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DISCOVERY OF A NOVEL HIT COMPOUND TARGETING MYCOBACTERIUM TUBERCULOSIS LEUCYL-TRNA SYNTHETASE

Aim. The objective of this study was to develop novel hit compounds targeting *Mycobacterium tuberculosis* leucyl-tRNA synthetase (LeuRS). **Methods.** Molecular docking was carried out using DOCK 4.0 software. The recombinant mycobacterial LeuRS was expressed in *Escherichia coli* Rosetta (DE3) cells and subsequently purified. The inhibitory activity of compounds toward recombinant *M. tuberculosis* LeuRS was assessed using an aminoacylation assay with BIOMOL® Green reagent. The IC_{50} value of the most active compound was measured in aminoacylation assay using ¹⁴C-labeled L-leucine. **Results.** Based on our previously reported results of a 20 ns molecular dynamics (MD) simulation of the complex of *M. tuberculosis* LeuRS with a leucyl-adenylate analogue, the frame at 15,816 ps, corresponding to the lowest interaction energy of the complex, was selected for subsequent molecular docking studies. According to the molecular docking data for a library comprising 253,838 compounds, 70 compounds were selected for investigation of their inhibitory activity toward recombinant *M. tuberculosis* LeuRS. Among them 10 compounds inhibited activity of recombinant *M. tuberculosis* LeuRS by 30–65% at the concentration of 100 μM. The IC_{50} value of the most active compound – 4,4,4-trifluoro-2-[1-hydroxy-4-(2-oxo-1,2-dihydro-benzo[cd]indole-6-sulfonylamino)-naphthalen-2-yl]-3-oxo-butyric acid isopropyl ester was 49.6 μM. **Conclusions.** Therefore, using the combination of computer modeling

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techniques and biochemical screening experiments the novel hit compound targeting *M. tuberculosis* LeuRS was identified which can be used for further chemical optimization and biological research.

Keywords: *Mycobacterium tuberculosis*, leucyl-tRNA synthetase, LeuRS, molecular dynamics, molecular docking, inhibitor.

Introduction

Tuberculosis remains one of the most dangerous infectious diseases worldwide and represents a major public challenge. According to the World Health Organization (WHO), tuberculosis once again became the leading cause of death from infectious diseases globally, following several years during which COVID-19 held that position (<https://www.who.int/news-room/fact-sheets/detail/tuberculosis>).

A major limitation of current tuberculosis therapy is the emergence of multidrug-resistant strains of the causative agent, *Mycobacterium tuberculosis*, which exhibits resistance to many existing antibiotics, including recently developed antituberculosis drugs such as bedaquiline, delamanid, and pretomanid [1–14]. Therefore, the discovery of new antituberculosis agents with novel mechanisms of action is of critical importance.

Aminoacyl-tRNA synthetases (ARSases) are essential enzymes involved in protein biosynthesis and are considered promising molecular targets for antibiotic development. These enzymes catalyze the covalent attachment of amino acids to their corresponding transfer RNAs (tRNAs) through a two-step reaction. The first step involves the formation of aminoacyl-adenylate via condensation of an amino acid with adenosine triphosphate (ATP), while the second step consists of transferring the aminoacyl group to the 3'-end of the cognate tRNA. Inhibition of aminoacyl-tRNA synthetases disrupts protein biosynthesis, preventing bacterial growth [15, 16]. Leucyl-tRNA synthetase (LeuRS) has been recognized as a particularly promising target for the development of antituberculosis agents. Li *et al.* described a highly potent inhibitor of *M. tuberculosis* LeuRS, named GSK3036656, which is currently undergoing clinical trial [17]. In addition to this compound, seve-

ral classes of *M. tuberculosis* LeuRS inhibitors have been reported. These include 3-aminomethylbenzoxaborole derivatives that act on the editing domain [18, 19], as well as several scaffolds targeting the aminoacyl-adenylate binding site, which were previously developed by our group, such as 5-phenylamino-2H-[1,2,4]triazin-3-one derivatives [20], N-benzylidene-N'-thiazol-2-ylhydrazines [21, 22], 2-(quinolin-2-ylsulfanyl)acetamides [23], N-phenyl-2-(quinolin-2-ylsulfanyl)acetamides [24], [3-phenyl-5-(1-phenyl-1H-[1,2,3]triazol-4-yl)-[1,2,4]oxadiazoles] [24, 25], N-(5-benzylthiazol-2-yl)-2-(1-aryltetrazol-5-yl)sulfanylacetamides [26] and Benzo[b]oxepine-4-carboxylic acid (5-benzylthiazol-2-yl)-amide [27] derivatives. In this study in order to find novel *M. tuberculosis* LeuRS inhibitors, we conducted a search for inhibitors targeting the enzyme conformation exhibiting the highest substrate affinity during 20-ns molecular dynamics simulation.

