

Identification of Novel Candidate Inhibitors Targeting hBCATm for Acute Liver Failure by In Silico Methods

Remziye Azra Kartop

Üsküdar University

Serra Özışık

Üsküdar University

Vildan Enisoğlu Atalay

enisogluatalayv@itu.edu.tr

Istanbul Technical University

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3 **Liver Failure by In Silico Methods**

4 **Remziye Azra Kartop¹, Serra Özışık², Vildan Enisoğlu Atalay^{3*}**

5
6 ¹ *Biotechnology Program, Graduate School of Science, Üsküdar University, Üsküdar, 34672,*
7 *Istanbul, Turkey,*

8 ² *Bioinformatics Program, Graduate School of Science, Üsküdar University, Üsküdar, 34672,*
9 *Istanbul, Turkey,*

10 ³*Istanbul Technical University, Informatics Institute, Maslak, 34467, Istanbul, Turkey*

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12
13 *Corresponding author, e-mail: enisogluatalayv@itu.edu.tr

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Abstract

Acute liver failure (ALF) is a life-threatening disease characterized by severe metabolic dysregulation and limited treatment approaches. Human mitochondrial branched-chain aminotransferase (hBCATm), involved in branched-chain amino acid metabolism, plays a critical role as a key target in ALF treatment options. This study aimed to derivatize new molecules more effective against hBCATm, perform docking procedures, conduct structure-activity relationship (SAR) analyses, perform molecular dynamics simulations for prominent candidate molecules, and calculate MMGBSA binding free energy using a sequential workflow. In the derivatization process performed based on the molecule coded ZINC000013555421(L0) selected from the ZINC database, different substituted groups were attached to the determined positions, first to produce drugs, and then, in an iterative cycle, 98 new candidate derivative molecules were generated using binding affinity scores obtained from the docking. Among this generated set of molecules, L-78 and L-87 molecules, showing higher affinity compared to the reference compound Azathioprine, stood out due to their positive interactions and increased hydrophobic complementarity at the hBCATm active site. The stability of the aforementioned ligands in the hBCATm active site and the fundamental interactions of the complex formed over 300 ns were characterized by MD simulations. Finally, MMGBSA binding free energy calculations were performed, supporting the finding that molecules L-78 and L-87 have better binding potential compared to Azathioprine. In conclusion, this study identified two novel molecules with high predicted affinity for hBCATm, potentially relevant to ALF management using *in silico* methods. These computational findings warrant further *in vitro* and *in vivo* validation to confirm the therapeutic potential of the proposed leads.

Keywords: Acute Liver Failure; Aminotransferase; Molecular Docking; Molecular Dynamics Simulation; Drug Design; BCATm Inhibitors.

Introduction

Acute liver failure (ALF) is a rare but life-threatening clinical condition characterized by rapid deterioration of liver function and high mortality, particularly among young adults (Trey & Davidson, 1970). In ALF, hepatic drug-metabolizing capacity is markedly reduced, and severe impairment of systemic defense mechanisms may lead to hepatic encephalopathy, coagulopathy, and multi-organ failure (Jalan & Lee, 2002). ALF is defined in the literature with two main terms: The Asia-Pacific Union defines it as acute liver injury with hyperbilirubinemia developing over a 4-week period, regardless of the individual's prior medical history (Sarin et al., 2009), while the EASL-AASLD consensus defines it primarily as an increase in mortality rates resulting from liver failure caused by the underlying condition (Olson et al., 2011). Early diagnosis is critical in ALF patients; while treatment rates are quite good in patients diagnosed in the early stages, after the onset of cirrhosis, treatment outcomes result in an 80% mortality rate. The mortality resulting from the disruption of metabolic homeostasis is related to serious respiratory, circulatory, and brain dysfunction. Metabolic dysfunction in this process is defined as hypoglycemia and insulin-resistant non-ketotic hyperglycemia induced by high parenteral glucose administration in approximately 25% of cases (Ranek et al., 1976). Despite advancements in treatment protocols, drug therapy for ALF remains quite limited, as mentioned above, and organ transplantation is recommended as the only definitive treatment (Germani et al., 2012; Goldaracena et al., 2015; Krawczyk et al. 2012; Nyckowski et al., 2006). However, the limited number of donors (Matsunami et al., 1992; Mehtohra et al., 2018) and the unfavorable timeframe (Lee et al., 2012) clearly indicate that developing new drug molecules for ALF, at least those more effective in slowing down the metabolic dysfunction process, is crucial for improving survival rates and overall survival.

In this study, branched-chain aminotransferase (BCAT), which plays a significant role in the metabolic regulation of the liver, was chosen as the target to contribute to the improvement of ALF in therapeutic processes. Studies have shown that the human mitochondrial BCAT isoform (hBCATm) is effective in ligand positioning and catalysis through strong hydrogen bond interactions mediated by the CXXC motif (Hutson, 2001; Davoodi et al. 1998), and consequently, it has been determined to have a critical importance in enzymatic function and redox-dependent regulation (Yennawar et al. 2002). Furthermore, it has been stated that structural alterations in the CXXC motif also change substrate deprotonation and pyridoxamine-5'-phosphate orientation, affecting the catalytic reaction (Hall & Hutson, 1993). Human mitochondrial branched-chain aminotransferase (hBCATm, also known as

BCAT2) has been identified as a potential molecular target due to the importance of the BCAA pathway in modulating metabolic dysregulation via ALF and slowing the progression of liver damage (She et al., 2007). The strategic value of targeting hBCATm in ALF is rooted in its role in managing ammonia levels and glutamate balance (Holeček, 2018). This metabolic pathway is a key contributor to the neurological complications of ALF, including cerebral oedema and hepatic encephalopathy (Scott et al., 2013). During the acute phase of liver failure, the resulting hyperammonemia prompts hBCATm to drive the transfer of nitrogen from BCAAs to α -ketoglutarate, which subsequently triggers an abnormal accumulation of glutamate (Holeček, 2018; Scott et al., 2013). Therefore, by limiting the nitrogen flux, it is possible to restore systemic equilibrium and mitigate the severe complications of liver failure. It may also be possible to slow down the metabolic stress through activity modulation predicted via the binding affinity of ligand-hBCATm complex. ALF is now recognized as a metabolically driven, ferroptosis-prone syndrome in which amino acid and iron metabolism play a central role in hepatocyte death and pharmacological ferroptosis inhibitors significantly improve the disease in preclinical models (Liue et al., 2021; Lemmer et al., 2023; He et al., 2025). Mitochondrial BCAT has been successfully pharmacologically targeted to redirect BCAA efflux in human hepatocytes and mouse models, demonstrating that this enzyme is a drug-targetable gatekeeper of amino acid catabolism in liver tissue (Hemmingsen et al., 2025). When examined from the perspective of *in silico* studies, the crystal structure of hBCATm, coded PDB ID: 2HHF, is suitable for investigating ligand-enzyme interactions through docking studies. It may be possible to slow down the metabolic process in the ALF cycle and thus reduce metabolic stress through activity modulation predicted via the binding affinity of the ligand-hBCATm complex. Based on this idea, a holistic computational methodology was applied by designing novel molecules targeting hBCATm, subjecting them to drug potential pre-tests, calculating their binding affinities through docking studies, performing structure-activity relationship (SAR) analysis, molecular dynamics (MD) simulations for prominent molecules, and MMGBSA binding free energy calculations. As a starting point, a structure coded ZINC000013555421 from the ZINC database was selected, and numerous derivatized candidate molecular structures were generated through an iterative cycle based on the binding affinity (BE) obtained. The molecule was selected because it has been identified in the literature as a compound with high affinity for estrogen receptors (ER- α and ER- β). The liver is one of the main organs of steroid hormone metabolism and is directly responsible for the biotransformation of estrogens. When liver dysfunction develops, estrogen metabolism decreases significantly, resulting in increased levels of circulating active estrogen.

