented as red spiked arcs. Residues contributing to hydrogen bonds are depicted in green and atomic distance (Å) is given in
green number. The binding modes of compounds were obtained from the AutoDock Vina v1.1.2.

(TIF)

S1 Table. Binding free energies and drug-like properties of 19 compounds from virtual screening against OF-SBAT and IF-SBAT of *Clonorchis sinensis* using MTiOpenScreen. (DOCX)

S2 Table. Binding energy and toxicity risk of 25 compounds with high molecular weight (Mr > 500 Da) against OF-/IF-SBAT of *Clonorchis sinensis* and OF-/IF-ASBT of *Homo sapiens* using AutoDock Vina v1.1.2. (DOCX)

Acknowledgments

We thank Elmar Krieger at the YASARA Biosciences GmbH (http://www.yasara.org; Vienna, Austria) and the staff members of the Zhang Lab (https://zhanglab.dcmb.med.umich.edu) for their technical help and insightful suggestions.

Author Contributions

**Conceptualization:** Won Gi Yoo, Sung-Jong Hong, Jin-Ho Song.

**Data curation:** Won Gi Yoo, Fuhong Dai.

**Formal analysis:** Won Gi Yoo, Jin-Ho Song.

**Funding acquisition:** Won Gi Yoo, Fuhong Dai.

**Investigation:** Won Gi Yoo, Fuhong Dai.

**Methodology:** Won Gi Yoo, Fuhong Dai.

**Project administration:** Sung-Jong Hong, Jin-Ho Song.

**Resources:** Won Gi Yoo, Sung-Jong Hong.

**Software:** Won Gi Yoo, Jin-Ho Song.

**Supervision:** Sung-Jong Hong, Jin-Ho Song.

**Validation:** Won Gi Yoo, Jhang Ho Pak, Jin-Ho Song.

**Visualization:** Won Gi Yoo, Jin-Ho Song.

**Writing – original draft:** Won Gi Yoo.

**Writing – review & editing:** Jhang Ho Pak, Sung-Jong Hong, Jin-Ho Song.

References

1. Yoo WG, Sohn WM, Na BK. Current status of *Clonorchis sinensis* and clonorchiasis in Korea: epidemiological perspectives integrating the data from human and intermediate hosts. Parasitology. 2022; 149 (10):1296–305.

2. Schistosomes, liver flukes and *Helicobacter pylori*. IARC Monogr Eval Carcinog Risks Hum. 1994; 61:1–241.

3. Choi MH, Park SK, Li Z, Ji Z, Yu G, Feng Z, et al. Effect of control strategies on prevalence, incidence and re-infection of clonorchiasis in endemic areas of China. PLoS Negl Trop Dis. 2010; 4(2):e601. https://doi.org/10.1371/journal.pntd.0000601 PMID: 20169061
4. Tinga N, De N, Vien HV, Chau L, Toan ND, Kager PA, et al. Little effect of praziquantel or artemisinin on clonorchiasis in Northern Vietnam. A pilot study. Trop Med Int Health. 1999; 4(12):814–8. https://doi.org/10.1046/j.1365-3156.1999.00499.x PMID: 10632989

5. Fallon PG. Schistosome resistance to praziquantel. Drug Resist Updat. 1998; 1(4):236–41. https://doi.org/10.1016/s1368-7646(98)80004-6 PMID: 16904406

6. Kim TI, Yoo WG, Li S, Hong ST, Keiser J, Hong SJ. Efficacy of artesunate and arteether against Clonorchis sinensis in rabbits. Parasitol Res. 2009; 106(1):153–6.

7. Li S, Song JH, Kim TI, Yoo WG, Won MH, Dai F, et al. Chemotactic migration of newly excysted juvenile Clonorchis sinensis is suppressed by neuro-antagonists. PLoS Negl Trop Dis. 2013; 13(8):e0007573.

8. Guimaeraes MA, de Oliveira RN, Veras LM, Lima DF, Campelo YD, Campos SA, et al. Anthelmintic activity in vivo of epispastolurarine against juvenile and adult worms of Schistosoma mansoni. PLoS Negl Trop Dis. 2015; 9(3):e0003656.