Materials and Methods

Molecular docking

Semi-flexible molecular docking was performed using the DOCK 4.0 software package [28–32]. The homology model of *M. tuberculosis* LeuRS was used as the receptor [21]. Ligand geometries were optimized using the YFF force field [33], and partial atomic charges were assigned using the Kirchhoff method [34].

Docking parameters were used as described previously [35], with minor modifications. In this study, the active site was defined by amino acid residues located within an 8 Å radius around the ligand. Active site spheres for ligand docking were generated using the *sphgen* module of DOCK, and spheres located outside the binding site were manually removed. Contact, energy, and bump grid maps of the receptor were generated using the *grid*

module of the DOCK package, with a grid spacing of 0.3 Å. The protein was represented using an all-atom model. For virtual screening, the multiple anchor search option was applied, with a minimum of 6 heavy atoms per anchor and a maximum of 1000 orientations considered for each ligand.

Molecular dynamics

Molecular dynamics (MD) simulations of the complex of *M. tuberculosis* leucyl-tRNA synthetase (LeuRS) with the ligand — 4,4,4-trifluoro-2-[1-hydroxy-4-(2-oxo-1,2-dihydro-benzo[cd]indole-6-sulfonylamino)-naphthalen-2-yl]-3-oxo-butyric acid isopropyl ester, were carried out using the GROMACS v.4.5 software package [36–39]. A homology model of *M. tuberculosis* LeuRS was generated with the SWISS-MODEL web server (<https://swissmodel.expasy.org/>), using as a template the crystal structure of *Thermus thermophilus* LeuRS with PDB ID: 2V0C [40].

The ligand topology file (.ITP) was generated using the ATB server (<https://atb.uq.edu.au/>), and the ligand structure in GRO format was obtained by converting the PDB file with Open Babel [41]. The initial ligand position in the active site of *M. tuberculosis* LeuRS was taken from the docking complex. The receptor structure was converted into the GROMACS internal .GRO format.

All simulations were carried out using the GROMOS 54a7 force field [42] and the SPC (Single Point Charge) solvation model [36]. Protonation states of amino acid residues were assigned using the *pdb2gmh* module of GROMACS. The receptor-ligand complex was centered in a cubic box using *editconf* program, ensuring a minimum distance of 1 nm between the complex and the box boundaries. The system was solvated using *genbox* program, which automatically determined the number of water molecules. Charge neutrality was achieved by replacing water molecules with 34 Na⁺ ions. Energy minimization was performed using the steepest descent algorithm for 1000 steps. The minimized system was then subjected to restrained molecular dynamics, where harmonic position re-

straints were applied to the atoms of both the receptor and ligand. This step allowed relaxation of the solvent environment and the removal of unfavorable contacts. Water relaxation was conducted for approximately 10 ps, followed by restrained MD for 40 ps.

The equilibrated system was then subjected to an unrestrained molecular dynamics (MD) simulation for 20 ns. The simulation was performed at 300 K within the canonical ensemble (NVT) using the leap-frog integrator. No barostat was applied during the simulation. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method [43, 44].

Hydrogen bond analysis between ligand and receptor donor-acceptor pairs was performed using the *g_hbond* module of GROMACS, applying geometric criteria of ≤0.35 nm for donor-acceptor distance and ≤30° for the angle. Root mean square deviation (RMSD) analysis was carried out using *g_rmsd*, selecting the ligand group relative to the protein group. The sum of electrostatic and van der Waals interaction energies between the receptor and ligand was calculated using the *g_energy* module of GROMACS.

