Biotecnología Aplicada

Elfos scientiae
http://elfosscientiae.cigb.edu.cu/

RESEARCH

Fasciola hepatica cathepsin L3 off-targets interactions with two potential inhibitors

✍Dany Naranjo Feliciano[1*], Adrian González Travieso[1*], Romel Calero Ramos[2], Jesús Bouza Figueroa[2], Yoanna María Alvarez-Ginarte[3], Humberto Andrés Carrillo Calvet[3]

[1] Departamento de Microbiología, Centro Nacional de Sanidad Agropecuaria (Censa) Carretera de Jamaica y Autopista Nacional, Km 22 1/2, San José de las Lajas, Mayabeque, Cuba
[2] Centro de Ciencias de la Complejidad (C3), Universidad Nacional Autónoma de México (UNAM), México
[3] Facultad de Química, Universidad de La Habana, Cuba
✍dany@censa.edu.cu

ABSTRACT

Fascioliasis is an emerging disease caused by the trematode *Fasciola hepatica*. Cathepsin L3 (FhCatL3), is an endoprotease decisive for the virulence and infection of juvenile flukes in many mammals, mainly cattle and man. The compounds BTB03218 and HTS12701 were previously predicted as new potential inhibitors against FhCatL3, using the CH3-AGTRNAA-CH3 substrate. However, the binding of these candidates to the homologous proteins of definitive hosts, as off-targets, is still unknown. Therefore, the protein-ligand interactions were evaluated at the substrate specificity subsites of human and bovine cathepsins L1 by molecular modeling. It was predicted that the compound HTS12701 is target-specific selective to the parasitic enzyme, as substrate. The HTS12701 aromatic bicycle structure is in π-π stacking interactions preferentially with FhCatL3 Trp69 at the substrate specificity subsite S4. The differences at S4 – FhCatL3 Trp69 x Leu - and S3´ - FhCatL3 Tyr143 x Leu or Gln -, destabilize the mammalian protein-ligand complexes with the substrate and HTS12701. The compound BTB03219 is not target-specific selective. BTB03219 established stable complexes through multiple hydrogen bonds at the substrate specificity subsite S1, especially for polar amino acids, FhCatL3 Thr161 or Asp, in mammalian enzymes. Understanding the molecular interactions of the FhCatL3-HTS12701 complex could help to design new fasciolicidal drugs of low toxicity for antiparasitic therapy.

Keywords: Fasciola hepatica, Cathepsin L, inhibitor, off-targets, molecular modeling

RESUMEN

Interacciones de las dianas moleculares no específicas de la catepsina L3 de *Fasciola hepatica* con dos potenciales inhibidores. La fascioliasis es una enfermedad emergente causada por el trematodo *Fasciola hepatica*. La catepsina L3 (FhCatL3), es una endoproteasa decisiva para la virulencia y la infección de los estadios juveniles en muchos mamíferos, principalmente el ganado vacuno y el hombre. Los compuestos BTB03218 y HTS12701, fueron predichos previamente como potenciales inhibidores contra la FhCatL3, con el uso del sustrato CH3-AGTRNAA-CH3. Sin embargo, la unión de estos candidatos a fármacos antiparasitarios a las proteínas homólogas de los hospederos definitivos, como dianas moleculares no específicas, es aún desconocida. Aquí, se evaluó las interacciones proteína-ligando en los subsitios de especificidad de sustrato de las catepsinas L1 humana y bovina, mediante modelación molecular. Se predijo que el compuesto HTS12701 es selectivo y sustrato blanco específico de la enzima parasitaria. La estructura bicíclica aromática del HTS12701 se encuentra en interacciones de apilamiento π-π, preferentemente con FhCatL3 Trp69 en el subsitio de especificidad de sustrato S4. Las diferencias en S4 - FhCatL3 Trp69 x Leu - y S3' - FhCatL3 Tyr143 x Leu o Gln -, desestabilizan los complejos proteína-ligando de mamíferos con el sustrato y el HTS12701. El compuesto BTB03219 no es blanco específico ni selectivo. BTB03219 estableció complejos estables mediante la formación de múltiples enlaces de hidrógeno en el subsitio de especificidad de sustrato S1, especialmente para aminoácidos polares, FhCatL3 Thr161 o Asp, en las enzimas de mamífero. La comprensión de las interacciones moleculares del complejo FhCatL3-HTS12701 podría ser valiosa, para diseñar un nuevo fármaco fasciolicida de baja toxicidad, para la terapia antiparasitaria.

Palabras clave: Fasciola hepatica, catepsina L, inhibidor, blanco molecular no específico, modelación molecular

How to cite (Vancouver style):
Naranjo Feliciano D, González Travieso A, Calero Ramos R, Bouza Figueroa J, Alvarez-Ginarte YM, Carrillo Calvet HA. *Fasciola hepatica* cathepsin L3 off-targets interactions with two potential inhibitors. Biotecnol Apl. 2025;42(1):1211-8.

Introduction

Parasitic diseases are responsible for morbidity and mortality higher than those generated by any other class of infectious organisms [1]. Among these infections is fascioliasis or hepatic distomatosis. *Fasciola hepatica* and *F. gigantica* are liver flukes causing fascioliasis, a worldwide zoonotic, complex disease [2]. Infection caused by the trematode *F. hepatica* is also considered a disease of veterinary medical importance. This parasitosis is responsible for substantial economic losses in livestock worldwide and poses a threat to human health in endemic areas [3].

Several drugs with fasciolicidal activity have been used for the control of fascioliasis for more than a century, mainly on the adult forms of the parasite. Only Triclabendazole has been effective on the immature (intrahepatic) and adult stages (in the bile ducts), given its high affinity on the beta tubulin of *F. hepatica*, which causes its immobilization and death [4]. Indeed, triclabendazole (TCBZ) treatment is the cornerstone of fascioliasis control in livestock and the only drug licensed for human use [2]. However, TCBZ resistance has been reported on every continent and threatens

1. World Health Organization. Foodborne trematode infections. 2021 [cited 2022 Nov 11]. Available from: https://www.who.int/es/news-room/fact-sheets/detail/foodborne-trematodiases.

2. Mas-Coma S, Valero MA, Bargues MD. One Health for fascioliasis control in human endemic areas. Trends Parasitol. 2023;39(8):650-67.

CC BY NC SA

Publicación libre de costo para el autor
No article processing charges

* These authors contributed equally to this work

effective control of fasciolosis in many parts of the world, in both cattle and humans [5]. Therefore, there is an increasing need for other target molecules, or fasciolicidal drugs that act through new inhibitory pathways to which the parasite has not shown resistance.