9. Wangchuk P, Pearson MS, Giacomini PR, Becker L, Sotillo J, Pickering D, et al. Compounds derived from the Bhutanese daisy, Ajania rubigena, demonstrate dual anthelmintic activity against Schistosoma mansoni and Trichuris muris. PLoS Negl Trop Dis. 2016; 10(8):e0004908.

10. Xie L, Jiang B, Duan JH, Zhuang SF, Liu YC, Zhu SQ, et al. Efficacy and safety of praziquantel, tribendimidine and mebendazole in patients with co-infection of Clonorchis sinensis and other helminths. PLoS Negl Trop Dis. 2014; 8(8):e3046.

11. Shoichet BK. Virtual screening of chemical libraries. Nature. 2004; 432(7019):862–5. https://doi.org/10.1038/nature03197 PMID: 15602552

12. Cavasotto CN, Ory AJ, Murgolo NJ, Czarnecki MF, Kocsi SA, Hawes BE, et al. Discovery of novel chemotypes to a G-protein-coupled receptor through ligand-strewn homology modeling and structure-based virtual screening. J Med Chem. 2008; 51(3):581–8. https://doi.org/10.1021/jm070759m PMID: 18198821

13. Hernandez Alvarez L, Naranjo Feliciano D, Hernandez Gonzalez JE, Soares RO, Barreto Gomes DE, Pascutti PG. Insights into the interactions of Fasciola hepatica cathepsin L3 with a substrate and potential novel inhibitors through in silico approaches. PLoS Negl Trop Dis. 2015; 9(5):e0003759.

14. Hariprasad G, Kaur P, Srinivasan A, Singh TP, Kumar M. Structural analysis of secretory phospholipase A2 from Clonorchis sinensis: therapeutic implications for hepatic fibrosis. J Mol Model. 2012; 18(7):3139–45.

15. Kim YJ, Yoo WG, Lee MR, Kang JM, Na BK, Cho SH, et al. Molecular and structural characterization of the tegumental 20.6-kDa protein in Clonorchis sinensis as a potential druggable target. Int J Mol Sci. 2017; 18(3):557.

16. Lu Y, Yoo WG, Dai F, Lee JY, Pak JH, Sohn WM, et al. Characterization of a novel organic solute transporter homologue from Clonorchis sinensis. PLoS Negl Trop Dis. 2018; 12(4):e0006459.

17. Dai F, Yoo WG, Lee JY, Lu Y, Pak JH, Sohn WM, et al. Multidrug resistance-associated protein 4 is a bile transporter of Clonorchis sinensis simulated by in silico docking. Parasit Vectors. 2017; 10(1):578.

18. Li S, Yoo WG, Song JH, Kim TI, Hong SJ. Bile acids drive chemotaxis of Clonorchis sinensis juveniles to the bile duct. PLoS Negl Trop Dis. 2018; 12(10):e0006818.

19. Uddin MH, Li S, Bae YM, Choi MH, Hong ST. In vitro maintenance of Clonorchis sinensis adult worms. Korean J Parasitol. 2012; 50(4):309–15.

20. Claro da Silva T, Polli JE, Swaan PW. The solute carrier family 10 (SLC10): beyond bile acid transport. Mol Aspects Med. 2013; 34(2–3):252–69. https://doi.org/10.1016/j.mam.2012.07.004 PMID: 23506899

21. Baghdasaryan A, Fuchs CD, Osterreicher CH, Lemberger UJ, Hallibasic E, Pahlman I, et al. Inhibition of intestinal bile acid absorption improves cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis. J Hepatol. 2016; 64(3):674–81. https://doi.org/10.1016/j.jhep.2015.10.024 PMID: 26529078

22. West KL, Zern TL, Butteiger DN, Keller BT, Fernandez ML. SC-435, an ileal apical sodium co-dependent bile acid transporter (ASBT) inhibitor lowers plasma cholesterol and reduces atherosclerosis in guinea pigs. Atherosclerosis. 2003; 171(2):201–10. https://doi.org/10.1016/j.atherosclerosis.2003.08.019 PMID: 14644388