Expression and purification of recombinant *M. tuberculosis* LeuRS

The plasmid vector pET28a-LeuRS (kindly provided by Prof. S. Cusack, European Molecular Biology Laboratory, Grenoble, France) was transformed into *Escherichia coli* Rosetta (DE3) cells. An overnight culture of *E. coli* Rosetta (DE3) cells harboring the pET28a-LeuRS plasmid was grown at 37 °C in LB medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. A 1:1000 dilution of the overnight culture was used to inoculate 1 L of LB medium containing kanamycin and chloramphenicol and was incubated at 20 °C for 14–16 hours at 1 mM IPTG under vigorous aeration. Cells were harvested by centrifugation at 4000 g for 20 min at +4 °C. The cell pellet obtained from 1 L of culture was resuspended in 25 mL of buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl,

5 mM MgCl_2 , 1 mM β -mercaptoethanol, and 10 mM imidazole) supplemented with 0.5 mM PMSF, 0.5 mg/ml lysozyme and half of one tablet of EDTA-free protease inhibitor cocktail). Cells were lysed by sonication (6 cycles of 30 s sonication followed by 60 s cooling) and were centrifuged at 14,000 g for 30 min. The obtained lysate was incubated with pre-equilibrated with buffer A Ni-NTA (GE Healthcare Life Sciences, Cytiva) for 45 min at +4 °C under gentle mixing. After that the suspension was washed by buffer A and buffer A supplemented with 900 mM NaCl and 10 mM imidazole. The protein was eluted using buffer A with final concentration of imidazole 250 mM.

The purified protein was concentrated using Amicon Ultra-50K centrifugal filter devices (Millipore). 10 mg of protein was obtained from 1 L of culture.

Expression and purification of recombinant human LeuRS

The overnight culture of *E. coli* Rosetta (DE3) cells harboring the pET16a-HSLeuRS plasmid was grown at 37 °C in LB medium supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol. A 1:1000 dilution of the overnight culture was used to inoculate 1 L of 2xTY medium containing kanamycin and chloramphenicol and was incubated at 18 °C for ON at 0.6 mM IPTG under vigorous aeration.

Cells were harvested by centrifugation at 4000 g for 20 min at +4 °C. The cell pellet obtained from 1 L of culture was resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 10 mM Na-P pH 7.8, 300 mM NaCl, 5 mM imidazole and 1 mM β -mercaptoethanol) supplemented with 1 mM PMSF, 1 mg/ml lysozyme and EDTA-free protease inhibitor cocktail. Cells were lysed by sonication (6 cycles of 15 s sonication followed by 60 s cooling) and were centrifuged at 14,000 g for 45 min. The obtained lysate was incubated with pre-equilibrated with buffer A Ni-NTA (GE Healthcare Life Sciences, Cytiva) for 60 min at +4 °C under gentle mixing. After that the suspension was washed by buffer A and buffer A

supplemented with 200 mM NaCl. The protein was eluted using buffer A with final concentration of imidazole 250 mM and then dialyzed against buffer B (20 mM Tris pH 8.0, 5 mM MgCl_2 , 5 mM β -mercaptoethanol) during overnight. After that the protein was loaded onto DEAE-sepharose (Pharmacia Biotech), washed by buffer B and eluted into gradient concentration of NaCl 0–400 mM. The purified protein was concentrated using Amicon Ultra-30K centrifugal filter devices (Millipore). 1.5 mg of protein was obtained from 1 L of culture.

Aminoacylation assay

The assays were performed in 96-well flat-bottom plates at 25 °C. The reaction was carried out in a total volume of 50 μL containing 100 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)-NaOH (pH 7.5), 10 mM MgCl_2 , 15 mM KCl, 1 mM dithiothreitol (DTT), and 50 $\mu\text{g}/\text{mL}$ bovine serum albumin (BSA). The mixture was supplemented with 200 μM ATP (Sigma), 1 mg/mL total *E. coli* MRE 600 tRNA (Boehringer Mannheim), 50 nM recombinant *M. tuberculosis* LeuRS, 1 mM leucine (Sigma), and 0.25 U/mL inorganic pyrophosphatase (Sigma).

All tested compounds were dissolved in 100% dimethyl sulfoxide (DMSO) (Merck) to obtain a stock concentration of 10 mM. The test compounds at the required concentrations (2.5 μL in 100% DMSO) were pre-incubated with the enzyme in 25 μL of reaction buffer for 30 min. The aminoacylation reaction was initiated by adding 25 μL of a 2x reaction mixture containing ATP, leucine, tRNA, and pyrophosphatase, followed by incubation for 10 min at room temperature.

