ORIGINAL ARTICLE

Discovery of Novel Pyruvate Kinase Inhibitors Against *Leishmania major* Among FDA Approved Drugs Through System Biology and Molecular Docking Approach

Sistem Biyolojisi ve Moleküler Yerleştirme Yaklaşımı Yoluyla FDA Onaylı İlaçlar Arasında *Leishmania major*'a Karşı Yeni Piruvat Kinaz İnhibitörlerinin Keşfi

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ABSTRACT

Objectives: Leishmaniasis is one of the common forms of neglected parasitic diseases that cause a worldwide disease burden without any effective therapeutic strategy. Control of the disease currently relies on chemotherapy because most of the available drugs have toxic side-effects and drug-resistant strains have emerged. Therefore, the development of new therapeutic strategies to treat patients for leishmaniasis has become a priority. The first step in drug discovery is to identify an effective drug target by methods such as system biology. Protein kinases are a promising drug target for different diseases. Due to lack of a functional krebs cycle in *Leishmania* species, they use glycolysis as the only source of ATP generation. Pyruvate kinase is the enzyme involved in the last step of glycolysis and considered as essential enzyme for the *Leishmania* survival.

Materials and Methods: This study sought to discover FDA approved compounds against the leishmanial pyruvate kinase protein. Our approach involved using quantitative proteomics, protein interaction networks and docking to detect new drug targets and potent inhibitors.

Results: Pyruvate kinase was determined as the potential drug target based on protein network analysis. The docking studies suggested trametenib and irinotecan with high binding energies of -10.4 and -10.3 kcal/mol, respectively, as the potential chemotherapeutic agents against *L. major.*

Conclusion: This study demonstrated the importance of integrating protein network analysis and molecular docking to identify new anti-leishmanial drugs. These potential inhibitors constitute novel drug candidates that should be tested *in vitro* and *in vivo* to determine their potential as an alternative chemotherapy in the treatment of leishmaniasis.

Key words: *Leishmania major*, pyruvate kinase, protein network, drug target, docking

ÖZ

Amaç: Leishmaniasis, herhangi bir etkili tedavi stratejisi olmadan dünya çapında bir hastalık yüküne neden olan ihmal edilmiş paraziter hastalıkların yaygın formlarından biridir. Mevcut ilaçların çoğu toksik yan etkilere sahip olduğundan ve ilaca dirençli suşlar ortaya çıktığından, hastalığın kontrolü şu anda kemoterapiye dayanmaktadır. Bu nedenle, leishmaniasis hastalarını tedavi etmek için yeni terapötik stratejilerin geliştirilmesi bir öncelik

haline gelmiştir. İlaç keşfinde ilk adım, sistem biyolojisi gibi yöntemlerle etkili bir ilaç hedefi belirlemektir. Protein kinazlar, farklı hastalıklar için umut verici bir ilaç hedefidir. *Leishmania* türlerinde fonksiyonel bir krebs döngüsü olmaması nedeniyle, ATP üretiminin tek kaynağı olarak glikoliz kullanırlar.

Gereç ve Yöntemler: Bu çalışma, leishmania piruvat kinaz proteinine karşı Gıda ve İlaç İdaresi onaylı bileşikleri keşfetmeyi amaçlamıştır. Yaklaşım, nicel proteomikler, protein etkileşim ağları ve yeni ilaç hedeflerini ve güçlü inhibitörleri tespit etmek için yerleştirmeyi içermiştir.

Bulgular: Protein ağı analizine dayalı olarak potansiyel ilaç hedefi olarak piruvat kinaz belirlenmiştir. Docking çalışmaları, *L. major*'a karşı potansiyel kemoterapötik ajanlar olarak sırasıyla -10,4 ve -10,3 kcal/mol'lük yüksek bağlanma enerjilerine sahip trametinib ve irinotekanı önermiştir.

Sonuç: Bu çalışma, yeni anti-leishmanial ilaçları tanımlamak için protein ağı analizini ve moleküler yerleştirmeyi entegre etmenin önemini göstermiştir. Bu potansiyel inhibitörler, leishmaniasis tedavisinde alternatif bir kemoterapi olarak potansiyellerini belirlemek için *in vitro* ve *in vivo* olarak test edilmesi gereken yeni ilaç adaylarını oluşturmaktadır.