23. Park J, Al-Hilal TA, Jeong JH, Choi J, Byun Y. Design, synthesis, and therapeutic evaluation of poly (acrylic acid)-tetraDOCA conjugate as a bile acid transporter inhibitor. Bioconjug Chem. 2015; 26(8):1597–605. https://doi.org/10.1021/bacs.bioconjchem.5b00230 PMID: 26086474

24. Dai F, Yoo WG, Lu Y, Song JH, Lee JY, Byun Y, et al. Sodium-bile acid co-transporter is crucial for survival of a carcinogenic liver fluke Clonorchis sinensis in the bile. PLoS Negl Trop Dis. 2020; 14(12):e0008952.
25. Hu NJ, Iwata S, Cameron AD, Drew D. Crystal structure of a bacterial homologue of the bile acid sodium symporter ASBT. Nature. 2011; 478(7369):408–11. https://doi.org/10.1038/nature10450 PMID: 21976025

26. Zhou X, Levin EJ, Pan Y, McCoy JG, Sharma R, Kloss B, et al. Structural basis of the alternating-access mechanism in a bile acid transporter. Nature. 2014; 505(7484):569–73. https://doi.org/10.1038/nature12811 PMID: 24317697

27. Moore RH, Chotpe, Swaan PW. Transmembrane domain V plays a stabilizing role in the function of human bile acid transporter SLC10A2. Biochemistry. 2013; 52(30):5117–24. https://doi.org/10.1021/bi400028q PMID: 23815591

28. Sabit H, Mallajosyula SS, MacKerell AD Jr., Swaan PW. Transmembrane domain II of the human bile acid transporter SLC10A2 coordinates sodium translocation. J Biol Chem. 2013; 288(45):32394–404. https://doi.org/10.1074/jbc.M113.518555 PMID: 24045943

29. Hussainzada N, Banerjee A, Swaan PW. Transmembrane domain VII of the human apical sodium-dependent bile acid transporter ASBT (SLC10A2) lines the substrate translocation pathway. Mol Pharmacol. 2013; 80(6):1484–91. https://doi.org/10.1124/mol.113.103104 PMID: 23815591

30. Hussainzada N, Claro Da Silva T, Swaan PW. The cytosolic half of helix III forms the substrate exit route during permeation events of the sodium/bile acid cotransporter ASBT. Biochemistry. 2009; 48(36):8528–39. https://doi.org/10.1021/bi900616w PMID: 19653651

31. Hussainzada N, Khandewal A, Swaan PW. Conformational flexibility of helix VI is essential for substrate permeation of the human apical sodium-dependent bile acid transporter. Mol Pharmacol. 2008; 73(2):305–13. https://doi.org/10.1124/mol.107.041640 PMID: 17971420

32. Khantwal CM, Swaan PW. Cytosolic half of transmembrane domain IV of the human bile acid transporter hASBT (SLC10A2) forms part of the substrate translocation pathway. Biochemistry. 2008; 47(12):3606–14. https://doi.org/10.1021/bi702498w PMID: 18311924

33. Jenuth JP. The NCBI. Publicly available tools and resources on the Web. Methods Mol Biol. 2000; 132:301–12. https://doi.org/10.1385/1-59259-192-2:301 PMID: 10547843

34. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 2014; 42(Web Server issue):W252–8. https://doi.org/10.1093/nar/gku340 PMID: 24782522

35. McGuffin LJ, Atkins JD, Salehe BR, Shuid AN, Roche DB. IntFOLD: an integrated server for modelling protein structures and functions from amino acid sequences. Nucleic Acids Res. 2015; 43(W1):W169–73. https://doi.org/10.1093/nar/gkv236 PMID: 25820431

36. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015; 10(6):845–58. https://doi.org/10.1038/nprot.2015.053 PMID: 25950237

37. Kallberg M, Margaryan G, Wang S, Ma J, Xu J. RaptorX server: a resource for template-based protein structure modeling. Methods Mol Biol. 2014; 1137:17–27. https://doi.org/10.1007/978-1-4939-0366-5_2 PMID: 24573471