In this scenario, proteases emerge as a biological target to consider, due to their pivotal role in many parasitism-related biological processes and their tight regulation by parasite anti-proteases (protease inhibitors) [6]. Cathepsins L of *F. hepatica* are cysteine-type endopeptidases that are similar in structure to papain [7]. Phylogenetic analyses performed on a large number of gene sequences deposited in public databases have separated this enzyme family into five groups, comprised from cathepsin L1 to cathepsin L5 [8]. This enzyme has been the subject of genetic engineering and crystallography experiments in the presence of various cathepsin L1- inhibitors [9, 10]. It has been shown that together with some cathepsin B variants, it is involved in the biological cycle of *F. hepatica*, propitiating parasitic colonization through tissue invasion, evasion of the immune response as well as its development and replication [11].

Specifically, *F. hepatica* cathepsin L3 (FhCatL3) is an excretion-secretion product of juvenile states (NEJ) [8]. FhCatL3 is a potential therapeutic target, since it favors the mobilization of the parasite through the bile ducts of the infected animal causing severe lesions and even death. According to the MEROPS database (https://www.ebi.ac.uk/merops/cgi-bin/pepsum?id=C01.129) [12], FhCatL3 is able to accommodate residues such as Gly and Pro in the substrate specificity subsite S2, which mediates its preference for intestinal collagen degradation and causing cysts in the intestinal epithelium. Moreover, cathepsin L is also present in definitive hosts. Both, cattle (bCatL1) and human (hCatL1) proteases play a key role in protein degradation and catabolism in the lysosome, as well as in antigen processing [13]. Therefore, the study of these mammalian proteins may lead to the development of selective fasciolicides for the control of this parasitosis. Research on cathepsins L has also been booming due to their involvement in the binding mechanisms of coronaviruses (SARS-CoV-2, SARS-CoV) to host receptor cells [14, 15].

In this line, two compounds named BTB03219 and HTS12701 were predicted [16], showing inhibitory potential for the parasitic enzymatic activity of cathepsin L3. They were identified through virtual screening of 13 000 molecules from the Maybridge Hitfinder database (https://uk.vwr.com/cms/maybridge). The selection criterion was the results of the molecular docking with the parasitic enzyme with respect to the interaction with the definitive hosts enzymes hCatL1 and bCatL1. Despite, molecular dynamics simulations, the change in the Gibbs free energy calculations, and per-residue contribution were not performed for both enzymes' protein-ligand complexes.

Nonetheless, *in silico* information regarding FhCatL3, such as the mechanism of action of a peptide substrate (CH3-AGTRNAA-CH3) and with the compounds BTB03218 and HTS12701 [16], has not provided information of bovine cathepsin L1 (bCatL1) as an off-target. Therefore, in this work, a binding pocket and binding mode for the bovine enzyme is proposed, by performing a comprehensive computational study applying homology modeling, molecular docking and molecular dynamics simulations.

Materials and methods

Structures and sequences

The ligands used as inhibitor candidates are: BTB03219 (IUPAC name: 1-N-[3,5-bis (trifluoromethyl) phenyl]-2-N-(1-ethynylcyclohexyl) benzene-1,2-dicarboxamide) and HTS12701 (IUPAC name: 2-{[4-benzyl-5- (pyridin-4-yl)-4H-1,2,4-triazol-3-yl] sulfanyl} 2-1-(1,2,3,4-tetrahydroquinolin-1-yl)ethan-1-one) (Figure 1). These compounds were obtained from virtual screening of the FheCatl3 enzyme [16], with the Maybridge British Company HitFinder database (https://uk.vwr.com/cms/maybridge). The structure of the peptide mimetic (MUT) of amino acidic sequence ACE-AGPR↓NAA-NME (Figure 1), analogous to the natural substrate of the parasitic enzyme, was obtained as previously described [16].

The amino acid sequences of FhCatL3 and bCatL1 were obtained from the UNIPROT sequence bank (https://www.uniprot.org/) under IDs Q9GRW6 and P25975, respectively. Using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/), homologous enzymes of highest sequence identity with resolved crystallographic structure were identified from the protein data bank (PDB) (https://www.rcsb.org/). FhCatL1 (PDB ID: 2O6X) and hCatL1 (PDB ID: 3OF8) were chosen as templates for the parasitic and bovine enzyme, respectively, in homology modeling.

Homology modeling

Sequence alignment of FhCatL3 and bCatL1 with the sequences of their FhCatL1 (PDB ID: 2O6X) and hCatL1 (PDB ID: 3OF8) templates was performed on the CLUSTAL OMEGA server (https://www.ebi.ac.uk/Tools/msa/clustalo). The homology models

3. Beesley NJ, Cwiklinski K, Allen K, Hoyle RC, Spithill TW, La Course EJ, *et al*. A major locus confers triclabendazole resistance in *Fasciola hepatica* and shows dominant inheritance. PLoS Pathog. 2023;19(1):e1011081.

4. Gandhi P, Schmitt EK, Chen CW, Samantray S, Venishetty VK, Hughes D. Triclabendazole in the treatment of human fascioliasis: a review. Trans R Soc Trop Med Hyg. 2019;113(12):797-804.

5. Patrick J, Anderson F, Wilson K, McCormick I, Skuce P, O'Roarke J. Triclabendazole-resistant liver fluke: issues and strategies. Livestock. 2018;23(Sup5):4-14.

6. Cwiklinski K, Dalton JP. Exploiting comparative omics to understand the pathogenic and virulence-associated protease: anti-protease relationships in the zoonotic parasites *Fasciola hepatica* and *Fasciola gigantica*. Genes (Basel). 2022;13(10):1854.

7. Norbury LJ. Structure, function and evolutionary studies of *Fasciola* Cathepsin L-like Proteases. [Dissertation]. Melbourne: Royal Melbourne Institute of Technology; 2008.

8. Cwiklinski K, Donnelly S, Drysdale O, Jewhurst H, Smith D, De Marco Verissimo C, *et al*. The cathepsin-like cysteine peptidases of trematodes of the genus *Fasciola*. Adv Parasitol. 2019;104:113-64.

9. Ferraro F, Merlino A, Gil J, Cerecetto H, Corvo I, Cabrera M. Cathepsin L inhibitors with activity against the liver fluke identified from a focus library of Quinoxaline 1,4-di-N-oxide derivatives. Molecules. 2019;24(13):2348.

10. Moran BW, Anderson FP, Ruth DM, Fágáin CO, Dalton JP, Kenny PT. Fluorobenzoyl dipeptidyl derivatives as inhibitors of the *Fasciola hepatica* cysteine protease cathepsin L1. J Enzyme Inhib Med Chem. 2010;25(1):1-12.

11. Kramer L, Turk D, Turk B. The future of cysteine Cathepsins in disease management. Trends Pharmacol Sci. 2017;38(10):873-98.