Anahtar kelimeler: *Leishmania major*, piruvat kinaz, protein ağı, ilaç hedefi, docking

INTRODUCTION

Leishmaniasis is a complex infectious disease caused by various species of the genus *Leishmania, and* is a serious public health problem in many tropical and subtropical regions of the world.[1,2] The disease currently occurs in 12 million people and is threatening about 350 million in 98 countries worldwide.[3] At least 20 different species of *Leishmania* are responsible for a wide spectrum of human leishmaniasis forms.[4] This infectious disease is spread by the bites of the female *Phlebotomus* infected with metacyclic (infective) forms. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis in humans mainly caused by *L. major* and *L. tropica*.[1]

Currently, despite worldwide efforts to develop new drugs and vaccines, there are not effective vaccines against leishmaniasis. The main line of leishmaniasis treatment is chemotherapy. Pentavalent antimonials are the first choice for leishmaniasis treatment but have many limitations such as toxicity and emergence of drug resistance.[5,6] Alternative chemotherapeutic drugs such as amphotericin B and paromomycin are limited due to their high toxicity and cost.[7] The challenges of toxicity, drug resistance and cost make it difficult to design an effective drug system. Therefore, the main challenge in the treatment of leishmaniasis is to discover highly effective drugs. Drug target identification is the first step in the complex drug discovery process.[8] An ideal target in a pathogen should be one that is necessary for survival of the pathogen, and should not be present in the mammalian host or must differ from its host homolog. Protein kinases are the main regulators of several cellular processes and have attracted much attention as potential drug targets to treat a wide range of infectious diseases. Although trypanosomatids generally use glycolysis as a primary source of ATP, *Leishmania,* in their amastigote form, use both glycolysis and aerobic oxidation. They are just less dependent on oxygen than their other form.[9] In addition, *Leishmania* grow without glucose *in vitro* indicating that glucose is not their primary energy substrate. Therefore, inhibiting the glucose pathway may not have an effect on parasite growth.[10] Energy metabolism during *Leishmania* growth and metacyclogenesis is affected by regulated enzymes that probably respond to changes in glucose and amino acid levels in the culture medium.[10]

The unique location of glycolytic enzymes in glycosomes in *Leishmania* provides them with specific features.[7]

Pyruvate kinase, an enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis, plays a role in regulating cell metabolism. In these organisms pyruvate kinase plays a key regulatory role, and is unique in responding to fructose 2,6-bisphosphate as an allosteric activator.[11] Given the importance of the energy production cycle and the important role of pyruvate kinase in this cycle, this enzyme could be considered as a potentially important pharmaceutical target. Also, this enzyme is necessary for survival of the parasite in the host and is different from the host homolog.[7,12]

Computational techniques increase the chance of success in drug discovery and in the design of desirable lead compounds. There are several computational approaches such as protein-protein interaction network analysis to select novel promising drug targets in different diseases.[13-15] In this work, we profiled the proteomic pattern of L. *major* metacyclic (infective stage) by using a label-free quantitative proteomic technique (sequential window acquisition of all theoretical fragment ion spectra mass spectrometry), and predicted the protein network of L. *major* in the STRING database. We analyzed the predicted protein network with centrality metrics to identify essential proteins as potential drug targets using the Cytoscape software. We identified drug targets evaluated with molecular docking to present new anti-leishmanial compounds (Figure 1). The objectives of this study were to: 1) identify appropriate target protein for discovery of drugs against leishmaniasis by system biology approach, and 2) identify potential lead compounds [Food and Drug Administration (FDA) compounds] for inhibition of the target enzyme by the docking method for studying protein- ligand binding interactions.
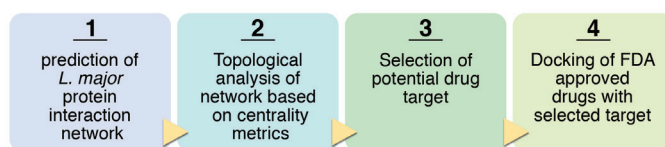

Figure 1. The framework used in the protein network and target prediction of Iranian isolate of *L. major*

FDA: Food and Drug Administration

MATERIALS AND METHODS

Prediction of protein network (system biology) and selection of target

The protein profile of L. *major* metacyclic was used for construction of protein-protein interactions (PPIs) through text mining, co-occurrence and co-expression in the STRING database. We calculated power law fit through Network Analyzer for predicted protein network. Topological analysis of predicted protein network, based on degree and betweenness centrality, were used to identify potential drug targets using CytoHubba plugin tool in Cytoscape software.