The reaction was terminated by adding 100 μL of BIOMOL® Green reagent (Enzo Life Sciences) and incubated for 25 min. BIOMOL® Green allows quantification of inorganic phosphate released during the pyrophosphatase reaction. Absorbance was measured at $\lambda = 630 \text{ nm}$ using a BioTek microplate reader (BioTek Instruments).

The inhibitory activities of the compounds were evaluated in two independent experiments.

The IC_{50} value was measured in aminoacylation assay using ^{14}C -labeled L-leucine as described earlier [26].

Results and Discussion

According to our previously reported data on molecular dynamics (MD) simulations of the *M. tuberculosis* LeuRS homology model complexed with 5'-sulfamoyladenosine-[(2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl sulfamate] [45], the protein conformation at 15,816 ps, which exhibited the lowest interaction energy during the 20 ns simulation, was selected for molecular docking studies. The complex of the 5'-sulfamoyladenosine with the amino acid residues of the synthetic site of *M. tuberculosis* LeuRS at 15,816 ps of MD simulation is shown in Fig. 1.

Molecular docking of the compound library comprising 253,838 ligands was performed into the synthetic site of the *M. tuberculosis* LeuRS conformation obtained at 15,816 ps of MD simulation using the DOCK 4.0 software package. Based on the scoring function values and visual inspection of the best-scored complexes of ligands with the aminoacyl-adenylate acceptor site of *M. tuberculosis* LeuRS, 70 compounds were selected for biochemical screening of inhibitory activity against recombinant *M. tuberculosis* LeuRS. Among them 10 compounds inhibited activity of recombinant *M. tuberculosis* LeuRS by 30–65% at the concentration of 100 μ M (Table). The most active compound — 4,4,4-trifluoro-2-[1-hydroxy-4-(2-oxo-1,2-dihydro-benzo[cd]indole-6-sulfonylamino)-naphthalen-2-yl]-3-oxo-butyric acid isopropyl ester (compound **1**), inhibited the activity of recombinant *M. tuberculosis* LeuRS with IC_{50} value of 49.6 μ M. This compound was tested for inhibitory activity toward human leucyl-tRNA synthetase. It was found that compound **1** inhibits human enzyme by 18.6% at the concentration of 100 μ M. Therefore, inhibitor **1** appears to demonstrate selectivity toward *M. tuberculosis* LeuRS.

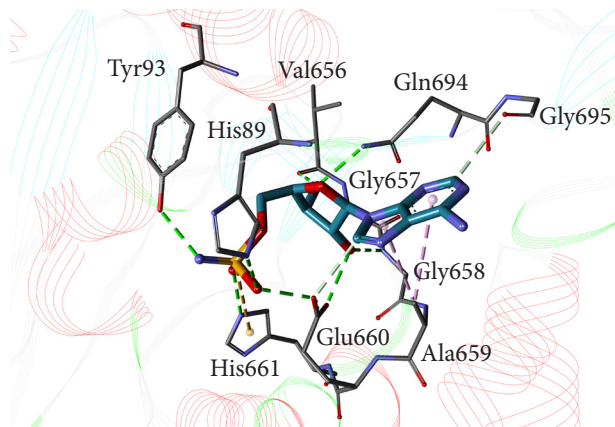


Fig. 1. The complex of 5'-sulfamoyladenosine with the amino acid residues of the active site of *M. tuberculosis* LeuRS. Hydrogen bonds are shown by green dashed lines, hydrophobic interactions are presented by purple dashed lines and π -sulfur interaction is indicated by yellow dashed line

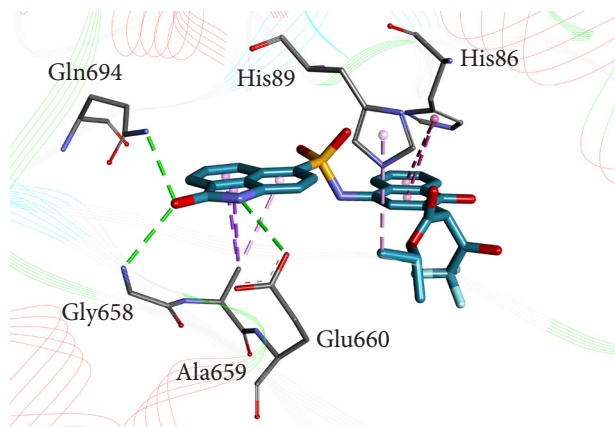


Fig. 2. The binding mode of compound **1** in the active site of *M. tuberculosis* LeuRS. Hydrophobic interactions are indicated by magenta dashed lines and hydrogen bonds are presented by green dashed lines