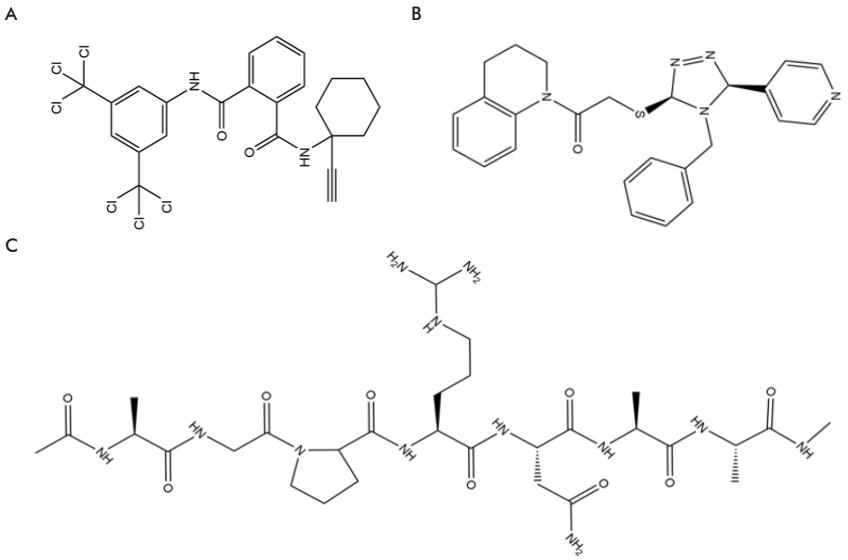

Figure 1. 2D structure of the three ligands used to analyze the *Fasciola hepatica* cathepsin L3 off-targets interactions with two potential inhibitors. A) The BTB02319 non-peptide inhibitor. B) The HTS12701 non-peptide inhibitor. C) The peptide mimetic (MUT) of amino acidic sequence ACE-AGPR↓NAA-NME

were calculated using Modeller v9.22 software [17]. The stereochemistry of the obtained models was evaluated with the Procheck (Ramachandran Plotting) server [18]. The models were visually inspected with the programs PyMOL v1.7 [19] and Chimera v1.11.2 [20], and their geometries optimized through energy minimization with the GROMACS v5.1.4 package [21]. Finally, they were evaluated with the UCLA-SAVS online server tools (ERRAT [22], VERIFY_3D [23], PROVE [24] and WHAT_CHEK [25]).

Molecular docking

A semi-rigid molecular docking was performed using the Autodock-Vina v4.2 program [26], in order to obtain the binding energy (*Svina*) data corresponding to the calculation for the three enzymes (FhCatL3, hCatL1 and bCatL1) with the three ligands (BTB03219, HTS12701, MUT), for a total of nine complexes. Visual inspection of the catalytic pocket was supported by the web server Prankweb (https://prankweb.cz/help) [27]. A box with X, Y and Z dimensions of 16.5, 21 and 15 Å, respectively, was used for the BTB03219 and HTS12701 ligands, while for the peptide the box dimensions were 26, 21 and 15 Å. The center of the boxes was attached to the sulfhydryl group of the catalytic cysteine. Ten solutions per complex were selected to be obtained. All other program parameters were run by default.

Molecular dynamics (MD) and Gibbs free energy calculations

Prior to the molecular dynamics runs the ligands were parameterized through the ambertools packages (antechamber, parmchk, tleap). For the case of the receptors a protonation study of the ionizable residues (His, Lys, Asp and Glu) was performed with the H++ and PDB-2PQR servers [28]. MD runs were performed with the GROMACS v5.1.4 package for a time of 50 ns. The AMBER99SB-ILDN force field was used, the water model used was TIP3P and the electro-neutrality of the medium was guaranteed by the addition of Na^+ and Cl^- ions in an appropriate ratio to reach a final concentration of 0.15 mol/L (ionic strength). The root-mean-square deviation in distance (RMSD) of the atoms of the protein-ligand complexes and the root root-mean-square fluctuation (RMSF) per residues were also calculated with the GROMACS v5.1.4 package.

For the calculations of Gibbs Free Energy (ΔG_{eff}) by the MMPBSA method [29], energy decomposition by residue and the calculation of configurational entropy (ΔS_{conf}) the program MMPBSA.py from the Amber14 package was used [30]. The implicit solvent model GBn2 (*igb* = 8) and radius mbondi3 were used to calculate the ΔG_{GB} term of each system. The energetic contributions were calculated for the intervals of highest stability for each simulation. For the entropic calculations the NMODE (Normal Mode) method was used, this term ($-T\Delta S_{conf}$) was subtracted from the previously calculated effective energy (ΔG_{eff}) to finally obtain the affinity energy (ΔG_{bind}). The Gibbs free energy calculation by the LIE-D method was performed using the protocol by Miranda *et* al. [31]. For ligands similar to small drugs, such as BTB03219 ($\alpha = 0.18$, $\beta = 0.39$) and HTS12701 ($\alpha = 0.18$, $\beta = 0.41$) and [32, 33] by assigning the neutral charge to the ligands. This method was not implemented for the peptide substrate MUT, since it is not parameterized for amino acids.

Calculation of the selectivity index

The ΔG_{bind} values obtained by the MMPBSA method were used. The affinity energies of each ligand for its target to inhibit were divided by the energies obtained with the enzymes to not inhibit [34] through the equation:

$$SI = \frac{\left(\sum_{i=1}^{N_{target}} \Delta G_{target_i}\right) / N_{target}}{\left(\sum_{i=1}^{N_{target}} \Delta G_{off\text{-}target_i}\right) / N_{off\text{-}target}}$$

Where, N_{target} is the number of energies obtained from a ligand by its targets to inhibit, and $N_{off\text{-}target}$ is the number of energies of the same ligand by its targets not to inhibit.

Results and discussion

Homology modeling and binding site prediction

Models of the three-dimensional structures of the enzymes FhCatL3 and bCatL1 were calculated, through the comparison modeling protocol. Validation showed the high quality of the models, given the sequence identity (~71 % for FhCatL3 and ~80 % for bCatL1) with respect to their templates. The sequence-structure-function relationship analysis for all three enzymes aid to corroborate that most of the amino acids comprising the subsites of the catalytic pocket (Table 1) are preserved, characteristic of these proteases in which the active site is highly conserved [35]. Despite, there were important variations in some residues of the parasitic enzyme such as tryptophan 69 (S4), threonine 161 (S1) and tyrosine 143 (S3'), substituted by leucine, aspartic and leucine/glutamine, respectively, in mammalian enzymes. Noteworthy, most of these residues in these subsites showed hydrophobic character [36].