Selection of ligand dataset

The 1948 FDA approved compounds data were retrieved from the DrugBank database (www.drugbank.ca). Refinements of the 3D structure of compounds were done by performing energy minimization by the HyperChem software using the molecular mechanics approach (Hypercube Inc. Gainesville, FL, USA).

Target and ligand preparation

As the protein target chosen in our study was pyruvate kinase, the most suitable 3D we used was *Leishmania* pyruvate kinase enzyme in interaction with Suramin drug [Protein Data Bank (PDB) ID: 3PP7] downloaded from the PDB database (www.pdb.org). The resolution and R-value of this structure were 2.35 A° and 0.23, respectively. Energy optimization and correction of target protein and ligands were done using Chimera and HyperChem softwares, respectively. Briefly, water molecules and heteroatoms including ligand, cofactor, and carbohydrates were removed from the structure. Polar hydrogen atoms were then added before Kollman charges were assigned. The active sites of the target protein were evaluated based on prediction servers, inspection of 3D structure surface and literature review. These estimations provide the probable pocket for the binding of the substrate, which plays critical and significant roles in the enzyme function. Achieved residues in the binding pocket included: Arg_{49}, Thr_{26}, Pro_{29}, Tyr_{59}, Lys_{335}, His_{54}, and Asn_{51}.
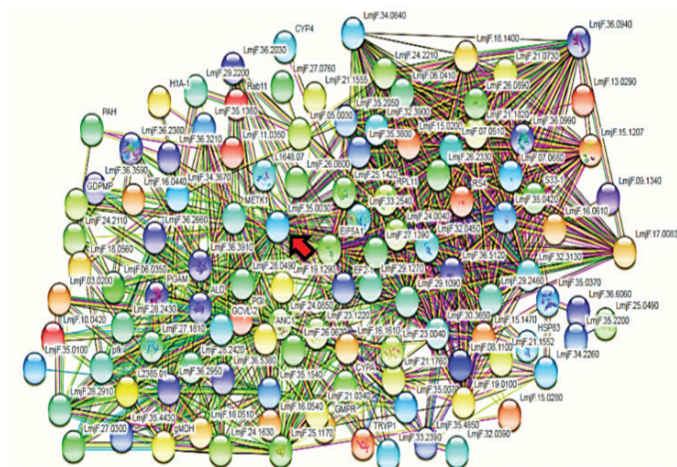
Docking studies

In the docking study phase, compounds were searched for leishmanial pyruvate kinase inhibition activity. After the target protein and ligands preparation had been performed, docking was performed by the AutoDockVina software.[16] The ligands and protein format were required to convert to the PDBQT as input format of AutoDockVina. The 3D structures of ligands were docked with the 3D structure of the pyruvate kinase enzyme and their affectivity was ranked on the basis of their binding energy and hydrogen bond interactions. The docking approach uses an energy-based scoring method in which low binding energy scores represent premier protein-ligand bindings.[17] Grid box parameters were set as follows: (center_x = 27, center_y = 18, center_z = 40, size_x = 28, size_y = 32, size_z = 3), which cover the whole protein. The Ligplot+ software was used for automatic generation of schematic diagram of protein- ligand interaction.[18] LigPlot+ is a successor to the original LIGPLOT program for automatic generation of 2D ligand-PPI diagrams. It is run from an intuitive java interface which allows on-screen editing of the plots via mouse click-and-drag operations. The results showed some ligands which can be taken for further study on drug discovery.

RESULTS AND DISCUSSION

Target selection

We constructed a PPI map, from the results generated by mass spectrometry of L. *major* metacyclic protein profiling. From 144 detected proteins (results not published) in the Iranian isolate of L. *major* metacyclic (infective stage), we predicted a protein network with 135 nodes and 1051 interactions using the STRING database (Figure 2). Based on analysis of the topology of the network with connectivity (node degree) and betweenness centrality, we predicted potential drug targets. A measure of these centrality values has also been proposed as a new way for investigating potential drug targets. Proteins with high connectivity and betweenness centrality values are called hubs and bottlenecks, respectively. In protein networks, the proteins with high betweenness centrality value are referred to as scaffold proteins and are associated with protein essentiality more than connectivity. It has been reported that connectivity[19] and betweenness centrality[20] are important indices for the identification of essential proteins in protein- protein interaction networks.[21] In the L. *major* network, we have provided a putative list of five essential (hub-bottleneck) proteins with high connectivity and betweenness centrality. Among the top five hub-bottlenecks on the basis of its major role in ATP generation in *Leishmania* pathogen, we selected pyruvate kinase as target for a docking study (Table 1). The 3D structure of the pyruvate kinase is available in the PDB database. Kinases are of special