Gynecomastia, vascular changes, and hormonal imbalances observed clinically in liver failure are a consequence of this mechanism. Therefore, estrogen receptors play an indirect but critical role in the pathophysiology of liver failure. In this context, characterizing the binding profile of the selected ligand to estrogen receptors at the molecular level in this study can contribute to understanding the hormonal signaling pathways associated with liver failure. Furthermore, some studies show that estrogen receptor subtypes may have regulatory effects on hepatic fibrosis, inflammation, and hepatocyte regeneration. Therefore, the inclusion of this ligand, which exhibits high affinity for estrogen receptors, in the docking analysis is scientifically justifiable for modeling potential molecular interactions associated with liver failure. On the other hand, the clinically relevant Azathioprine (Azat) compound was chosen as the reference molecule, allowing for a comparative evaluation of the calculated binding affinities. Selection of Azat as a reference molecule stems from its dual relevance: its clinical application in liver diseases and its documented impact on nitrogen-related metabolic pathways (Maltzman & Koretzky, 2003; Hoofnagle, 2026; DrugBank, 2026; Sterneck et al., 1991; Björnsson et al., 2017). Since Azathioprine is known to indirectly modulate the nitrogen balance and interfere with specific hepatic enzymatic pathways (Maltzman & Koretzky, 2003; Hoofnagle, 2026), it provides a rigorous standard for assessing the binding affinity and inhibitory potential of our hBCATm-targeted derivatives. This approach ensures a clear validation of the candidate molecules' stronger affinity for specific mitochondrial targets over standard clinically relevant compounds.

Experimental

Generation of Candidate Molecules Through Derivatization

This study aimed to design a more effective inhibitor of the aminotransferase enzyme than Azat (Figure 1.a) for ALF. In this context, as mentioned above, the molecule coded ZINC000013555421 (Figure 1.c), which exhibits high affinity for ER- α and ER- β , was determined as the starting (L0) structure. In this study, the selection of the structure coded ZINC000013555421 (L0) from the Zinc database as the starting skeleton for derivatization was made in the context of both disease context and physicochemical evaluations. This is due to the fact that estrogen receptors (ERs) have an important role in liver physiology and pathogenesis of liver diseases and show high affinity (Kasarinaite et al., 2023; Xu et al., 2022; Della Torre, 2020). ER ligands are structurally lipophilic and aromatic, which is advantageous for the hBCATm active site to interact with hydrophobic sub-pockets, and structure-activity studies

have shown that increased hydrophobicity and molecular volume increase binding to the BCATm active site (Khan et al., 2022; Toyokawa et al., 2021; Arao & Korach, 2011). Therefore, the aim was not to model a known BCATm ligand, but rather to provide a liver-related, drug-like scaffold whose physicochemical profile could be systematically optimized for hBCATm binding, by using an ER-affinity scaffold, with the selection of L0 due to both suitability in the disease context (liver-targeting pharmacophores) and structural compatibility with the hydrophobic pocket of BCATm. The resulting outcome is consistent with the increased complementarity of the derivative structures to this hydrophobic pocket.) A rational design was achieved in a sequential cycle by attaching different substituted groups to the positions indicated in Figure 1.c of the L0 molecule. During the derivatization process, for each ligand created, a web-based screening tool, SwissAdme (Daina et al., 2017), was used to evaluate compatibility with Lipinski's 5 rules (Lipinski et al., 2001) and drug potential criteria. The processes of docking and interaction mapping, along with the improvement of binding affinities, were carried out iteratively. Interaction maps have been particularly helpful in this process.

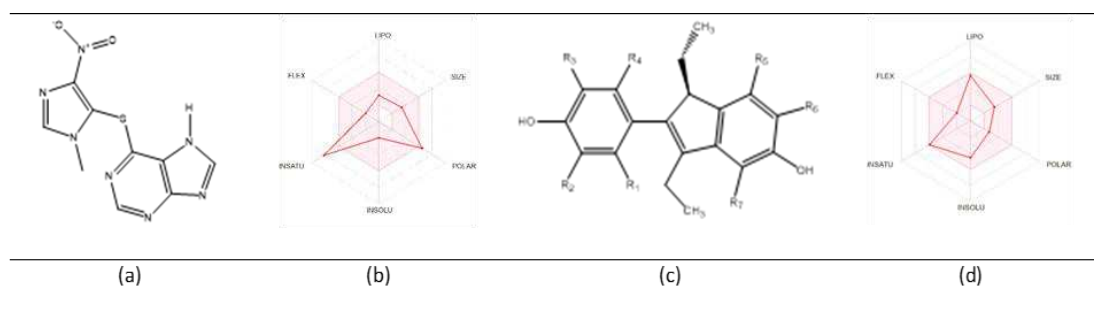


Fig. 1. Chemical structures and drug-likeness profiles of the reference compounds. (a) 2D structure of Azat, the clinical reference drug. (b) SwissAdme bioavailability radar chart for azathioprine, showing its drug-likeness profile. (c) 2D structure of the database hit ZINC000013555421 (L0), with R-group positions (R2, R3, R5, R6, R7) indicated where modifications were made for derivatization. (d) SwissAdme bioavailability radar chart for ZINC000013555421.

In the derivatization process of new candidate molecules, the basic skeletal structure of the L0 molecule was preserved, and substituent groups such as halogens, hydroxyl, phenyl, and fluorine were cyclically attached to the R2, R3, R5, R6, and R7 positions, creating 98 modified molecules that met the requirements. Groups attached to the R1 and R4 positions were not

included in the list because they yielded negative results in terms of affinity. The modification results were evaluated according to criteria such as binding affinities, SAR analyses, and interaction maps, and the most promising molecules were determined. The 30 molecules with the best binding affinities are listed in Table 1 below.

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Table 1. Functional groups and binding positions attached to the ZINC000013555421(L0) molecule

Ligand	R2	R3	R5	R6	R7
L-0					
L-6	PH ₂			OH	CF ₂ OCCHCH ₂
L-27				FCHOCHOHCH ₃	CH ₂ OH
L-32				CHSHOCOCHCHF	
L-38				CF ₂ OCOCHCH ₂	
L-40	Br		CF ₂ OCOCHCH ₂		
L-45		Cl	CF ₂ OCHCO		
L-50			CF ₂ OCOCHCH ₂		PH ₂
L-51				CF ₂ OCOCH ₂	
L-56		Cl	CF ₂ CHO	CHCH ₂	
L-62	F	PH ₂	CF ₂ OCOCHCH ₂		
L-67	F			CF ₂ OCOCHCHPH ₂	
L-69			Cl	FCHOCHOCHCHOH	
L-71	F	PH ₂	FCHOCHOCHCH ₂		
L-72			Br	FCHOCHOCHCHOH	
L-73	Br			FCHOCHOCHCHOH	
L-75			CH ₂ F	FCHOCHOCHCHSH	
L-77			FCHOH	FCHOCHOCHCHSH	
L-78			FCOHCl	FCHOCHOCHCHSH	
L-79	FCHOH			FCHOCHOCHCHSH	
L-80		FCHOH		FCHOCHOCHCHSH	
L-82		PH ₂	FCHOCHOCHCH ₂		F
L-85		PH ₂	FCHOCHOCHCH ₂		Cl
L-87			FCOHCl	FCHOCHOCHCHPH ₂	
L-88			FCHF	FCHOCHOCHCHOH	
L-89			FCHF	FCHOCHOCHCH ₂	
L-93			FCHOCHOCHCH ₂	FCHF	
L-94		FCHF	FCHOCHOCHCH ₂		
L-96		FCHF	FCHOCHOCHCHPH ₂		
L-97		FCHCl	FCHOCHOCHCHPH ₂		
L-98			FCHOCHOCHCH ₂		FCHCl