38. Meier A, Soding J. Automatic prediction of protein 3D structures by probabilistic multi-template homology modeling. PLoS Comput Biol. 2015; 11(10):e1004343. https://doi.org/10.1371/journal.pcbi.1004343 PMID: 26496371

39. Yang J, Zhang Y. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res. 2015; 43(W1):W174–81. https://doi.org/10.1093/nar/gkv342 PMID: 25883148

40. Xu D, Zhang Y. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. Biophys J. 2011; 101(10):2525–34. https://doi.org/10.1016/j.bpj.2011.10.024 PMID: 22098752

41. Zhang J, Liang Y, Zhang Y. Atomic-level protein structure refinement using fragment-guided molecular dynamics conformation sampling. Structure. 2011; 19(12):1784–95. https://doi.org/10.1016/j.str.2011.09.022 PMID: 22153501

42. Heo L, Park H, Seok C. GalaxyRefine: Protein structure refinement driven by side-chain repacking. Nucleic Acids Res. 2013; 41(Web Server issue):W384–8. https://doi.org/10.1093/nar/gkt458 PMID: 23737448

43. Laskowski RA, Rullmann JA, MacArthur MW, Kaptein R, Thornton JM. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR. 1996; 8(4):477–86. https://doi.org/10.1007/BF00228148 PMID: 9008363

44. Wiederstein M, Sippl MJ. ProSa-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 2007; 35(Web Server issue):W407–10. https://doi.org/10.1093/nar/gkm290 PMID: 17517781
45. Colovos C, Yeates TO. Verification of protein structures: patterns of nonbonded atomic interactions. Proteins Sci. 1993; 2(9):1511–9. https://doi.org/10.1002/pro.556002190 PMID: 8401235

46. Lovell SC, Davis IW, Arendall WB, 3rd, de Bakker PI, Word JM, Prisant MG, et al. Structure validation by Co geometry: ψ, ψ, and φ deviation. Proteins. 2003; 50(3):437–50.

47. Rose PW, Bi C, Bluhm WF, Christie CH, Dimitropoulos D, Dutta S, et al. The RCSB Protein Data Bank: new resources for research and education. Nucleic Acids Res. 2013; 41(Database issue):D475–82. https://doi.org/10.1093/nar/gks1200 PMID: 23193259

48. Huang CC, Meng EC, Morris JH, Pettersen EF, Ferrin TE. Enhancing UCSF Chimera through web services. Nucleic Acids Res. 2012; 40(Web Server issue):W478–84. https://doi.org/10.1093/nar/gks306 PMID: 24861624

49. Walsh I, Martin AJ, Di Domenico T, Vullo A, Pollastrì G, Tosatto SC. CSpritz: accurate prediction of protein disorder segments with annotations for homology, secondary structure and linear motifs. Nucleic Acids Res. 2011; 39(Web Server issue):W190–6. https://doi.org/10.1093/nar/gkr411 PMID: 21646342

50. Yang J, Roy A, Zhang Y. Protein-ligand binding site recognition using complementary binding-specific structure comparison and sequence profile alignment. Bioinformatics. 2013; 29(20):2588–95. https://doi.org/10.1093/bioinformatics/btt447 PMID: 23975762

51. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev. 2001; 46(1–3):23–26. https://doi.org/10.1016/S0169-409X(00)00129-0 PMID: 11259830

52. Labbe CM, Rey J, Lagorce D, Vavrusa M, Becol J, Sperandio O, et al. MTOpenScreen: a web server for structure-based virtual screening. Nucleic Acids Res. 2015; 43(W1):W448–54. https://doi.org/10.1093/nar/gkw306 PMID: 25855812

53. Bardou P, Mariette J, Escudie F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer. BMC Bioinformatics. 2014; 15:293. https://doi.org/10.1186/1471-2105-15-293 PMID: 25176396

54. Kim S, Thiesen PA, Bolton EE, Chen J, Fu G, Gindulyte A, et al. PubChem Substance and Compound databases. Nucleic Acids Res. 2016; 44(D1):D1202–13. https://doi.org/10.1093/nar/gkv951 PMID: 26400175