Characterization of the model binding site

Molecular docking and MD simulations, together with Gibbs free energy calculations provide an immediate and scientifically sound basis for designing highly specific inhibitors against viral proteins [37]. In this study, an energetic characterization of the interaction interface of the substrate peptide MUT was performed (Figure 2). The MUT peptide was used as control, given its high affinity for the parasitic enzyme, which facilitates the comparison of its selectivity index with that of the selected inhibitor candidates (HTS12701 and BTB03219). This substrate is a segment of collagen and it has been degraded by FhCatL3 in *in vivo* experiments [8]. MUT scans all subsites of the catalytic pocket from S4 to S3', where the hydrogen bonds are formed and a large number of apolar contacts occur.

Table 1. Subsites that make up the catalytic pocket of *Fasciola hepatica* cathepsin L3 (FhCatL3), human cathepsin L1 (hCatL1) and bovine cathepsin L1 (bCatL1)*

	Subsites						
Enzyme	S4	S3	S2	S1	S1'	S2'	S2'
FhCatL3	W69	G68 V160	W26 M70 A163	G67 T161 H162	G23 C25	Q19 W184	Y143
hCatL1	L70	G69 M162	W27 M71 G165	G68 D163 H164	G24 C26	Q20 W190	L145
bCatL1	L65	G64 L158	W22 M66 G161	G63 D159 H160	G19 C21	Q15 W186	Q141

* Different residues among subsites are colored in red.

12. Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Res. 2018;46(D1):D624-32.

13. Norbury LJ, Basałaj K, Bąska P, Kalinowska A, Zawistowska-Deniziak A, Yap HY, *et al*. Construction of a novel phage display antibody library against *Fasciola hepatica*, and generation of a single-chain variable fragment specific for *F. hepatica* cathepsin L1. Exp Parasitol. 2019;198:87-94.

14. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci U S A. 2005;102(33):11876-81.

15. Gomes CP, Fernandes DE, Casimiro F, da Mata GF, Passos MT, Varela P, *et al*. Cathepsin L in COVID-19: From pharmacological evidences to genetics. Front Cell Infect Microbiol. 2020;10:589505.

16. Hernández Alvarez L, Naranjo Feliciano D, Hernández González 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.

17. Webb B, Sali A. Comparative protein structure modeling using MODELLER. Curr Protoc Bioinformatics. 2016;54:5.6.1-37.

18. Laskowski RA, Moss DS, Thornton JM. Main-chain bond lengths and bond angles in protein structures. J Mol Biol. 1993;231(4):1049-67.

19. The PyMOL Molecular Graphics System. New York: Schrodinger L.L.C. Version 1.7. 2010.

20. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, *et al*. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004;25(13):1605-12.

21. Pronk S, Páll S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, *et al*. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics. 2013;29(7):845-54.

22. Colovos C, Yeates TO. Verification of protein structures: patterns of nonbonded atomic interactions. Protein Sci. 1993;2(9):1511-9.

23. Lüthy R, Bowie JU, Eisenberg D. Assessment of protein models with three-dimensional profiles. Nature. 1992;356(6364):83-5.

24. Pontius J, Richelle J, Wodak SJ. Deviations from standard atomic volumes as a quality measure for protein crystal structures. J Mol Biol. 1996;264(1):121-36.

25. Hooft RWW, Vriend G, Sander C, Abola EE. Errors in protein structures. Nature. 1996;381(6580):272.

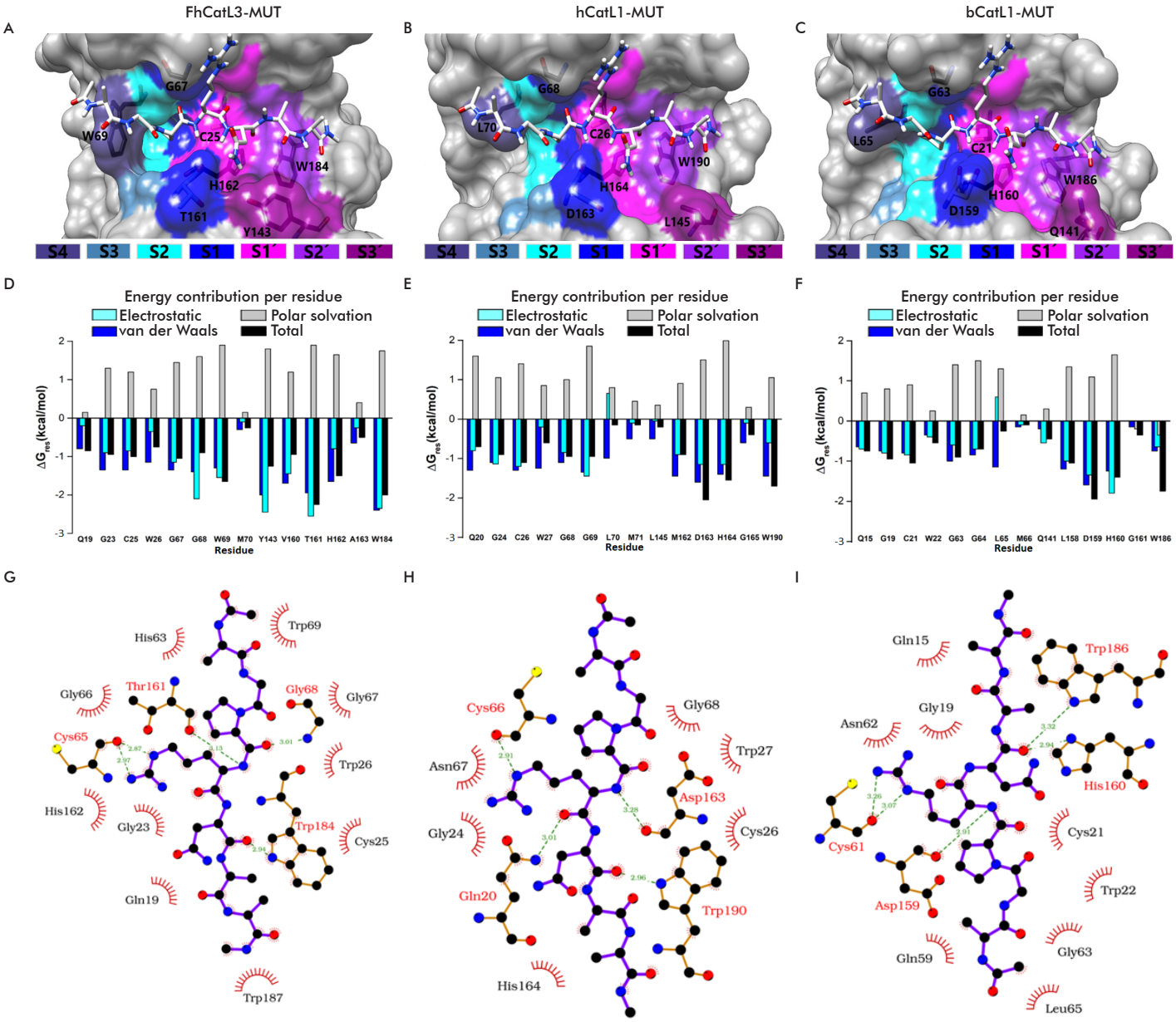

Figure 2. Representation of the interaction interface of compound MUT with *Fasciola hepatica* cathepsin L3 (FhCatL3; A, D, G), human cathepsin L1 (hCatL1; B, E, H) and bovine cathepsin L1 (bCatL1; C, F, I). A-C) The subsites were colored according to the legend. D-F) The energy decomposition per residue was calculated considering the van der Waals, electrostatic, polar solvation and total contributions. G-I) The respective protein-ligand interaction diagrams obtained in LigPlot show apolar contacts as red jagged arcs and polar (H-bonds) as green dashed lines.