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190 Docking Calculations

Docking studies were performed between the derivative forms of the molecules coded
 pdb id: 2HHF and ZINC000013555421, which were selected as macrostructures representing
 branched-chain amino acid (BCAA) metabolism in liver mitochondria with a resolution of 1.80
 Å. SwissAdme images of Azat, selected as the reference molecule, and its L0 ligand are given
 in Figures 1.b and 1.d. All docking operations were performed using AutoDock Vina (Trott &
 Olson, 2010), and the obtained interaction maps were visualized using BIOVIA Discovery
 Studio Visualizer (Dassault Systèmes BIOVIA, 2016). In the docking studies, the active region
 grid box coordinates were determined as 40x40x40 Å³ and the center coordinates were
 determined as x=16.000773, y=-10.087533, z=19.142467, referencing the BCATm catalytic
 region defined in the literature¹⁵. At the beginning of the docking studies, the RMSD was
 measured to be < 2.0 Å in the docking process performed with the crystal structure's own ligand,
 and the same criterion was observed for all subsequent processes performed with 3 repetitions
 for the derivatized candidate ligand molecules (Trott & Olson, 2010). A standard protocol was
 followed when preparing the protein for the docking processes: first, water molecules were
 removed, then hydrogen atoms and then Kollmann charge were added. The process was
 completed with 3 replicates. The 98 candidate structures, for which derivatization processes
 were performed, were first screened for compliance with Lipinski rules via SwissAdme, then
 conformer screening and geometry optimizations were carried out with 60° of rotational
 freedom in single bonds using the semi-empirical PM6 method (Stewart, 2008; Stewart, 2009)
 and the Spartan'16 program (Wavefunction Inc., 2016). The physicochemical parameters of the
 optimized candidate molecules, such as molecular weight (M_w), volume (V), surface area (Å²),
 lipophilicity (logP), dipole moment (μ), lowest unoccupied molecular orbital energy (E_{LUMO}),
 highest occupied molecular orbital energy (E_{HOMO}), electrophilic index (ω), and
 electronegativity (χ), were calculated, and the correlations between the resulting binding
 affinities and the molecules were examined.

Molecular Dynamics Simulations

MD simulations were performed to investigate the stability and dynamic movements of
 complexes formed by proteins with molecules that are prominent in molecular docking studies.
 MD studies were conducted for complexes formed between the apo hBCAT enzyme with the
 pdb id: 2HHF structure and the L-78 and L-87 derivative molecules, which have the most stable
 affinity with the reference ligand Azat. MD simulations were carried out using the OPLS4 force
 field and the Schrödinger Maestro program Desmond module (v. 2022.1) (Bowers et al., 2006).

System Preparation: Using the Protein Preparation Wizard, the B lobe, water molecules, and ligand molecule of the hBCATm structure were removed, leaving only the A lobe in the system. hBCATm enzyme exists as a functional homodimer (Hindy et al., 2014; Islam et al., 2007). So that, isolating the A lobe enhanced the processing speed while preserving the catalytic site's structural integrity during MD simulations. Since each monomer functions independently at the molecular level (Islam et al., 2007), this monomeric model provides a valid representation of the ligand's behavior within the binding pocket. Active site definition was performed using the Receptor Grid Generation tool. Possible ionization states, tautomers, and stereoisomers for Azat, L-78, and L-87 molecules were prepared using the LigPrep tool with the Epic Classic protocol at a target pH of 7.0 ± 2.0 . MD simulations were initiated with the most stable conformation obtained via Ligand Docking for the three different ligands studied.

Simulation Setup: Each protein-ligand complex selected with the System Builder tool was dissolved along with the apo protein using the System Builder tool. The prepared systems for each complex were placed in an orthorhombic box with an SPC water model and a 10 Å buffer spacing on all axes. The system charge was neutralized by adding Na^+ ions, followed by the addition of 0.15 M NaCl, an ionic medium modeling the physiological environment. Box volume was minimized for process efficiency.

Simulation Protocol: Energy minimization and system equilibration were performed using the default relaxation protocol in Desmond under NPT ensemble conditions (300 K, 1.01325 bar). Production MD simulations were then carried out for 300 ns per system. The integration time step was 2 fs, and trajectory frames were recorded every 300 ps, resulting in approximately 1000 square per simulation for analysis.

Trajectory Analysis: The stability of the simulations was evaluated by monitoring the root mean square deviation (RMSD) of the protein backbone ($\text{C}\alpha$ atoms) and the ligand heavy atoms (after fitting to the protein backbone). A simulation was considered equilibrated once RMSD values stabilized without directional drift. Local flexibility was assessed by calculating the root mean square fluctuation (RMSF) per residue ($\text{C}\alpha$ atoms). Furthermore, protein-ligand interaction profiles and secondary structure stability over the simulation time were analysed to gain insights into the binding dynamics and complex stability.

Binding Free Energy Calculations with MMGBSA

To obtain a more accurate and thermodynamically rigorous estimation of the binding affinity for the top-ranked candidates, the Molecular Mechanics/Generalized Born Surface Area (MMGBSA) method (Genheden & Ryde, 2015) was employed. This approach integrates

molecular mechanical energies in the gas phase with continuum solvation models, providing a physically grounded estimation of the binding free energy (ΔG_{bind}) that often correlates better with experimental data than docking scores alone.

Trajectory Sampling and Energy Decomposition: MMGBSA calculations were performed on equilibrated portions of MD trajectories of 300 ns. For the calculated apo hBCATm, Azat, L-78, and L-87 complexes, 500 uniform snapshots were taken at 200 ps intervals from the last 100 ns of the simulation period. These specified time intervals were chosen based on RMSD stability analyses to ensure that all specified systems reached conformational equilibrium. The binding free energy was calculated using the standard MMGBSA equation (Genheden & Ryde, 2015; Kollman et al., 2000).

$$\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} - T\Delta S$$

where:

ΔE_{MM} represents the change in molecular mechanical energy in the gas phase, comprising bonded (bond, angle, torsion) and non-bonded (electrostatic and van der Waals) terms:

$$\Delta E_{\text{MM}} = \Delta E_{\text{bond}} + \Delta E_{\text{angle}} + \Delta E_{\text{torsion}} + \Delta E_{\text{elec}} + \Delta E_{\text{vdW}}$$

ΔG_{solv} denotes the change in solvation free energy upon binding, which is further decomposed into polar (ΔG_{GB}) and non-polar (ΔG_{SASA}) contributions:

$$\Delta G_{\text{solv}} = \Delta G_{\text{GB}} + \Delta G_{\text{SASA}}$$

$T\Delta S$ is the entropic contribution, which arises from changes in translational, rotational, and conformational degrees of freedom.

Computational Details and Assumptions: All MMGBSA calculations were conducted using the Prime MMGBSA module integrated within the Schrödinger Maestro Suite 2022-1. The OPLS4 force field was used for both protein and ligand parameters. Solvent effects were modeled with the VSGB 2.0 solvation model (Li et al., 2011), which employs a variable-dielectric generalized Born approach for the polar component. The following parameters were applied: an internal dielectric constant (ϵ_{in}) of 4.0 for the protein and ligand, and an external dielectric constant (ϵ_{out}) of 80.0 for the implicit aqueous solvent. The non-polar solvation term (ΔG_{SASA}) was estimated using a linear relation to the solvent-accessible surface area (SASA) with a surface tension coefficient $\gamma = 0.0072 \text{ Kcal/mol/\AA}$.

