Identification of selective \textit{LdDHFR} inhibitors using quantum chemical and molecular modeling approach

Vishnu Kumar Sharma\textsuperscript{a}, Deepika Kathuria\textsuperscript{b,c} and Prasad V. Bharatam\textsuperscript{b}

\textsuperscript{a}Department of Pharmacoinformatics, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Punjab, India; \textsuperscript{b}Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Punjab, India; \textsuperscript{c}University Center for Research and Development, Chandigarh University, Gharuan, Punjab, India

Communicated by Ramaswamy H. Sarma

\textbf{ABSTRACT}

Among the various known targets for the treatment of Leishmaniasis, dihydrofolate reductase (DHFR) is an essential target which plays an important role in the folate metabolic pathway. In the current study, pharmacoinformatics approaches including quantum chemistry methods, molecular docking and molecular dynamics simulations have been utilized to identify selective \textit{Leishmania donovani} DHFR (\textit{LdDHFR}) inhibitors. Initially, for the design of new \textit{LdDHFR} inhibitors, a virtual combinatorial library was created by considering various head groups (scaffolds), linkers and tail groups. The scaffolds utilized in the library design were selected on the basis of their proton affinity (PA) estimated using quantum chemical methods, required to make a strong H-bond interaction with negatively charged \textit{LdDHFR} active site. Later on, molecular docking-based virtual screening was performed to screen the designed library. Selectivity of the chosen hits toward the \textit{LdDHFR} was established through re-docking in the human DHFR enzyme (\textit{HsDHFR}). Best five hits were subjected to molecular dynamics (MD) simulations to validate their selectivity as well as stability in \textit{LdDHFR}. Out of the five hits, four were found to be energetically more favorable and promising for selective binding toward \textit{LdDHFR} in comparison to \textit{HsDHFR}.

\textbf{Introduction}

Leishmaniasis is a parasitic disease which is caused by protozoan parasites. The burden of leishmaniasis is increasing annually and thus making it an area of concern. Visceral leishmanias (known as Kala-azar in India) is caused by \textit{L. donovani} and is the most fatal among all forms of leishmaniasis. To combat this situation many drugs are being used which include amphotericin B, miltefosine, paromomycin, pentamidine, and pentavalent antimonials, but the only FDA (Food and Drug Administration) approved medications for VL are intravenous liposomal amphotericin B (L-AmB) and oral miltefosine. Dihydrofolate reductase (DHFR) is a validated drug target and its main function is to convert dihydrofolate (DHF) to tetrahydrofolate (THF) in the presence of NADPH cofactor (Knighton et al., 1994; Schnell et al., 2004; Vickers & Beverley, 2011). During this conversion, one hydride ion from the NADPH gets transferred to the protonated DHF to yield THF which further participates in the biosynthesis of nucleotides and amino acids (Abbat et al., 2015; Sharma et al., 2017; Tjong et al., 2019).

The structure of the substrate (DHF) molecule can be divided in to three sub-structures which are 1) pteridine ring moiety, 2) benzamide moiety, and 3) glutamate moiety. Similarly, the active site (the substrate binding domain) of \textit{LdDHFR} can be divided in to three sub-pockets according to the accommodation of DHF in the active site. The sub-pockets of \textit{LdDHFR} include a) pteridine sub-site (head part), b) benzamide sub-site (linker part), and the glutamate sub-site (tail part/solvent accessible region) (Sharma & Bharatam, 2021). The pteridine binding site contains negatively charged amino acid residue Asp52 with side chain ‘O’ atoms which binds with the positively charged pteridine moiety and form strong H-bond interactions. Apart from that, backbone ‘N’ and ‘O’ atoms of the Val30 and Val31 residues are also involved in the formation of strong H-bond interactions with the same moiety (Gilbert, 2002; Sharma & Bharatam, 2021). Followed by pteridine sub-site, benzamide sub-site is present which accommodates the benzamide moiety by making π-π interaction with Phe56 residue. Benzamide sub-site of \textit{LdDHFR} is highly hydrophobic in nature and wider than that of human DHFR (\textit{HsDHFR}) due to the presence of Met53 residue in place of Phe31 (\textit{HsDHFR}) (Gilbert, 2002). Benzamide sub-site further opens as a solvent accessible region to accommodate glutamate moiety. Glutamate sub-site of the \textit{LdDHFR} enzyme is similar to the \textit{HsDHFR} enzyme, the major difference is the presence of Phe91 (hydrophobic ring amino acid...
residue) in LdDHFR in place of Asn64 (polar amino acid) (Gilbert, 2002; Sharma et al., 2017; Zuccotto et al., 1998). In the previous reports, it was clearly indicated that the targeting Phe91 residue along with the Glu43, Ser44, Met53 and Lys57 are important for achieving the high selectivity toward LdDHFR enzyme (Sharma et al., 2017; Zuccotto et al., 1998).