The stability monitored during the MD simulations highlight minimal variations of the RMSD in FhCatL3, somewhat expected since MUT is a natural substrate analog (Figure 3). In contrast, an unstable behavior of the MUT ligand was observed in mammalian enzymes bCatL1 and hCatL1, causing variations of its RMSD and RMSF that exceeded 10 Å, with the exit of the catalytic cavity of bCatL1 after 30 ns of simulation. The energetic analysis by residue showed an interaction with the 14 amino acids of the active site, mainly with Q19, G23, G25, W26, G67, W69, Y143, T161, H162 and W184. The presence of the non-conserved residues W69 (S4) and Y143 (S3') in the parasitic enzyme is critical in substrate stabilization in these subsites. Their substitution by leucines in the case of hCatL1 and glutamine in bCatL1, which are amino acids of bad energetic contribution, destabilizes the molecule in these systems.

Compound interactions

Non-peptide inhibitors are regarded as the best strategy for *in vivo* inhibition since they escape from protease degradation [38]. The molecular docking of compound HTS12701, which is a non-peptide inhibitor, in the three enzymes yielded the starting configuration for MD (Figure 4). The affinity energy (*Svina*) with FhCatL3 of this molecule was in agreement with the reported value of –8.0 kcal/mol [16].

26. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010;31(2):455-61.

27. Jakubec D, Skoda P, Krivak R, Novotny M, Hoksza D. PrankWeb 3: accelerated ligand-binding site predictions for experimental and modelled protein structures. Nucleic Acids Res. 2022;50(W1):W593-7.

28. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G, *et al*. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res. 2007;35(Web Server issue):W522-5.

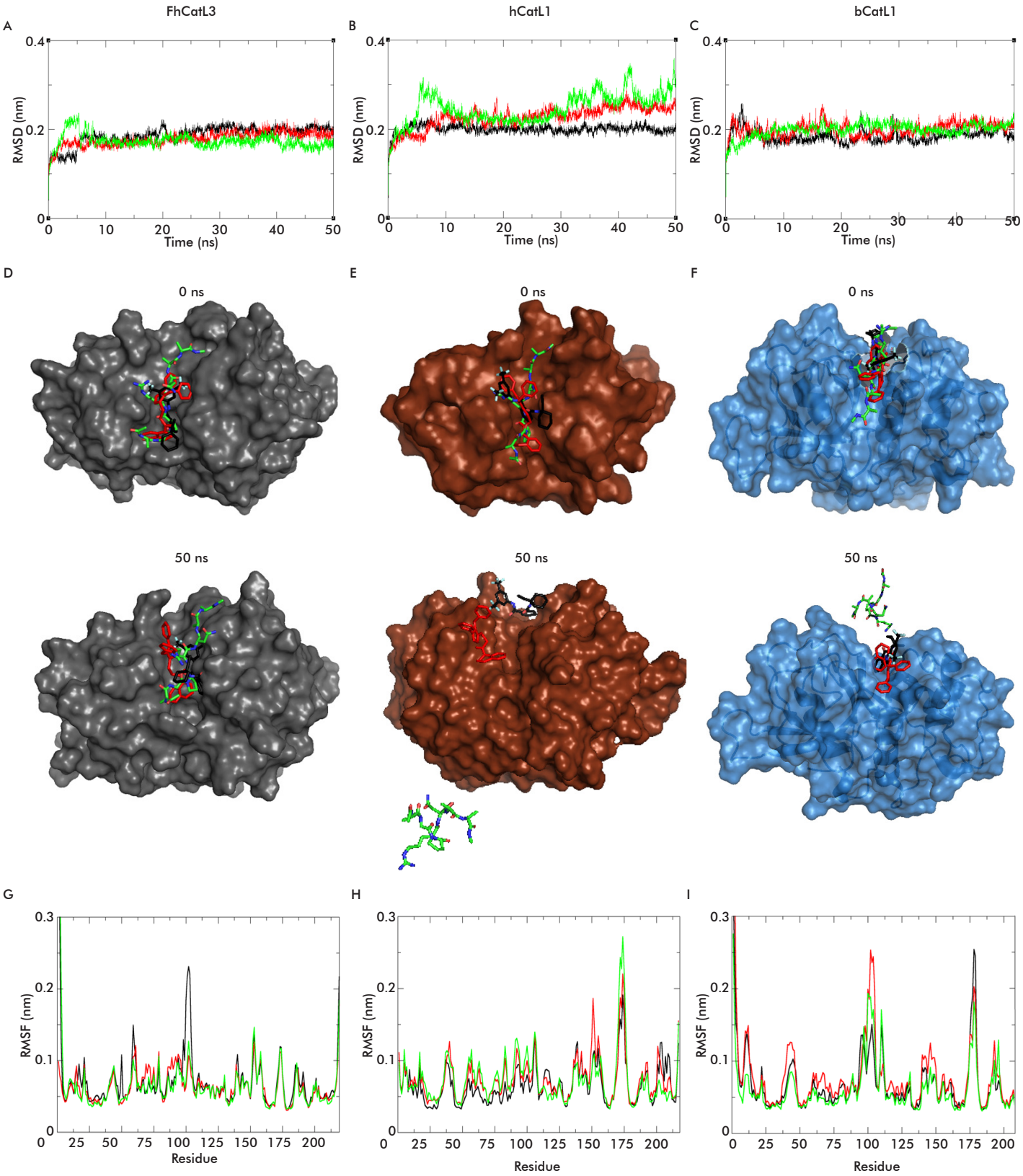

Figure 3. Variations of the root-mean-square deviation in distance (RMSD) of the atoms of the protein-ligand complexes and the root root-mean-square fluctuation (RMSF) per residue for the nine systems. A-C) RMSD analysis from 0 to 50 ns. D-F) Molecular dynamics of the protein-ligand complex and the inhibitor at 0 and 50 ns. G-I) RMSF analysis. Ligands are color-coded (A-C, G-I): BTB03219 (black), HTS12701 (red), and MUT peptide (green).