55. Forlì S, Huey R, Pique ME, Sanner MF, Goodsell DS, Olson AJ. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nat Protoc. 2016; 11(5):905–19. https://doi.org/10.1038/nprot.2016.051 PMID: 27077332

56. Harris R, Olson AJ, Goodsell DS. Automated prediction of ligand-binding sites in proteins. Proteins. 2008; 70(4):1506–17. https://doi.org/10.1002/prot.21645 PMID: 17910060

57. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, et al. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem. 2009; 30(16):2785–91. https://doi.org/10.1002/jcc.21256 PMID: 19399780

58. O’Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. Open Babel: An open chemical toolbox. J Cheminform. 2011; 3:33. https://doi.org/10.1186/1471-2105-13-33 PMID: 21982300

59. Laskowski RA, Swindells MB. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. J Chem Inf Model. 2011; 51(10):2778–86. https://doi.org/10.1021/ci200227u PMID: 21919503

60. Drwai MN, Banerjee P, Dunkel M, Wettig MR, Preissner R. ProTox: a web server for the in silico prediction of rodent oral toxicity. Nucleic Acids Res. 2014; 42(Web Server issue):W53–8.

61. Xiong G, Wu Z, Yi J, Fu L, Yang Z, Hsieh C, et al. ADMETlab 2.0: an integrated online platform for accurate and comprehensive predictions of ADMET properties. Nucleic Acids Res. 2021; 49(W1):W5–W14. https://doi.org/10.1093/nar/gkab255 PMID: 33893803

62. Kang JM, Le HG, Na BK, Yoo WG, pH-Dependent structural dynamics of cathepsin D-family aspartic peptidase of Clonorchis sinensis. Pathogens. 2021; 10(9):1128.

63. Yang J, Zhang Y. Protein structure and function prediction using I-TASSER. Curr Protoc Bioinformatics. 2015; 52:5.8.1–15.

64. Fu LL, Liu J, Chen Y, Wang FT, Wen X, Liu HQ, et al. In silico analysis and experimental validation of azelastine hydrochloride (N4) targeting sodium taurocholate co-transporting polypeptide (NTCP) in HBV therapy. Cell Prolif. 2014; 47(4):326–35.

65. Kang JM, Yoo WG, Le HG, Lee J, Sohn WM, Na BK. Clonorchis sinensis MF6p/HDM (CsmMF6p/HDM) induces pro-inflammatory immune response in RAW 264.7 macrophage cells via NF-κB-dependent MAPK pathways. Parasit Vectors. 2020; 13(1):20.

66. Dai, F, Yoo WG, Lee JY, Lu Y, Pak JH, Sohn WM, et al. Molecular and structural characteristics of multidrug resistance-associated protein 7 in Chinese liver fluke Clonorchis sinensis. Parasitol Res. 2017; 116(3):953–62.
67. Yang Y, Faraggi E, Zhao H, Zhou Y. Improving protein fold recognition and template-based modeling by employing probabilistic-based matching between predicted one-dimensional structural properties of query and corresponding native properties of templates. Bioinformatics. 2011; 27(15):2076–82. https://doi.org/10.1093/bioinformatics/btr350 PMID: 21666270

68. Franca TC. Homology modeling: an important tool for the drug discovery. J Biomol Struct Dyn. 2015; 33(8):1780–93. https://doi.org/10.1080/07391102.2014.971429 PMID: 25266493

69. Ward AB, Szewczyk P, Grimard V, Lee CW, Martinez L, Doshi R, et al. Structures of P-glycoprotein reveal its conformational flexibility and an epitope on the nucleotide-binding domain. Proc Natl Acad Sci U S A. 2013; 110(33):13386–91. https://doi.org/10.1073/pnas.1309275110 PMID: 23901103

70. Murashita K, Yoshiura Y, Chisada S, Furuita H, Sugita T, Matsunari H, et al. Homologue gene of bile acid transporters ntcp, asbt, and ost-alpha in rainbow trout Oncorhynchus mykiss: tissue expression, effect of fasting, and response to bile acid administration. Fish Physiol Biochem. 2014; 40(2):511–25.