Figure 2. Protein-protein interaction network obtained by STRING v10.5. Nodes depict proteins and PPI are represented by edges in the network; interaction source of the PPI's are represented by various colors. The pyruvate kinase protein locus in PPIN is shown by arrows

PPI: Protein-protein interaction, PPIN: Protein-protein interaction network

interest as an important category of *Leishmania* proteins to be considered as sources of new drug targets.[12] In addition, several investigations compared the PyK crystal structure of the parasite with the PyK of other organisms. These studies reported important differences between these enzymes especially at the level of its effector site indicating distinctive regulatory attributes for the parasite enzyme, which could be a potential anti-leishmanial drug target.[11,22] For example Rebollo et al.[23] also selected pyruvate kinase along with other enzymes as a target protein for discovery of a new agent with potential leishmanicidal activity by virtual screening of chemical databases that all of them considered essential for the survival of *Leishmania*.[24] In fact, the parasite needs glycolysis enzymes because they are essential for the amastigote form of parasite. So the target would stop the intracellular parasite development in the host.

Docking studies

Structure-based virtual screening was carried out to predict the binding affinity of 1388 FDA approved compounds in order to identify new inhibitors of pyruvate kinase from *Leishmania*. This approach involved computational docking of ligands with a receptor, followed by scoring and ranking of ligands to discove potential leads. In previous studies, docking studies of some *Leishmania* enzymes such as Tryparedoxin peroxidase,[25] cysteine protease A,[26] N-myristoyltransferase,[27] 6-phosphoglucono-lactonase,[23] and trypanothione reductase[28] were done. Tryparedoxin peroxidase and trypanothione reductase as antioxidant enzymes play an important role in parasite survival, therefore, they can be suitable options for drugs against *Leishmania*. For example, Kothandan et al.[28] conducted a toxicology and docking study, and introduced Emetine as a potential lead compound against leishmaniasis. In the aforementioned studies, the several virtual screening databases have included Pubchem, ChemBridge and Pubchem BioAssay database. The various tools and softwares included Dock[29], Gold[30], Molegro Virtual Docker[17], SwissDock server[31], LibDock algorithm inbuild in DSv.5,[32] Sybyl 8.0 and AutoDockVina. In this study, we performed the docking assay for the first time by FDA approved compounds by AutoDockVina in order to identify pyruvate kinase inhibitors. After the compounds were screened

against pyruvate kinase protein, the results were ranked based on their docking scores and the top 10 high scoring compounds are listed in Table 2. These compounds with high affinity to receptors could be considered as potential leads, which must be tested *in vitro* or *in vivo* experiments to confirm their real potential as anti-leishmanial drugs. Our results indicated that trametinib (DB08911) and irinotecan (DB00762) had the highest binding affinity to the target with -10.4 and -10.3 kcal/mol, respectively, and could provide a scaffold for further drug design efforts. Hydrogen-bonds play a crucial role in determining the specificity of ligand binding. In potential drugs from our findings, trametinib has one hydrogen bonding with a length of 2.88 A° and Irinotecan has also two hydrogen bonds, one with a length of 2.99 A° and the other one 3.14 A°. The number of hydrogen bonds of other compounds are shown in Table 2. Also, among these potential inhibitors, no side-effects have been reported for nilotinib (DB04868, Dock score: -10.1), netupitant (DB09048, Dock score: -10.1) and conivaptan (DB00872, Dock score: -9.9) in DrugBank database, rendering them valuable candidates for drug designing in future.

The protein-ligand interaction plays a crucial role in structure-based drug discovery. Based on our *in silico* docking analysis we suggest the following leishmanial pyruvate kinase potential drugs (trametinib, irinotecan, nilotinib, netupitant, naldemedine, eltrombopag, vumon, conivaptan, valstar and lomitapide). In this study, we took the pyruvate kinase essential protein (target) that plays a significant role in leishmaniasis and proposed the potent drugs that were used against leishmaniasis to evaluate its efficacy. These drugs can be tested *in vivo* and *in vitro* and as a lead for further validation in clinical trials. The study results would help to develop the new drugs for the leishmaniasis treatment.