Entropic contribution $T\Delta S$ was not included in MMGBSA studies due to the computationally expensive and often noisy nature of entropy calculations of ΔG_{bind} values, a common practice in MMGBSA studies. This exclusion is specifically justified in this study because the identified lead candidates (L-78 and L-87) and the reference molecules share a consistent chemical scaffold and interact with the same rigid binding site of hBCATm.

Furthermore, relative differences in conformational entropy performed for similar ligand series bound to the same hard pocket, similar to this study, are expected to be small and systematic, ensuring that comparisons of ΔG_{bind} values remain meaningful for ranking purposes. This approach is consistent with numerous previous studies using MMGBSA for inhibitor screening and validation.

Data Analysis and Statistics: For each ligand-protein complex, the 500 calculated ΔG_{bind} values were averaged to yield the mean binding free energy and its standard deviation. The individual energy components ΔE_{vdW} , ΔE_{elec} , ΔG_{GB} , ΔG_{SASA} were similarly averaged to provide mechanistic insight into the driving forces of binding.

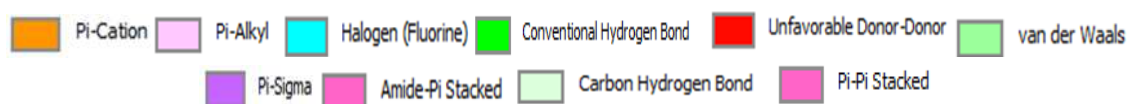
The statistical significance of differences in MMGBSA ΔG_{bind} values between ligand-protein complexes was assessed using the Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction for multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results and discussion

Comparative Analysis of Binding Affinities and Significance

In docking studies of structures obtained from the derivatization of the ZINC000013555421 coded structure, the structures giving the best binding affinity were found to be L-78 (-9.6 Kcal/mol) and L-87 (-9.5 Kcal/mol) when compared with molecules L0 (-7.8 Kcal/mol) and Azat (-7.6 Kcal/mol). This difference in affinity indicates a theoretically enhanced binding potential compared to the reference molecule. In the context of drug design, such a gain in binding energy suggests that the derivatized candidates may offer higher affinity towards the hBCATm active site (Kuntz et al., 1999) According to these results, the success of the derivatization process highlights the importance of targeting the hydrophobic sub-pockets of the BCATm active site, a feature noticed in previous inhibitor designs for aminotransferases but not fully utilized (Wilcken et al., 2013). Table 2 presents the interacting amino acids, interaction types, and binding distances obtained from the ligand-hBCATm macrocomplex during the docking process. It was determined that the L0 skeleton interacts with the peripheral residues Lys79 and Gly177, and that these interactions do not adequately cover the deep regions of the binding pocket, resulting in low binding affinity. The prominent molecules L-78 and L-87 showed improved binding interactions in the active site pocket through hydrogen bonds, hydrophobic and electrostatic contacts. It was interpreted that the hydrogen bonds between the candidate molecules and Gly204 play an important role in active site retention and stable

Ligand	Leu59	Ser71	Gln73	Phe75	Lys79	Trp94	Tyr141	Arg143	Val145	Phe164	Ala172	Phe174	Gly177	Gly204	Tyr207	Gly208	Thr210	Val225	Thr240	Ala314	Gln346	Lys350	Glu360	Trp361
Azat										5.87	5.74										3.98	5.38		
L-0					3.64								3.70											
L-6			6.05	5.23				8.12						3.70	4.66					5.03				
L-27				7.46													4.06	5.11	5.52					
L-32	4.60		3.10							6.26														
L-38	5.68							4.42	6.23	6.97				5.86										
L-40			5.99					8.11						3.73	4.66					5.09				
L-45			6.08	5.31				8.08						3.44	4.77	3.53				5.06	5.23			
L-50			5.83				7.34	7.93						3.69	4.87									
L-51				5.94											4.15									
L-56				5.51				8.13						3.32	4.65					4.95	5.09			
L-62			5.86				7.38	8.08						3.52	4.76					5.29				
L-67			5.92	6.11				6.55	6.82						5.73									
L-69		3.59		5.34			7.70	8.13							4.76					5.01				
L-71			5.81	5.17			7.29	8.01						3.36	4.77					5.42				
L-72		3.60	5.33					8.18							4.75					4.98				
L-73			5.97	4.79				6.77	6.83						4.58		4.30							
L-75				5.26				8.16							4.76					4.96				
L-77				5.28				8.17						3.67	4.73					4.94				
L-78			5.88	5.56				8.12						3.72	4.76					5.28				
L-79			5.92	6.17				6.73	6.82						5.60									
L-80			5.95	4.66				6.60	6.86						5.78		4.75							
L-82				5.41				8.13						3.73	5.72					4.98	5.08			
L-85				5.39				8.07						3.72	4.61					4.96	4.98			
L-87			5.41					8.17							4.75					4.98				
L-88						6.65																4.91	4.74	
L-89						6.60																4.15	4.62	
L-93				6.36				8.23												5.34				
L-94			5.04	5.73				8.28							4.82					5.27				
L-96				5.09			5.																	



334

335 On the other hand, in docking studies for the L-78 and L-87 constructs identified as
 336 potential candidates, a stronger interaction was detected between the active site and Gly204 in
 337 particular, including hydrogen bonds, hydrophobic contacts, electrostatic interactions, and π -
 338 based interactions. This interaction is consistent with the findings of Yennawar et al., 2002
 339 which highlight the functional importance of the Gly204 containing loop in substrate
 340 orientation and catalysis, where the stable hydrogen bond observed suggests that L-78 and L-
 341 87 can potentially occupy similar spatial regions as the substrate, possibly interacting with key
 342 residues involved in the catalytic cycle and thus enhance competitive inhibition. Another
 343 important data extracted from Table 2 is that the hydrophobic interactions observed with the
 344 amino acids Phe75 and Tyr207 support the retention of ligands in the aromatic and non-polar
 345 regions of the binding site, and also contribute to the stability of the complexes during MD
 346 simulation. Furthermore, it is thought that the π - π and π -alkyl interactions between the
 347 aromatic ring in the structure of Tyr207 and the π -systems of the ligands also contribute to the
 348 binding energy. Furthermore, electrostatic and polar interactions identified with Arg143 and
 349 Gln73 were similarly found to be effective. The positively charged side chain of Arg143 was
 350 found to support the orientation and stability of molecules through interactions such as
 351 hydrogen bonds and salt bridges with the polar functional groups of ligands, while Gln73 acted
 352 as both a hydrogen bond donor and acceptor. Polar contacts with Thr240 were identified as
 353 secondary interactions supporting ligand positioning in the inner region of the active pocket.

354 A general conclusion drawn from Table 2 reveals a clear structure-activity relationship
 355 (SAR) in the interaction analysis. While the L0 base structure forms restricted and weak
 356 contacts, the high-affinity derivative structures L-78 and L-87 formed more effective
 357 interactions with Gln73, Phe75, Arg143, Gly204, Tyr207, and Thr240 residues, explaining
 358 consistent binding stability and affinity increase with placement scores and MD simulations.
 359 These findings suggest that L-78 and L-87 may be more potent and potentially more effective
 360 hBCATm inhibitor candidates compared to the main scaffold L0.