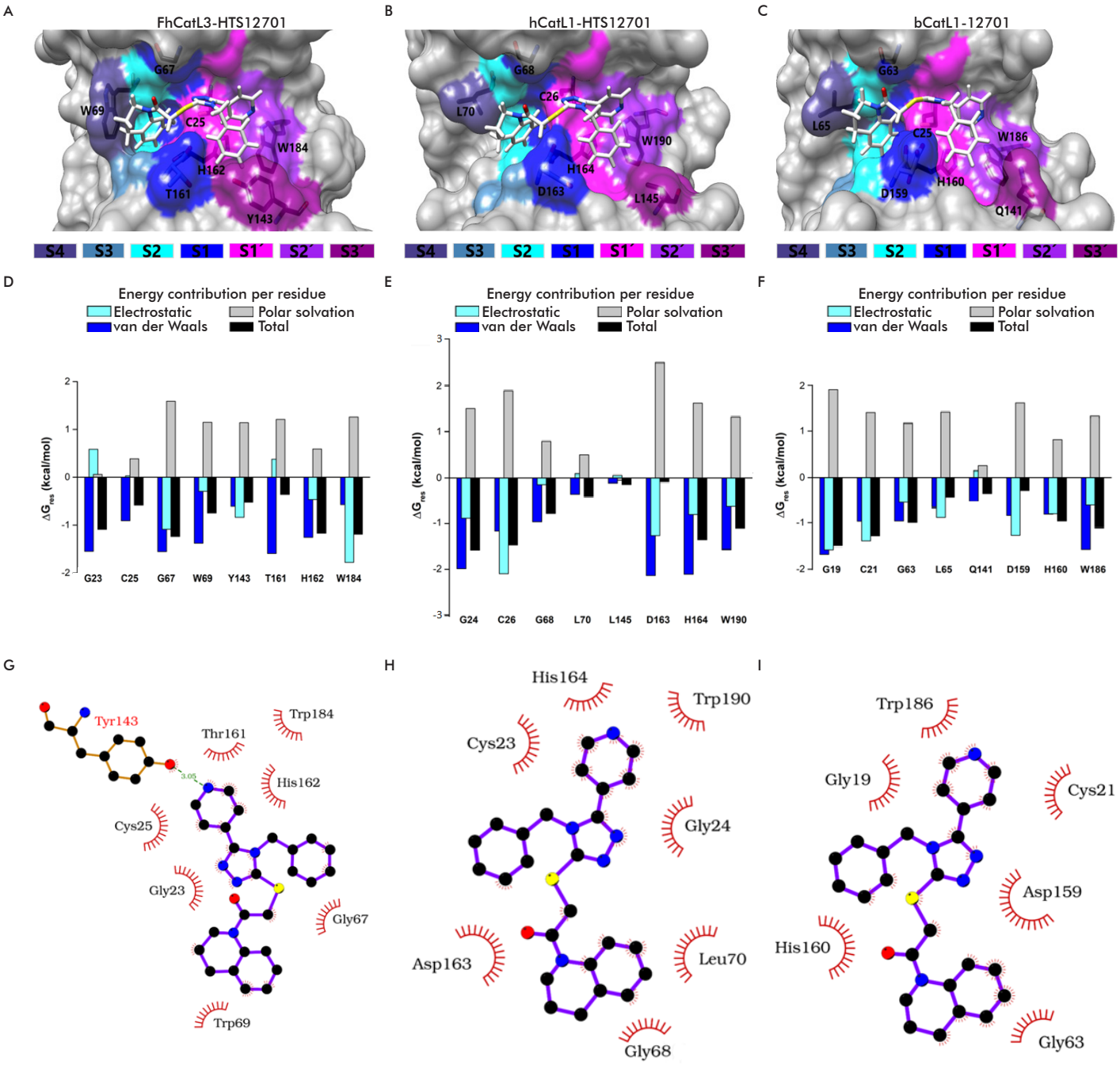

Figure 4. Representation of the interaction interface of compound HTS12701 with *Fasciola hepatica* cathepsin L3 (FhCatL3; A, D, G), human cathepsin L1 (hCatL1; B, E, H) and bovine cathepsin L1 (bCatL1; C, F, I). A, B and C) The subsites were colored according to the legend. D, E and F) The energy decomposition per residue was calculated considering the van der Waals, electrostatic, polar solvation and total contributions. G, H and I) The re-spective protein-ligand interaction diagrams obtained in LigPlot show apolar contacts as red jagged arcs and polar (H-bonds) as green dashed lines.

The non-covalent binding mode of this ligand is favored by hydrophobic interactions almost entirely with a large number of amino acidic residues, and only one hydrogen bond with tyrosine 143 (S3') is appreciated in the parasitic enzyme. The presence of several aromatic cycles and bicycles (benzene and naphthalene derivatives) in the compound increases the hydrophobicity and with this the number of apolar contacts in the catalytic cavity. The stability of the HTS12701-enzyme complexes was monitored over time through MD simulations (50 ns). Structural properties such as RMSD and RMSF showed high deviations in mammalian enzymes, into contrast with the high stability seen for the parasitic enzyme (Figure 3). This demonstrates the selectivity of this molecule to be used as a possible fasciolicide candidate, since it shows affinity for the parasite enzyme and does not affect, in turn, the enzymes of the definitive hosts.

29. Genheden S, Ryde U. How to obtain statistically converged MM/GBSA results. J Comput Chem. 2010;31(4):837-46.

30. Case DA, Babin V, Berryman JT, Betz RM, Cai Q, Cerutti DS, *et al*. AMBER 14. San Francisco: University of California; 2014.

The energetic analysis per residue by Gibbs Free Energy calculations evidenced the presence of eight amino acids involved in the binding mode: G23, C25, G67, W69, Y143, T161, H162 and W184 (according to the FhCatL3 enumeration). W69 (S4), Y143 (S3') and T161 (S1) are not conserved in mammalian enzymes, so the specificity of the molecule could be given by these residues. W69 is one of the residues with the highest van der Waals contribution, fundamental in anchoring the inhibitory potential in the S4 subsite. The indole group of W69 and the quinoline group of the HTS12701 ligand form a π-π stacking interaction, which strength have been previously addressed [39]. When W69 has been substituted by leucine in mammalian enzymes, a residue of much lower energetic contribution, HTS12701 tended to destabilize in these systems, causing a failure of inhibition.