The well-known DHFR inhibitors are Methotrexate (MTX), Pyrimethamine (PYR), Trimethoprim (TMP) and Cycloguanil (CYG). DHF and MTX carries 2,4-diaminopteridine moiety while Pyrimethamine (PYR) and Trimethoprim (TMP) contain 2,4-diaminopyrimidine moiety. Cycloguanil (CYG) possesses the 2,4-diaminotriazine moiety. The scaffolds present in the above mentioned drugs are highly basic and show high proton affinity in nature (Cocco et al., 1983). Cocco et al. (1981) performed a Carbon-13 Nuclear Magnetic Resonance (C-13 NMR) study on MTX, aminopterin, and folate to examine (i) the protonation of the pteridine ring, (ii) stability of these compounds at pH range 6–10 and (iii) the effect of protonation in the binding with DHFR. They suggested that the protonated state of these compounds are stable up to pH range 6–10 and binding of protonated form is more prominent as compared to non-protonated form. Later, in 1983, the same group reported C13-NMR based study on MTX, TMP and PYR to observe the co-relation between the protonation and the binding affinity (Cocco et al., 1981, 1983). The study was carried out on three different sources of dihydrofolate reductase for binding analysis and the results revealed that the protonated forms of these compounds (especially N1 protonated form) were most strongly bound in all three reductase at pH 7–10. It was also observed that N1 protonated inhibitors exhibit strong interaction with the carboxyl group of Asp/Glu residue of DHFR active site and maintained this strong association with DHFR up to pH 10. The authors also mentioned that the known antifolates get protonated at physiological pH before exhibiting drug action.

Our lab has been extensively working in the design and synthesis of Plasmodium falciparum DHFR (PfDHFR) inhibitors employing various pharmacoinformatic strategies such as quantum chemical methods, molecular docking, virtual screening, and molecular dynamics simulation (Abbat et al., 2015; Abbat & Bharatam, 2016; Adane et al., 2014; Adane & Bharatam, 2011; Bhagat et al., 2019; Jaladanki et al., 2020). Considering the limited availability of drugs for the treatment of Leishmania, there is a pressing need to develop effective anti-leishmanial drugs to reduce the disease burden. In the present study, we attempt to design a virtual combinatorial library of LdDHFR inhibitors by considering the proton affinity of the various scaffolds using Density Functional Theory (DFT) study. The designed combinatorial library was screened for LdDHFR using a molecular docking strategy. The selectivity and binding potential of the designed molecules toward the LdDHFR with respect to HsDHFR were ensured through the re-docking of selected hits in the active site of the HsDHFR. In the next step, MD simulations were carried out on selected LdDHFR hits to validate their selectivity and binding toward LdDHFR.

Computational methods

Data collection

In this study, 20 scaffolds (head group), 20 different linkers and 32 tail groups were considered. All the chosen fragments are part of the various reported DHFR inhibitors which were collected from ChEMBL database and literature (https://www.ebi.ac.uk/chembl; Abbat et al., 2015; Adane et al., 2014; Adane & Bharatam, 2008, 2011, 2016; Bhagat et al., 2019; Tawari et al., 2011).

Quantum chemical analysis

In order to find a suitable scaffold that binds to the LdDHFR at pteridine binding sub-site, a highly basic functional unit is required. To estimate the possible stable protonation state of all the twenty scaffolds, proton affinity (PA) was calculated using the Density Functional Theory (DFT) (Bartolotti & Fluchick, 1996; Bhagat et al., 2017, 2019; Laird et al., 1996; Parr & Yang, 1989). All the quantum chemical calculations were carried out using Gaussian09 software (Frisch et al., 2009). Initially, all the twenty scaffolds were optimized using the B3LYP (Becke3, Lee, Yang, Parr) method (Becke, 1993; Lee et al., 1988) with 6–31 + G(d) basis set. Proton affinity was estimated at various ‘N’ atoms of all the scaffolds considered under the study. All the structures were optimized in the gas phase using the above cited method.

On the basis of proton affinity, 13 scaffolds were selected for further study. For selection criteria, minimum PA was chosen as 230 kcal/mol as this value is close to the super base character >240 kcal/mol. The structures of the selected head groups are pointed in Table S1 (Supplementary material). Selected scaffolds were manually linked with the chosen tail groups using various linkers as mentioned in Table S1 (Supplementary material). From this exercise, a virtual combinatorial library (Huc & Lehn, 1997; Lehn, 1999; Ramström & Lehn, 2002) of ~8300 molecules was designed for screening to identify possible LdDHFR inhibitors.