361

362 **Structure-Activity Relationship (SAR) and Interaction Profile**

363 Table 3 presents the SAR data, including the calculated physicochemical parameters
 364 and binding affinities for all molecules studied. Molecules exhibiting the best binding affinity

were found to have high logP and V values, indicating good interaction with the hydrophobic active pocket of the macrostructure. Modifications, particularly at positions R5 and R6 of L0, were observed to shift towards a strong affinity with this region through van der Waals contacts and π - π /alkyl interactions. This finding is reflected in the LogP-BA correlation with $R^2 = 0.8648$.

On the other hand, halogens added as substituted groups modulate stability in metabolic systems, particularly fluorine, through lipophilicity regulation via "halogen binding" (Wilcken et al., 2013). The added halogen groups facilitate supportive interactions with the carbonyl oxygen and aromatic side chains in the protein main chain, thus affecting affinity and selectivity. This situation is also supported in the L-78: FCOHCl; L-87: FCOHCl structures, which are among the most stable molecules.

Table 3. Calculated physicochemical parameters for the ZINC000013555421 ligand and its derivatives (A, V, M_w , logP, μ , χ , ω , E_{LUMO} , E_{HOMO} and BE(Kcal/mol))

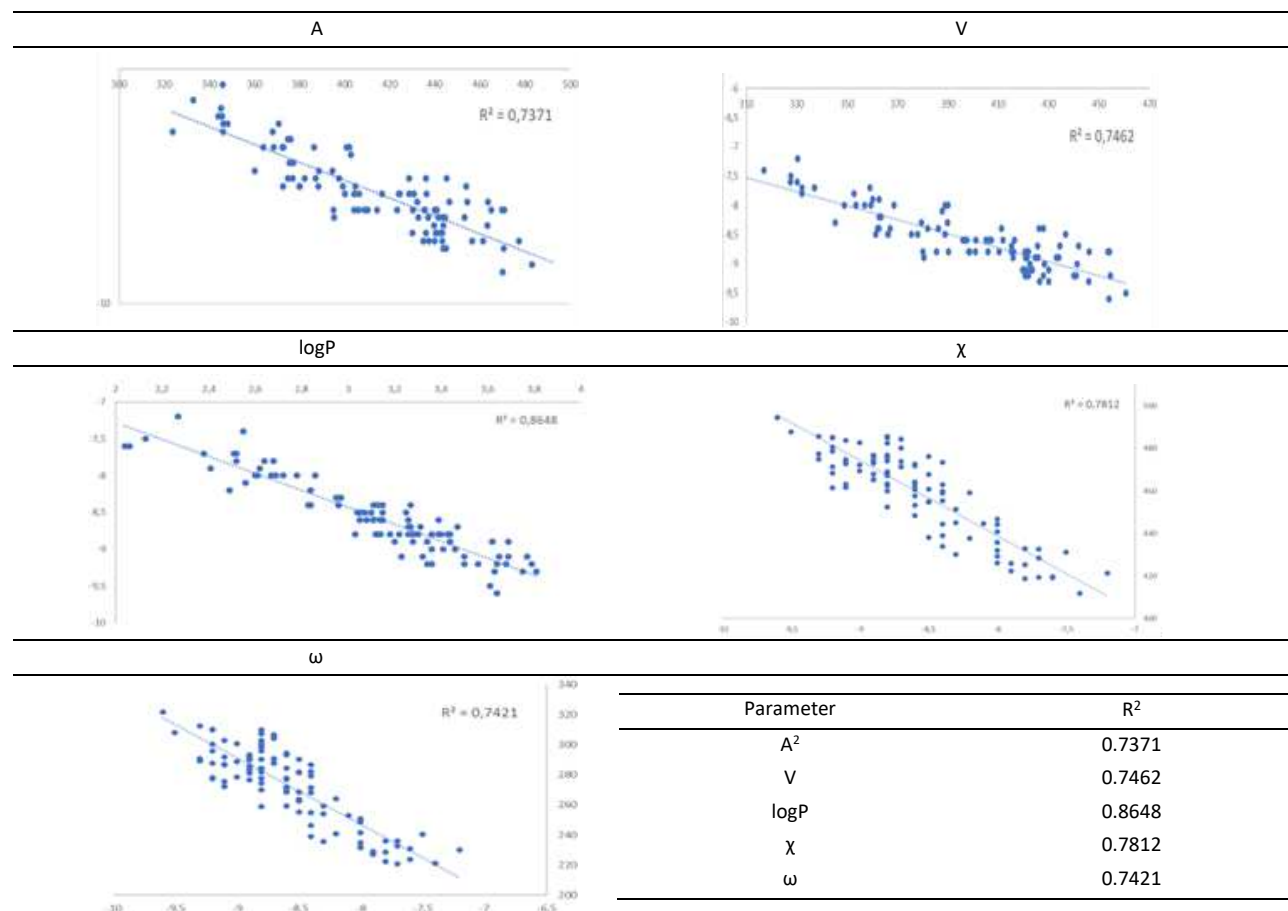
	A	V	M_w	LogP	χ	ω	E_{LUMO}	E_{HOMO}	μ	BE	K_i
Azat.	257.70	234.64	277.26	0.97	561.29	398.13	-165.63	-956.95	5.22	-7.6	2.68×10^{-6}
L-0	323.75	308.33	280.36	2.64	425.22	228.32	-29.29	-821.18	1.43	-7.8	1.91×10^{-6}
L-6	443.36	427.95	448.40	3.64	480.52	300.19	-95.93	-865.11	5.49	-9.2	1.80×10^{-7}
L-27	430.31	410.22	402.46	3.28	452.41	258.71	-56.85	-847.97	4.85	-8.8	3.54×10^{-7}
L-32	430.63	411.88	414.49	3.15	463.99	284.26	-85.32	-842.66	2.67	-8.6	4.96×10^{-7}
L-38	414.35	398.71	400.42	3.18	468.26	281.69	-79.07	-857.45	3.34	-8.8	3.54×10^{-7}
L-40	436.00	420.81	479.31	3.69	474.78	286.74	-81.71	-867.85	3.50	-9.1	2.13×10^{-7}
L-45	395.41	380.37	408.82	3.20	474.18	291.34	-88.30	-860.05	3.18	-8.9	2.99×10^{-7}
L-50	437.06	420.93	432.40	3.28	467.92	280.70	-77.92	-857.91	5.61	-8.9	2.99×10^{-7}
L-51	406.20	385.48	388.40	3.03	462.46	274.33	-72.66	-852.25	4.80	-8.8	3.54×10^{-7}
L-56	410.16	400.97	418.86	3.30	469.14	283.45	-80.91	-857.36	1.74	-8.8	3.54×10^{-7}
L-62	444.92	426.47	450.39	3.75	477.38	290.54	-85.19	-869.57	2.78	-9.3	1.52×10^{-7}
L-67	444.43	425.73	450.39	3.69	476.08	292.46	-88.59	-863.57	3.27	-8.9	2.99×10^{-7}
L-69	436.91	415.86	432.87	3.44	474.07	289.78	-86.29	-861.84	1.17	-8.9	2.99×10^{-7}
L-71	439.77	422.01	432.40	3.79	468.47	280.00	-73.75	-863.19	1.13	-9.2	1.80×10^{-7}
L-72	440.35	420.34	477.32	3.65	463.27	275.17	-73.30	-853.23	1.21	-9.1	2.13×10^{-7}
L-73	443.28	423.02	477.32	3.77	461.63	271.97	-69.86	-853.40	5.22	-9.1	2.13×10^{-7}
L-75	453.04	434.06	446.51	3.62	465.37	283.19	-83.00	-847.74	4.53	-8.9	2.99×10^{-7}
L-77	461.30	440.95	460.53	3.68	478.65	295.54	-91.04	-866.26	3.07	-9.2	1.80×10^{-7}
L-78	470.01	453.88	496.95	3.64	494.57	321.57	-114.25	-874.89	4.79	-9.6	9.17×10^{-8}
L-79	456.55	439.98	462.51	3.36	485.16	309.56	-104.98	-865.34	1.89	-9.2	1.80×10^{-7}
L-80	463.22	441.39	462.51	3.36	472.15	288.47	-85.75	-858.55	2.84	-9.0	2.53×10^{-7}
L-82	437.11	421.40	432.40	3.56	471.24	287.66	-85.23	-857.32	4.01	-9.2	1.80×10^{-7}
L-85	443.71	430.03	448.85	3.81	474.86	289.00	-84.73	-864.99	4.04	-9.3	1.52×10^{-7}
L-87	482.92	460.61	496.87	3.61	487.70	307.64	-101.13	-874.27	2.93	-9.5	1.09×10^{-7}
L-88	443.64	428.37	448.43	3.41	482.68	300.57	-95.12	-870.23	3.25	-9.0	2.53×10^{-7}
L-89	432.76	421.36	432.43	3.62	473.03	285.61	-81.31	-864.74	3.29	-8.9	2.99×10^{-7}
L-93	430.08	419.75	432.43	3.50	472.80	286.47	-82.64	-862.96	3.01	-9.1	2.13×10^{-7}
L-94	439.66	422.49	432.43	3.46	469.40	278.33	-73.58	-865.21	2.11	-9.0	2.53×10^{-7}
L-96	470.56	445.86	464.41	3.63	485.67	312.28	-108.00	-863.34	4.08	-9.3	1.52×10^{-7}
L-97	477.12	454.43	454.83	3.50	484.95	310.01	-105.65	-864.25	4.82	-9.2	1.80×10^{-7}