The other molecule, BTB03219, presents polar chemical groups such as carbonyl, amide and fluorine atoms. Therefore, its binding mode (Figure 5) is favored by the formation of several hydrogen bonds in all enzymes. This compound acts preferentially with the subsite S1 position, occupied by threonine 161 in FhCatL3, a polar residue that favors electrostatic contacts. This residue varies in mammalian enzymes to aspartic acid, an amino acid that exerts a similar functionality. Other residues involved in the stability of the molecule are the catalytic pair histidine-cysteine.

31. Miranda WE, Noskov SY, Valiente PA. Improving the LIE method for binding free energy calculations of protein-ligand complexes. J Chem Inf Model. 2015;55(9):1867-77.

32. Aqvist J, Medina C, Samuelsson JE. A new method for predicting binding affinity in computer-aided drug design. Protein Eng. 1994;7(3):385-91.

33. Punkvang A, Saparpakorn P, Hannongbua S, Wolschann P, Beyer A, Pungpo P. Investigating the structural basis of arylamides to improve potency against *M. tuberculosis* strain through molecular dynamics simulations. Eur J Med Chem. 2010 Dec;45(12):5585-93.

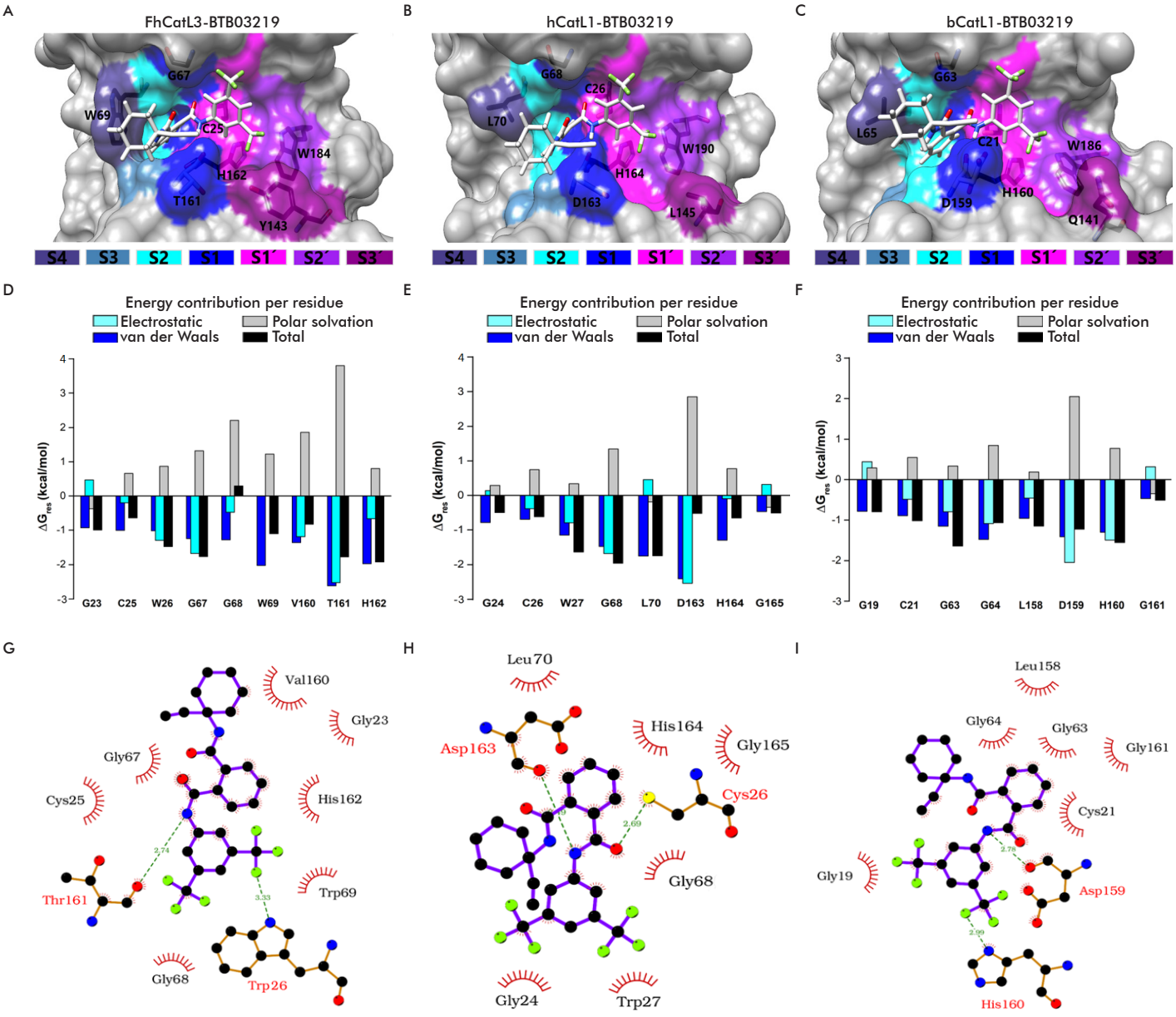

Figure 5. Representation of the interaction interface of compound BTB03219 with *Fascola hepatica* cathepsin L3 (FhCatL3; A, D, G), human cathepsin L1 (hCatL1; B, E, H) and bovine cathepsin L1 (bCatL1; C, F, I). A, B and C) The subsites were colored according to the legend. D, E and F) The energy decomposition per residue was calculated considering the van der Waals, electrostatic, polar solvation and total contributions. G, H and I) The respective protein-ligand interaction diagrams obtained in LigPlot show apolar contacts as red jagged arcs and polar (H-bonds) as green dashed lines.

Then, this compound establishes stable complexes in all enzymes, so that a low selectivity could be inferred. The most stable configuration obtained by molecular docking in the parasitic enzyme yielded an energetic value (ΔS_{vina}) similar to that reported of –8.1 kcal/mol [38].

MD simulations corroborated the stability of compound BTB03219 in all systems, yielding highly favorable binding free energy values by both LIE-D and MMPBSA methods. Residue-based energy decomposition highlighted the presence of nine amino acids involved in the interaction core: G23, C25, W26, G67, G68, W69, V160, T161 and H162 (enumeration according to FhCatL3). In this case, the S1 subsite makes the greatest contribution to ligand stability with T161 and H162. The functionality of the S1 subsite is conserved in all three enzymes, and with it the stability of the molecule. The contribution of this subsite is lower for bCatL1, but it is counterbalanced by the high contribution in S2 (W27, G68 and L70).

The energetic calculations for the nine complexes are shown in Table 2. The reference value for these systems is –12 kcal/mol. This coincides with the experimental inhibitory constant of the hCatL1 crystallographic structure with the inhibitor dipeptidyl glioxal, this last interacting similar to the tested compounds.