71. Alhadef R, Ganoth A, Arkin IT. Mechanistic studies of the apical sodium-dependent bile acid transporter. Proteins. 2015; 83(6):1107–17. https://doi.org/10.1002/prot.24796 PMID: 25820648

72. Weinman SA, Carruth MW, Dawson PA. Bile acid uptake via the human apical sodium-bile acid cotransporter is electrogenic. J Biol Chem. 1998; 273(52):34691–5. https://doi.org/10.1074/jbc.273.52.34691 PMID: 9856990

73. Bastug T, Heinzelmann G, Kuyucak S, Salim M, Vandenbergh RJ, Ryan RM. Position of the third Na+ site in the aspartate transporter Glt1pm and the human glutamate transporter, EAAT1. PloS one. 2012; 7(3):e33058.

74. Zomot E, Gur M, Bahar I. Microseconds simulations reveal a new sodium-binding site and the mechanism of sodium-coupled substrate uptake by LeuT. J Biol Chem. 2015; 290(1):544–55. https://doi.org/10.1074/jbc.M114.617555 PMID: 25381247

75. Balakrishnan A, Sussman DJ, Polli JE. Development of stably transfected monolayer overexpressing the human apical sodium-dependent bile acid transporter (hASBT). Pharm Res. 2005; 22(8):1269–80. https://doi.org/10.1007/s11095-005-5274-8 PMID: 16078136

76. Ferraro F, Merlino A, Dell Oca N, Gil J, Tort JF, Gonzalez M, et al. Identification of chalcones as Fasciola hepatica cathepsin L inhibitors using a comprehensive experimental and computational approach. PLoS Negl Trop Dis. 2016; 10(7):e0004834.

77. McKerrow JH, Lipinski CA. The rule of five should not impede anti-parasitic drug development. Int J Parasitol Drugs Drug Resist. 2017; 7(2):248–9. https://doi.org/10.1016/j.ijpddr.2017.05.003 PMID: 28623818

78. Loukine E, Keiser MJ, Whitebread S, Mikhailov D, Hamon J, Jenkins JL, et al. Large-scale prediction and testing of drug activity on side-effect targets. Nature. 2012; 486(7403):361–7. https://doi.org/10.1038/nature11159 PMID: 2272194

79. Sofia MJ, Bao D, Chang W, Du J, Naragarthnam D, Rachakonda S, et al. Discovery of a β-D-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. J Med Chem. 2010; 53(19):7202–18. https://doi.org/10.1021/jm100863x PMID: 20845908

80. Jonckers TH, Vandyck K, Vandekerckhove L, Hu L, Tahri A, Van Hoof S, et al. Nucleotide prodrugs of 2′-deoxy-2′-spirooxetane ribonucleosides as novel inhibitors of the HCV NS5B polymerase. J Med Chem. 2014; 57(5):1836–44. https://doi.org/10.1021/jm4015422 PMID: 24345201

81. Esser CK, Bugianesi RL, Caldwell CG, Chapman KT, Durette PL, Girotra NN, et al. Inhibition of stromelysin-1 (MMP-3) by P1′-biphenylylethyl carboxyalkyl dipeptides. J Med Chem. 1997; 40(6):1026–40. https://doi.org/10.1021/jm960465t PMID: 9083493

82. Stetler-Stevenson WG, Liotta LA, Brown PD. Role of type IV collagenases in human breast cancer. Cancer Treat Res. 1992; 61:21–41. https://doi.org/10.1007/978-1-4615-3500-3_2 PMID: 1360233

83. Okada Y, Shinmei M, Tanaka O, Naka K, Kimura A, Nakanishi I, et al. Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. Lab Invest. 1992; 66(6):680–90. PMID: 1602738

84. Gorvin JH, Raison CG, Solomon W, Standen OD, Walls LP. The action of substances analogous to Schistosoma mansoni. Br J Pharmacol Chemother. 1957; 12(3):329–35. https://doi.org/10.1111/j.1476-5381.1957.tb00143.x PMID: 13460239