L-98	442.37	429.98	448.89	3.32	483.88	302.75	-97.20	-870.55	1.43	-9.1	2.13x10 ⁻⁷
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379

380 The correlations of the physicochemical parameters calculated for the 98 derivative
 381 molecules with their binding affinities, R^2 values, are given in Table 4. It can be seen that the
 382 best correlation against BA was obtained for LogP with $R^2=0.8648$, and the other calculated
 383 correlations also made a significant positive contribution.

384 **Table 4.** Correlation graphs for the binding affinities of 2HHF and ZINC000013555421 ligand
 385 and its derivatives versus A, V, logP, χ , ω and calculated R^2 values



386

387 Validation by Molecular Dynamics: Beyond Static Docking

388 To verify the stability of the complex structure formed by the most stable molecules
 389 obtained from the docking processes with the protein, MD simulations were performed for 300
 390 ns for the L-78 and L-87 complexes using the apo hBCATm enzyme. Looking at the protein
 391 skeleton RMSD value, it is observed that a stable complex is reached after approximately 2Å
 392 of oscillation after reaching equilibrium. When comparing the heavy atom RMSD values of the
 393 derivatized L-78 and L-87 ligands with the RMSD values of the Azat molecule studied as the
 394 reference structure, it is thought that the derivative structures are more stable, which is related
 395 to the stability in the binding region (Figure 2). RMSF profiles showing reduced fluctuations

in active site residues (Gln73, Phe75, Arg143, Gly204, Tyr207) in ligand-bound states compared to apo, indicating pocket stabilization. Theoretically, molecular docking generates data on the instantaneous interaction between the ligand and the protein, while MD simulations demonstrate the stability of the complex formed over a selected time interval.

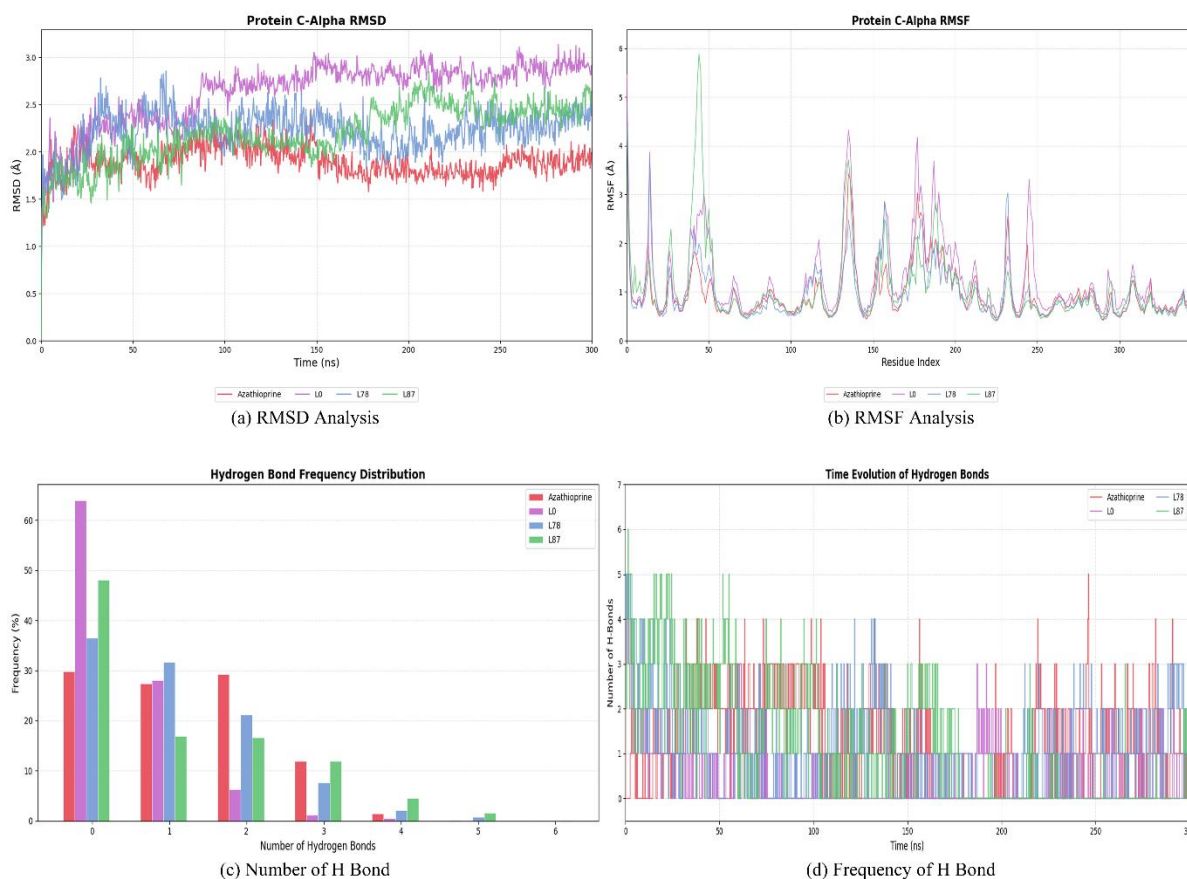


Fig. 2. Molecular dynamics simulation results from 300 ns trajectories for apo hBCATm and its complexes with Azathioprine, L-78 and L-87. (a) Protein RMSD and ligand RMSD (Lig fit Prot), (b) RMSF profiles of C α atoms, (c) Number of H bonds, (d) Frequency of H bond.