From these results, it can be stated that molecular docking shows limitations [40], since it records the interactions for only one configuration of the system and does not consider the action of the solvent or entropic contributions. Therefore, the values obtained by this method do not correspond to the LIE-D and MMPBSA calculations. Recent studies using these methods on homologous enzymes such as 3CLpro, the SARS-CoV-2 coronavirus replication protease, have shown significant differences between the results obtained from molecular docking and Gibbs free energy calculations [37]. The results achieved in MD were corroborated with binding Gibbs free energy calculations, the BTB03219 ligand showing high energetic values in all enzymes while HTS12701 and MUT peptide only achieved good results in the parasitic enzyme.

Selectivity indexes of the compounds

The selectivity index is a quantitative indicator that provides a comparative measure of the affinity of a ligand for a given target [41]. The value obtained for the MUT peptide (1.79) was used as reference, with 1.24 for the BTB03219 ligand and 1.43 for HTS12701. This last value was the closest to that of the control (MUT). This makes of HTS12701 the most promising compound analyzed to be further studied as antiparasitic therapeutic molecule against fascioliasis, both in humans and mainly in cattle, where the greatest affectations are concentrated worldwide.

Noteworthy, the estimated selectivity index values from MM-PBSA results, while valid *in silico* to computationally rank compounds, require to be validated by including the analysis considering the solvent entropy and the protein flexibility. Therefore, *in vitro* testing will corroborate the calculated selectivity values, based on K_d or IC_{50}.

Table 2. Affinity energies obtained in molecular anchoring and by the LIE-D and MMPBSA methods*

Complexes	S_{vina}	LIE-D ΔG_{bind}	MMPBSA ΔG_{bind}
FhCatL3-MUT	–7.2	–	–11.23 ± 0.56
bCatL1-MUT	–8.0	–	–5.27 ± 0.45
hCatL1-MUT	–7.3	–	–7.21± 0.51
FhCatL3-HTS12701	–7.4	–9.89 ± 0.34	–10.16 ± 0.44
bCatL1-HTS12701	–7.1	–6.71 ± 0.30	–7.32 ± 0.63
hCatL1-HTS12701	–7.4	–7.22 ± 0.19	–6.89 ± 0.50
FhCatL3-BTB03219	–8.2	–11.28 ±	–11.95 ± 0.72
bCatL1-BTB03219	–7.2	–9.36 ± 0.26	–9.11 ± 0.43
hCatL1-BTB03219	–7.4	–8.72 ± 0.23	–10.02 ± 0.56

* FhCatL3: *Fasciola hepatica* cathepsin L3. bCatL1: bovine cathepsin L1. hCatL1: bovine cathepsin L1. Complexes were analyzed, formed by the three proteases with the peptide MUT and synthetic HTS12701 and BTB03219 substrate inhibitors. All the energies were expressed in kcal/mol. MMPBSA ΔG_{bind} was calculated as: ΔG_{eff} -$T\Delta S_{conf}$. All ΔG_{bind} values were expressed as the mean ± standard deviation of the mean. The experimental inhibition constant (Ki_{exp})for the crystal (PDB ID: 3OF8) was 0.6 nM. The experimental binding energy (ΔG_{exp}), calculated by the formula -RTln(Ki_{exp}) was ≈ –12,0 kcal/mol. Values within ~3.5 kcal/mol of this reference, between –8.5 and –12.0 kcal/mol, were regarded as accurate predictions of strong affinities.

Conclusions

In summary, molecular docking and MD simulations were combined with Gibbs free energy calculations to evaluate the stability and *in silico* selectivity of the inhibitory molecules BTB03219 and HTS12701 on the target FhCatL3 and as non-specific molecular targets of hCatL1 and bCatL1. HTS12701 was the compound that achieved a selectivity index closer to that of the MUT natural substrate of the parasitic enzyme. This was based on the ligand mode of action on the W69 residue of the S4 subsite of the parasitic enzyme, which is not conserved in the mammalian enzymes. The prevalence of hydrophobic interactions in the FhCatL3-HTS12701 interaction core is critical in the stability of the complex. This *in silico* study could facilitate the development of new inhibitors, with stronger binding and of higher selectivity properties on the FhCatL3, for the effective therapeutic control of fascioliasis in humans and livestock.

Acknowledgements

This research was funded by the International Foundation for Science (IFS) (Grant B/4908-1), Coordenação de Aper-feiçoamento de Pessoal de Nível Superior, CAPES/MESCUBA PROJETOS 173/12 and Asociación Nacional de Universidades e Instituciones de Educación Superior (ANUIES) de la República Mexicana -2022-. This work was partially supported by Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED), through Red 2223RT0140 RENATEC. The authors thank to Pedro Alberto Valiente Flores, PhD, for his excellent technical assistance; Pedro Geraldo Pascutti, PhD, for the initial financial support and Mayelin Mirabal Sosa for her careful review and editing of the article. We are also grateful to the Project leaders, María del Carmen Travieso Novelles and Belkis Corona González, for their invaluable contributions and support while conducting this study.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Received in Noviembre, 2024.
Accepted in January, 2025.

34. Genheden S, Ryde U. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. Expert Opin Drug Discov. 2015;10(5):449-61.

35. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, *et al*. Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim Biophys Acta. 2012;1824(1):68-88.

36. Dana D, Pathak SK. A review of small molecule inhibitors and functional probes of human Cathepsin L. Molecules. 2020;25(3):698.

37. Mirza MU, Froeyen M. Structural elucidation of SARS-CoV-2 vital proteins: Computational methods reveal potential drug candidates against main protease, Nsp12 polymerase and Nsp13 helicase. J Pharm Anal. 2020 Aug;10(4):320-8.

38. Hernández-González JE, Salas-Sarduy E, Hernández Ramírez LF, Pascual MJ, Álvarez DE, Pabón A, *et al*. Identification of (4-(9H-fluoren-9-yl) piperazin-1-yl) methanone derivatives as falcipain 2 inhibitors active against *Plasmodium falciparum* cultures. Biochim Biophys Acta Gen Subj. 2018;1862(12):2911-23.

39. Deng JH, Luo J, Mao YL, Lai S, Gong YN, Zhong DC, et al. π-π stacking interactions: Non-negligible forces for stabilizing porous supramolecular frameworks. Sci Adv. 2020;6(2):eaax9976.

40. Aldeghi M, Bodkin MJ, Knapp S, Biggin PC. Statistical analysis on the performance of molecular mechanics Poisson-Boltzmann surface area versus absolute binding free energy calculations: Bromodomains as a case study. J Chem Inf Model. 2017;57(9):2203-21.

41. Kumar S, Sharma D, Narasimhan B, Ramasamy K, Shah SAA, Lim SM, *et al*. Computational approaches: discovery of GTPase HRas as prospective drug target for 1,3-diazine scaffolds. BMC Chem. 2019;13(1):96.