This study has potential limitations. The anti-leishmanial effect of FDA-approved ligands against pyruvate kinase enzyme estimates are based only on computational methods and molecular interaction mechanism (i.e., protein interaction network and docking study). Further research involving experimental screening of compounds with high affinity and their analogues, optimization, and validation could lead to discovery of novel drugs for leishmaniasis treatment.

Table 1. Putative drug target ranked according to connectivity and betweenness centrality values in Hubba plugin in Cytoscape software. Pyruvate kinase selected in order to perform docking assay (grey color)

Hub-bottleneck proteins

Rank	Gene name	Protein name
1	*LmjF.28.2780*	Putative heat-shock protein hsp70
2	*ENOL*	Enolase
3	*LmjF.35.0030*	Pyruvate kinase
4	*LmjF.36.2030*	Chaperonin HSP60, mitochondrial
5	*LmjF.25.1170*	ATP synthase subunit beta

Table 2. List of docking results of the FDA approved compounds with high binding affinity to the leishmanial pyruvate kinase inhibition
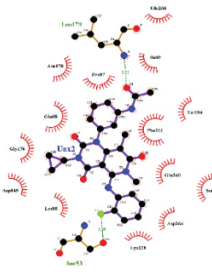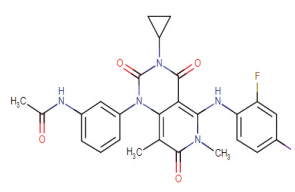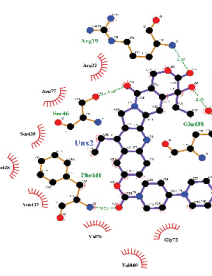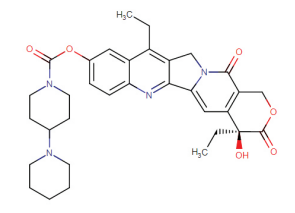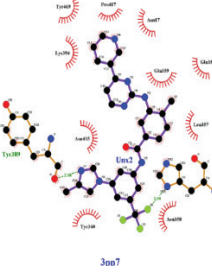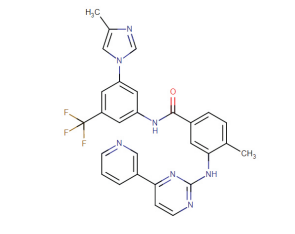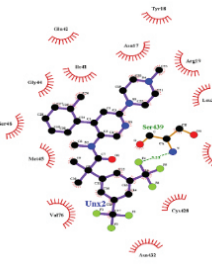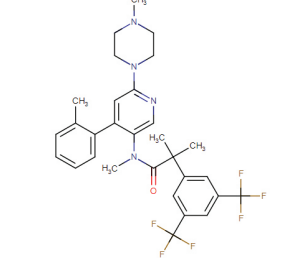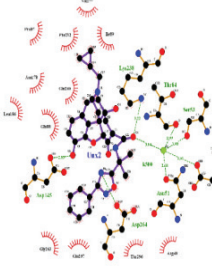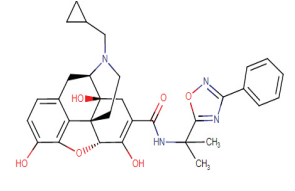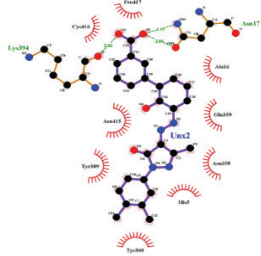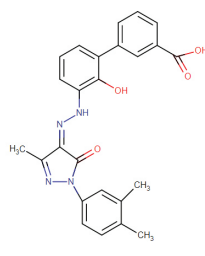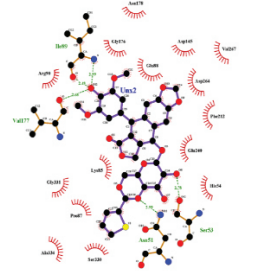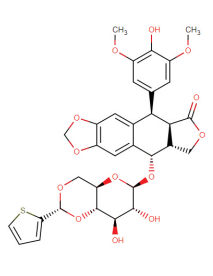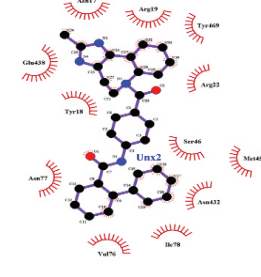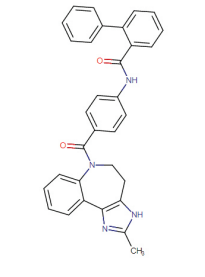
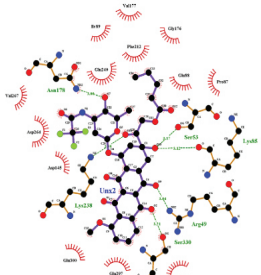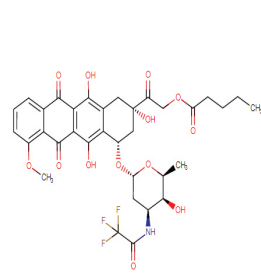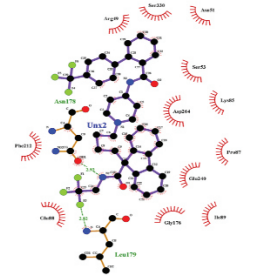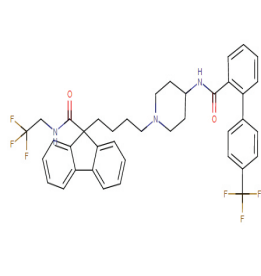| No.	Compound ID	Compound name	Binding energy (kcal/mol)	H-bond number (length:A°)	Target residues	Ligplot+ analysis	Drug structure
1	DB08911	Trametinib	-10.4	1 (2.88)	Glu268, Il89, Val184, Phe212, Glu240, Ser211, Asp264, Lys238, Lys85, Asp145, Gly176, Glu88, Asn178, Pro87		
2	DB00762	Irinotecan	-10.3	2 (2.99, 3.14)	Lys467, Phe463, Gly75, Val440, Val76, Asn432, Cys428, Ser439, Asn77, Arg22		
3	DB04868	Nilotinib	-10.1	-	Lys394, Tyr469, Pro417, Asn17, Glu359, Gln354, Leu357, Asn358, Try360, Asn415		
4	DB09048	Netupitant	-10.1	3 (3.11, 3.12, 3.23)	Asn17, Tyr18, Arg19, Leu351, Asn77,Cys428, Asn432, Val76, Met45, Ser46, Gly44, Ile41, Gln42		
5	DB11691	Naldemedine	-10.1	1 (3.09)	Arg49, Thr296,Gln297, Gly263, Glu88,Glu240, Leu186, Asn178,Pro87, Phe212, Val177, Ile89		