The binding mode of the most active compound **1**, which was predicted by molecular docking is presented in Fig. 2. The complexes of the remaining nine compounds **2**–**10** with amino acid residues in the aminoacyl-binding site of *M. tuberculosis* LeuRS are presented in Fig. S1 (Supplementary Material, available at <https://doi.org/10.6084/m9.figshare.32879819>).

Structures and *in vitro* inhibitory activity (%) of compounds at the concentration of 100 μ M toward *M. tuberculosis* LeuRS

№	Structure	Inhibitory activity, %	№	Structure	Inhibitory activity, %
1		65.0	6		36.7
2		46.7	7		36.4
3		46.3	8		35.7
4		44.4	9		31.3
5		36.8	10		30.0

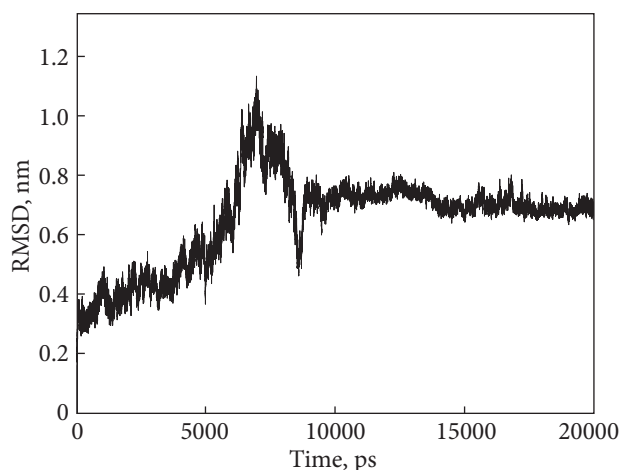


Fig. 3. The root-mean-square deviation (RMSD) of compound **1** from the starting position in the complex with *M. tuberculosis* LeuRS during 20 ns MD simulation

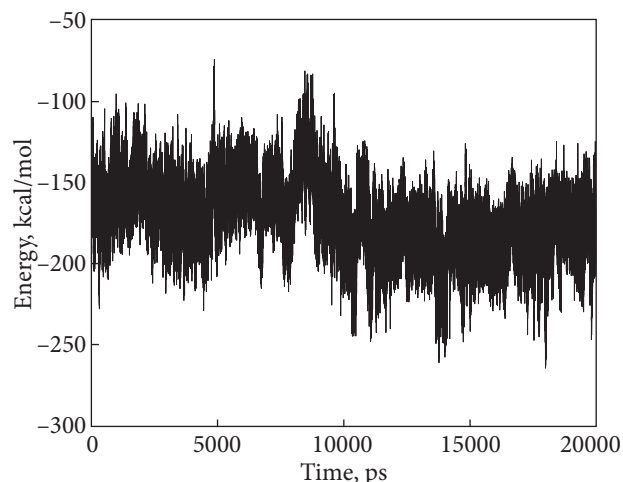
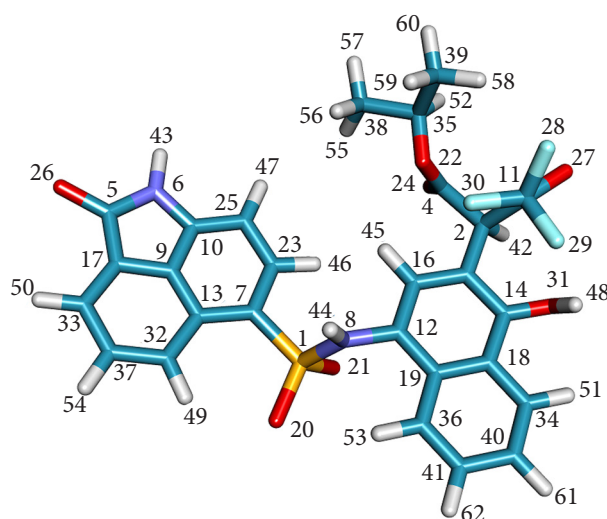
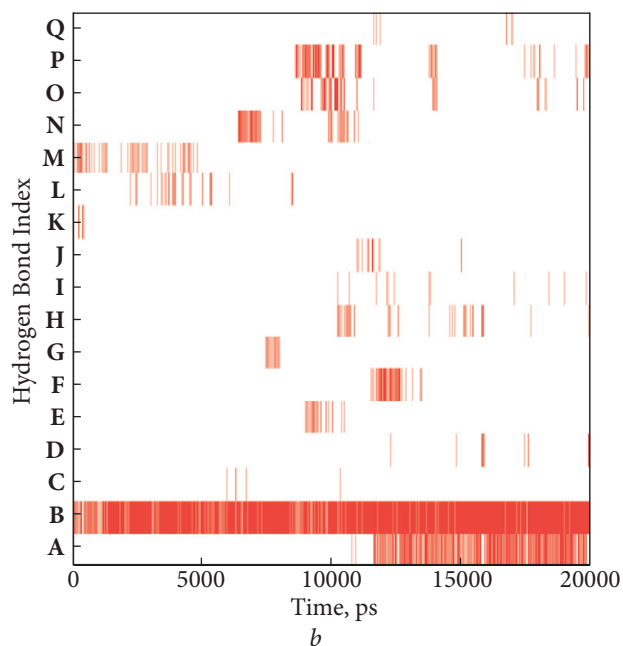