In this study, the stability of the L-78 and L-87 candidate molecules, selected from among the modified molecules based on the docking results in 300 ns simulations, was confirmed. The low and stable RMSD values of both the protein (<3 Å) and the ligands (<2 Å) indicate that the complexes studied are structurally stable, have not undergone significant conformational shift, and the binding position is preserved. More importantly, the RMSF analysis performed on the L-78 and L-87 structures in MD simulations showed a decrease in fluctuations, particularly in the Gln73, Phe75, Arg143, Gly204, and Tyr207 active site residues, compared to the apo state, suggesting ligand-induced active site stabilization. This reduction in

local conformational flexibility [VEA1.1] of the binding pocket is a classic indicator of stable ligand binding and is typically associated with high affinity inhibitors (Goto et al., 2003). These residues, critical for substrate positioning and catalysis involving the CXXC motif, showed lower $C\alpha$ RMSF values in ligand-bound states, suggesting that L-78 and L-87 stiffen the binding pocket and potentially restrict the conformational flexibility required for enzymatic activity. In contrast, the apo protein showed higher flexibility in these regions, indicating greater conformational freedom in the absence of ligands. This ligand-induced stiffening supports the stable binding modes observed in the RMSD trajectories and strengthens the potential of L-78 and L-87 as effective hBCATm modulators. As seen in Figure 2, the protein-ligand interaction analyses confirm the persistence of stable hydrophobic interactions, particularly with Phe75 and Tyr207 residues surrounding the binding pocket, in addition to the hydrogen bonds with Gly204 throughout the simulation. These analyses from MD simulations support the idea that the indicated candidate molecules form more stable complexes with hBCATm than with L0 under physiological conditions. In MD simulations, the conformational stability of the complexes was investigated by calculating the RMSD values of the protein backbone and the heavy atoms of the L-78 and L-87 ligands in the last 100 ns of the simulations (Table 5).

430

Table 5. RMSD statistics (mean $\pm$ SD) for protein backbone and ligand heavy atoms calculated over the last 100 ns of the 300 ns MD trajectories for apo hBCATm and its complexes with Azat, L-78, and L-87

System	Protein Backbone RMSD (Å) Mean $\pm$ SD	Ligand Heavy Atoms RMSD (Å) Mean $\pm$ SD	State
Apo hBCATm	1.82 $\pm$ 0.31	-	Stable
Azat	2.15 $\pm$ 0.42	2.48 $\pm$ 0.61	Stable
L-0	1.92 $\pm$ 0.33	2.35 $\pm$ 0.55	Stable
L-78	1.96 $\pm$ 0.29	1.81 $\pm$ 0.38	Highly Stable
L-87	2.03 $\pm$ 0.33	1.92 $\pm$ 0.41	Highly Stable

434

The calculated RMSD values were 1.81 $\pm$ 0.38 Å for L-78, 1.92 $\pm$ 0.41 Å for L-87, and 2.48 $\pm$ 0.61 Å for Azat. This indicates that our candidate molecules exhibit greater stability in the binding pocket due to having lower ligand RMSD values. The protein backbone RMSD values were found to be around < 2.1 Å for all systems studied, indicating that the overall structure of the protein was not disrupted by complex formation. RMSD and RMSF profiles obtained from 300 ns MD simulations performed for L-78–hBCATm and L-87–hBCATm complexes are presented in Figure 3. Another data point obtained from 300 ns MD simulations

is the rGyr and SASA graphs given in Figure 3. When candidate L-78 and L-87 complexes are compared with Azat and the initial structure L-0, indicating more compact and conformationally stable binding modes with lower rGyr values (Figure 3.a). Looking at the SASA values (Figure 3.b), the L-78 and L-87 complexes exhibit a significantly lower and less fluctuating surface area profile compared to Azat and L-0, indicating significantly reduced solvent exposure, limited access of the ligands to the solvent, and the formation of a stable, solvent-protected binding interface within the active site. When the results are evaluated collectively, it is understood that the complexes formed by L-78 and L-87 with hBCATm are protected in the active site, exhibiting structural stability and dynamic compatibility, and these results are consistent with the binding free energies calculated using MMGBSA.

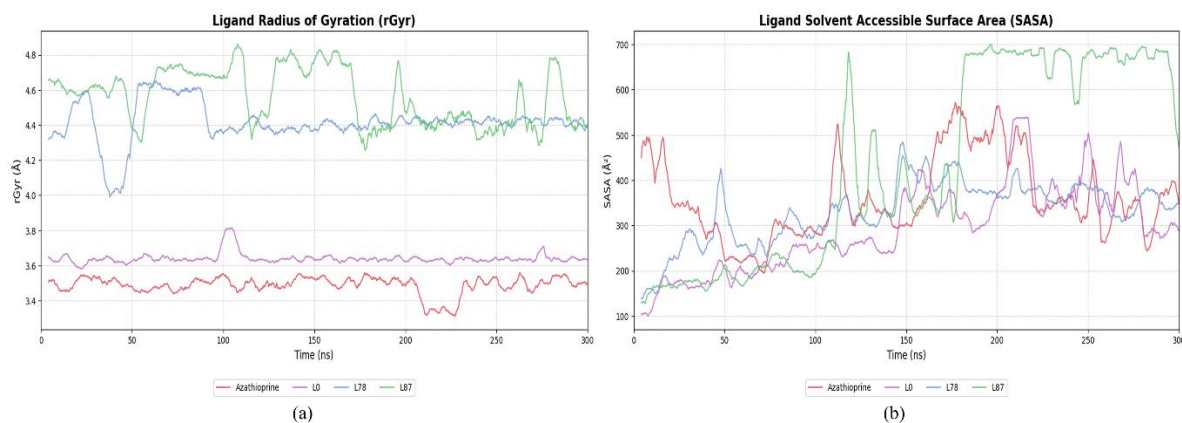


Fig. 3. Molecular dynamics simulation results from 300 ns trajectories for apo hBCATm and its complexes with Azat, L-0 L-78 and L-87, (a) rGyr, (b) SASA.

To validate the docking and MD simulations performed within the scope of the study, MMGBSA calculations were carried out to calculate the binding energies and elucidate the energetic determinants. As can be seen in Table 6, the binding free energies (ΔG_{bind}) for the prominent L-78 and L-87 molecules were calculated as -61.4 ± 5.1 and -59.5 ± 4.9 Kcal/mol, respectively. On the other hand, for the starting structure L-0, ΔG_{bind} was calculated as -44.3 ± 4.1 Kcal/mol, and for the reference compound Azat, ΔG_{bind} was calculated as -41.6 ± 3.8 Kcal/mol. Looking at the obtained results, it was observed that the rankings obtained from both the docking and MD results of the L-78 and L-87 molecules completely coincided with ΔG_{bind} .

Table 6. MMGBSA Binding Free Energies (Kcal/mol)

System	ΔE_{vdW}	ΔE_{elec}	ΔG_{GB}	ΔG_{SASA}	ΔG_{bind}	ΔG_{bind} (Docking)
Azat	-45.2 ± 3.1	-12.8 ± 2.5	20.5 ± 2.1	-4.1 ± 0.3	-41.6 ± 3.8	-7.6
L-0	-48.5 ± 3.5	-10.2 ± 2.2	18.9 ± 1.9	-4.5 ± 0.4	-44.3 ± 4.1	-7.8
L-78	-62.3 ± 4.2	-15.4 ± 2.8	22.1 ± 2.3	-5.8 ± 0.5	-61.4 ± 5.1	-9.6
L-87	-60.8 ± 4.0	-14.9 ± 2.7	21.8 ± 2.2	-5.6 ± 0.5	-59.5 ± 4.9	-9.5

468

469 Analysis of the energies acting on ΔG_{bind} reveals that ΔE_{vdW} contributions are
470 greater in L-78 and L-87 molecules compared to Azat, highlighting the hydrophobic
471 contributions in the hBCATm active site and the correlation between the ligand volume and the
472 active pocket. This result is consistent with V and logP for L-78 and L-87. Regarding
473 electrostatic interactions, ΔE_{elec} : -15.4 Kcal/mol for L-78 and ΔE_{elec} : -14.9 Kcal/mol for
474 L-87 suggest that hydrogen bonds, particularly those involving Gly204, contribute positively
475 to binding via F and Cl substituent groups, but these are partially offset by the polar dissolution
476 term (ΔG_{GB}). On the other hand, the non-polar solubility contribution of the hydrophobic
477 surface area (ΔG_{SASA}) was also slightly supportive for our candidate molecules, contributing
478 to their binding to the enzyme's hydrophobic pocket. Conversely, the polar solubility term
479 (ΔG_{GB}) was found to have a negative contribution for L-78 and L-87, but this energy increase
480 was compensated for by strong van der Waals and electrostatic gains.