Table 2. contiuned

No.	Compound ID	Compound name	Binding energy (kcal/mol)	H-bond number (length:A°)	Target residues	Ligplot+ analysis	Drug structure
6	DB06210	Eltrombopag	-10.0	1 (3.12)	Pro417, Ala16, Glu359, Asn358,His5, Tyr360, Try389, Asn415, Cys416		
7	DB00444	Vumon	-9.9	-	Asn178, Gly179, Glu88, Asp145, Val267, Asp264, Phe212, Glu240, His54, Ser330, Ala334, Pro87, Gly331, Lys85, Arg90		
8	DB00872	Conivaptan	-9.9	3 (2.81, 3.26, 3.23)	Asn17,Arg19, Tyr469, Arg22, Ser46, Met45, Asn432, Ile78, Val76, Asn77, Tyr18, Glu438		
9	DB00385	Valstar	-9.9	2 (3.01, 3.20)	Val177, Gly176, Ile89, Phe212, Glu240, Glu88, Pro87, Thr296, Gln297, Glu300, Asp145, Asp264, Val267,		
10	DB08827	Lomitapide	-9.8	1 (2.80)	Arg49, Ser330, Asn51, Ser53, Lys85, Asp264, Pro87, Glu240, Gly176, Glu88, Phe212		

CONCLUSION

In this study, we used molecular docking to identify the top potent pyruvate kinase inhibitors among different FDA approved compounds. Structure-based drug designing and lead discovery were used effectively for computer-aided drug discovery. We screened *in silico* a large library of FDA approved compounds against pyruvate kinase protein, a vital enzyme for *Leishmania* survival. Ligands bounded with higher affinity to the target protein ranked by AutoDockVina score. The results presented here require clinical evaluation before the identified therapeutic agents can be used as anti-leishmanial drugs. The identified leads with the higher possibility of binding to the target protein would reduce the cost of biological testing.

Code availability
Data were analysed using Cytoscape and AutoDock softwares.

ACKNOWLEDGMENTS

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