Fig. 4. The sum of electrostatic and van der Waals interactions of the complex of *M. tuberculosis* LeuRS with compound **1** during 20 ns MD simulation



a



b

Fig. 5. The map of intermolecular hydrogen bonds between amino acid residues in the aminoacyl-adenylate binding site of *M. tuberculosis* LeuRS with the atoms of compound **1** during 20 ns MD simulation. **A** — Ser81_OG — C3, **B** — Gly88_N — O21, **C** — His89_N — O21, **D** — Gln129_NE2 — S1, **E** — Ile697_N — H45, **F** — Ile710_N — H51, **G** — Lys742_N — S1, **H** — Ser81_O — C3, **I** — Gly82_N — C3, **J** — Gly82_O — C3, **K** — Ile697_O — C3, **L** — Ile740_O — C3, **M** — His86_ND1 — C23, **N** — His89_ND1 — C23, **O** — Glu660_OE1 — O31, **P** — Glu660_OE2 — O31, **Q** — Tyr696_OH — O31

According to molecular docking results the identified hit compound interacts with the amino acid residues in the adenine acceptor site of *M. tu-*

berculosis LeuRS, such as His86, Gln694 and with amino acid residues in the ribose binding region such as His89, Gly658, Ala659 and Glu660.

To evaluate the stability of the complex formed between compound **1** and *M. tuberculosis* LeuRS and to identify the key protein-ligand interactions for subsequent chemical optimization, molecular dynamics (MD) simulations were performed for 20 ns using the GROMACS 4.5 software package. As shown by the root-mean-square deviation (RMSD) profile (Fig. 3) and the combined electrostatic and van der Waals interaction energy plot (Fig. 4), the *M. tuberculosis* LeuRS-compound **1** complex remained stable throughout the simulation. A conformational reorganization of the complex was observed at approximately 6 ns, followed by stabilization after 8 ns, indicating the formation of a stable binding mode for the remainder of the simulation.

The hydrogen bond map indicates that the initial stabilization of the complex, prior to structural reorganization, is mediated by hydrogen bonds with Gly88 (**B**), Ile697 (**K**), Ile740 (**L**), and His86 (**M**) (Fig. 5). After approximately 6 ns of the MD simulation, compound **1** undergoes displacement within the synthetic site of *M. tuberculosis* LeuRS relative to its initial binding pose. This displacement is likely associated with the disruption of the initial hydrogen bonds and the concurrent formation of a new hydrogen bond with His89 (**N**). A subsequent, short-lived displacement is observed at approximately 8 ns (Fig. 3), potentially associated with the formation of a hydrogen bond with Lys742 (Fig. 5, **G**). This interaction is accompanied by a slight increase in the total electrostatic and van der Waals interaction energy of the receptor-ligand complex (Fig. 4). Thereafter, the complex reaches a more stable conformation through the establishment of an extensive hydrogen bond network involving Ser81 (**A**, **H**), Gly88 (**B**), Gln129 (**D**), Ile697 (**E**), Ile710 (**F**), Gly82 (**I**, **J**), His89 (**N**), Glu660 (**O**, **P**), and Tyr696 (**Q**) (Fig. 5). The formation of this hydrogen bond network correlates with

a decrease in the total electrostatic and van der Waals interaction energy of the receptor-ligand complex (Fig. 4), consistent with enhanced complex stability.