481

482 **MMGBSA Binding Free Energy Analysis**

483 Overall, the MMGBSA analysis not only supported the affinity ranking but also
484 contributed to explaining the binding through thermodynamic parameters. The strong affinity
485 of L-78 and L-87 molecules, the dominance of van der Waals and electrostatic terms, was
486 interpreted as a balanced combination of strong hydrophobic interactions and specific,
487 persistent polar contacts, making them potential leading candidates for hBCATm. To better
488 explain the accuracy of the ligand ranking, statistical comparisons were performed on the
489 ΔG_{bind} value obtained from MMGBSA calculations derived from 500 snapshots taken from
490 the last 100 ns equilibrium portion of 300 ns MD simulations. Due to the non-normal
491 distribution of the complexes studied, a non-parametric Kruskal-Wallis test followed by Dunn's
492 post-hoc test (with Bonferroni correction) was performed. The L-78 and L-87 constructs
493 showed significantly more favorable binding free energies than Azathioprine and L-0 ($p <$
494 0.001), while the difference between L-78 and L-87 was not statistically significant ($p = 0.084$).

495 These results, summarized in Table 7, support the improved binding potential of both candidate
 496 ligands.

497

498 **Table 7:** Statistical comparison of MMGBSA ΔG_{bind} values

Comparison	p-value (adjusted)	Significance
L-78 vs. Azat	< 0.001	***
L-87 vs. Azat	< 0.001	***
L-78 vs. L-0	< 0.001	***
L-87 vs. L-0	< 0.001	***
L-0 vs. Azat	0.083	ns
L-78 vs. L-87	0.084	ns
ns: not significant ($p \geq 0.05$) ***: $p < 0.001$		

499

500 According to the results obtained from the study, although L-78 and L-87 exhibit
 501 promising binding affinity and stability towards hBCATm, it is known that many
 502 transaminases, hBCATm and hBCATc share similar cofactor folding pockets and catalytic
 503 mechanisms, but differ in their subcellular localization and regulatory properties. ALT and AST
 504 belong to different aminotransferase families with different substrate specificities and active
 505 site residues. However, the proposed compounds may exhibit off-target inhibition against
 506 related enzymes such as the cytosolic isoform hBCATc, aspartate aminotransferase, or alanine
 507 aminotransferase. The interactions observed with the Gly204 cycle and the CXXC motif do not
 508 definitively confirm their relative specificity to hBCATm in terms of selectivity. Therefore, it
 509 is recommended that studies be conducted with a broad set of PLP-dependent enzymes to
 510 confirm selectivity and assess the risk of potential off-target adverse effects during the
 511 experimental validation phase of the obtained data (Hindry et al., 2014; Conway et al., 2015).
 512 The therapeutic potential of L-78 and L-87 ligands, obtained through successive computational
 513 steps in this study, must first be supported by in vitro and in vivo tests. Future studies should
 514 include experimental validation through enzyme inhibition tests, binding affinity
 515 measurements, and selectivity profiling, and ultimately in vivo efficacy and toxicity studies in
 516 animal models of acute liver failure.

517

518

Conclusions

519 The investigation of BCATm inhibitors for metabolic disorders is a new field. Although
 520 the role of BCATm in branched-chain amino acid (BCAA) metabolism and its dysregulation in
 521 liver disease are well known (Yennawar et al., 2002; She et al., 2007), few potent and selective

small molecule inhibitors have been reported. Several inhibitors for small molecule BCAT have been developed in the literature for oncology, metabolic disorders, and neurological diseases. Gabapentin has been reported to have low selectivity and efficacy, with an inhibition range of approximately 65 mM. BCAT-IN-2, developed by GlaxoSmithKline (GSK), exhibits moderate efficacy with $pIC_{50} = 7.3$ for BCATm and 6.6 for BCATc. BAY-069, which has the best efficacy with $IC_{50} = 31$ nM for BCAT1 and $IC_{50} = 153$ nM for BCAT2, has shown weak cellular activity and has not been applied in liver disease models. Therefore, when looking at existing inhibitors, the developed candidate molecules L-78 and L-87, with binding scores of -9.6 and -9.5 kcal/mol, utilize hydrophobic subpockets of the hBCATm active site via halogen substitutions and aromatic interactions; this is a design strategy not fully utilized in previous BCATm inhibitor developments.

Most research has focused on substrate analogs or mechanism-based inhibitors. Our study contributes by applying a rational, structure-based design approach to a novel chemical type (ZINC000013555421 skeleton) leading to molecules with predicted affinity that rivals or exceeds that of known inhibitors. Compared to Azat, an immunosuppressant with known hepatotoxicity risks, our candidates are designed for a specific metabolic target in liver failure. This represents a shift towards targeted metabolic therapy for ALF, consistent with the increasingly better understanding of ALF as a metabolic breakdown disease (Bémeur, et al., 2010). The compliance of L-78 and L-87 with Lipinski's Five Rules ($M_w < 500$, $\log P < 5$, $HBD < 5$, $HBA < 10$) further demonstrates their potential for oral bioavailability, a desirable characteristic for any therapeutic agent. In this context, the relevant study aimed to identify novel candidate molecules targeting human mitochondrial branched-chain aminotransferase (hBCATm) for acute liver failure. The following steps were followed: systematic derivatization, molecular docking, structure-activity relationship (SAR) analysis, MD simulations, and MMGBSA binding free energy calculations. The obtained results were compared with the skeleton L-0 and the reference compound Azat, and ultimately L-78 and L-87 emerged as the most promising potential molecules. The calculations performed throughout the study consistently supported each other, and consistency in the determined ranking was observed, highlighting the internal consistency and robustness of the applied multi-step computational workflow. However, it is important to acknowledge that the current findings are based entirely on *in silico* modeling and simulation. Although multi-step validation protocols ensure the internal consistency of data, these computational predictions cannot fully replicate the complex physiological and pharmacokinetic environment of liver. Therefore, the identified candidates should be considered as putative leads whose biological activity, selectivity over

other aminotransferases and actual therapeutic efficacy remain to be determined. As a final conclusion, this study identified L-78 and L-87 as promising computational lead candidates targeting hBCATm. However, these findings are based entirely on *in silico* methods and warrant further experimental validation (*in vitro* enzyme inhibition, selectivity profiling, and *in vivo* ALF models) to confirm their therapeutic potential.

561

562 **CRedit authorship contribution statement**

563 **Remziye Azra Kartop:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Serra Özışık:** Investigation, Formal analysis, Visualization, Writing - review & editing. **Vildan Enisoğlu Atalay:** Supervision, Methodology, Validation, Writing - original draft, Writing - review & editing.

567

568 **Declaration of Competing Interest**

569 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

571

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575

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579

580 **Supplementary data**

581 **Data Availability**

582 The datasets generated during the current study are available in a public repository at:
583 <https://doi.org/10.5281/zenodo.18419917>

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