Analysis of the binding mode of compound **1** in the complex with LeuRS revealed that the naphtholactam moiety forms key interactions within the enzyme binding site and therefore does not require further structural modification. Likewise, the sulfonamide group serves as an optimal linker and should be retained. Consequently, further optimization efforts can be focused exclusively on the naphthalene core. The proposed strategy involves cyclization of the β -ketoester with the naphtholic hydroxyl group to generate a rigid naphtho[b]furan scaffold. This conformational restriction is expected to reduce the entropic penalty upon binding while enabling an additional interaction with His86 within the LeuRS binding pocket. Furthermore, bioisosteric replacement of the trifluoromethyl group with a methyl group at the C2 position of the proposed furan ring is anticipated to improve the lipophilic properties of the compound.

Conclusion

Therefore, using computer modeling simulation and subsequent *in vitro* aminoacylation analysis we identified novel hit compound — 4,4,4-trifluoro-2-[1-hydroxy-4-(2-oxo-1,2-dihydro-benzo [cd]indole-6-sulfonylamino)-naphthalen-2-yl]-3-oxo-butyric acid isopropyl ester, inhibiting *M. tuberculosis* LeuRS with IC₅₀ value of 49.6 μ M. This chemical scaffold can be used for further chemical optimization and research.

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ПОШУК НОВИХ ІНГІБІТОРІВ ЛЕЙЦИЛ-ТРНК СИНТЕТАЗИ *MYCOBACTERIUM TUBERCULOSIS*

Мета. Метою дослідження було розробити нові інгібітори лейцил-трНК синтетази (ЛейРС) *Mycobacterium tuberculosis*. **Методи.** Молекулярний докінг здійснювали за допомогою програми DOCK 4.0. Рекombінантну мікобактеріальну ЛейРС експресували в клітинах *Escherichia coli* Rosetta (DE3). Інгібувальну активність сполук щодо рекombінантної ЛейРС *M. tuberculosis* визначали за допомогою реакції аміноацилювального з використанням реагенту BIOMOL[®] Green. Значення IC₅₀ для найбільш активної сполуки встановлювали за допомогою радіометричного методу з використанням ¹⁴C-міченого L-лейцину. **Результати.** За результатами раніше проведеного моделювання молекулярної динаміки комплексу ЛейРС *M. tuberculosis* із 5'-сульфамойладенозином для молекулярного докінгу було обрано конформацію ЛейРС *M. tuberculosis* на 15816 пс, що характеризується найнижчою енергією комплексу із аналогом аденілату. На основі даних молекулярного докінгу бібліотеки, що налічувала 253838 сполук, було відібрано 70 сполук для дослідження їх інгібувальної активності щодо рекombінантної ЛейРС *M. tuberculosis*. Серед них виявлено 10 речовин, що інгібували активність рекombінантної ЛейРС *M. tuberculosis* на 30—65% при концентрації 100 мкМ. Значення IC₅₀ найбільш активної сполуки — ізпропілового естеру 4,4,4-трифлуоро-2-[1-гідрокси-4-(2-оксо-1,2-дигідро-бензо[cd]індол-6-сульфоніламіно)нафтаден-2-іл]-3-оксо-бутирової кислоти становило 49,6 мкМ. **Висновки.** Таким чином, за допомогою комбінації методів комп'ютерного моделювання та біохімічного скринінгу ідентифіковано новий клас інгібіторів ЛейРС *M. tuberculosis*, що може бути використаний для подальшої хімічної оптимізації та біологічних досліджень.

Ключові слова: *Mycobacterium tuberculosis*, лейцил-трНК синтетаза, ЛейРС, молекулярна динаміка, молекулярний докінг, інгібітор.