Molecular docking

All the designed molecules were first screened against LdDHFR using the molecular docking method. For the molecular docking analysis, all the designed molecules were prepared using the LigPrep module of Schrödinger software (LigPrep 3.5, Schrödinger LLC, 2015). Likewise, protein preparation and grid generation for the LdDHFR active site were carried out using protein preparation wizard and receptor grid generation module, respectively, incorporated in Schrödinger software. Finally, the prepared molecules were docked in the active site of LdDHFR using the standard operating procedure with standard precision (SP) protocol in the Glide module of Schrödinger software (Glide 6.7, Schrödinger, LLC, 2015; Friesner et al., 2004). The Glide scoring employed in Glide 6.7 is based on the empirical ChemScore function reported by Eldridge et al. (1997) which is represented by GScore (Equation 1). The components of GScore are (i) Coulomb-van der Waals interaction-energy
score; (ii) lipophilic lipophilic term; (iii) hydrogen bonding which is separated in the different component based on the electronegativity and charges of participating atoms; (iv) metal-ligand interaction term; (v) penalty for freezing rotatable bonds; (vi) Site term include polar interaction (non-hydrogen bonding) in the active site and (vii) ‘Rewards’ in the equation refers to the penalties for various features such as buried polar groups, hydrophobic enclosure, correlated hydrogen bonds, and amide twists etc. (de Lima et al., 2016; Friesner et al., 2004; Kuca et al., 2018). For each ligand, 50 poses were generated and utmost 10 poses per ligand were saved to the sorted pose file. On the basis of Glide Gscore (Gscore/C20/C0 6 kcal/mol) and molecular recognition interactions with the key active site residues of \( \text{LdDHFR} \) and their accommodation in the active site, 100 best hits were selected.

\[
\text{GScore} = 0.05 \cdot \text{vdW} + 0.15 \cdot \text{Coul} + \text{Lipo} + \text{Hbond} + \text{Metal} + \text{Rewards} + \text{RotB} + \text{Site}
\]

PyMOL software was utilized to visualize ligand-protein interactions (DeLano, 2002). All the selected hits were re-docked in the active site of \( \text{HsDHFR} \) by following the above mentioned docking protocol. All the 100 hits (selected docked conformation) were complexed with the \( \text{LdDHFR} \) and \( \text{HsDHFR} \), respectively and were submitted for the prime-MMGBSA (Prime 4.0, Schrödinger LLC, 2015) analysis. The selectivity of the designed compounds was ensured through the comparative analysis of the Gscore and prime-MMGBSA energy contribution in the \( \text{LdDHFR} \) and \( \text{HsDHFR} \), respectively. The complete methodology utilized in this study is depicted in Figure 1.

**Molecular dynamics simulations**

To evaluate the stability as well as to estimate the binding affinity of the selected hits in the active site of \( \text{LdDHFR} \), molecular dynamics (MD) simulations were performed for the various ternary complexes (\( \text{LdDHFR-NADPH-Ligand} \)) using AMBER 12 package (Case et al., 2012). The first step of MD simulation is the preparation of ligand(s) (all in the protonated state) which was done using Antechamber program by incorporating GAFF (General Amber Force Field) force field (Wang et al., 2004). In the next step, Tleap program was utilized to generate initial parameter topology (prmtop) files and initial coordinate (inpcrd) files for ligands and complexes by implementing Amber GAFF and ff99SB force field parameters (Lindorff-Larsen et al., 2010). During this step, protein-ligand and ternary complexes were solvated using TIP3P water (Mark & Nilsson, 2001) with solvation box extended to 20 Å in each direction of the solute forming a cubic box. The solvated complexes were subjected to minimization, the gradual heating of the minimized system from 0 to 300 K, density equilibration under NPT ensemble, followed by constant pressure equilibration (NPT) of 1 ns at 300 K and 1 atm pressure (pressure relaxation time of 2 ps). In the final step, a production run for 50 ns was performed under NPT ensemble (with non-bonded interactions cutoff distance of 12 Å).

The relative binding free energy for the protein-ligand complex formation was estimated using Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method (Genheden & Ryde, 2015; Hou et al., 2011). The MM-GBSA calculations were performed on the last 5 ns trajectory obtained from MD simulations of protein-ligand complexes to ensure good conformational sampling and reliable binding free energy calculations. To estimate the stability of ligand and protein in protein-ligand complexes throughout the MD simulation runs RMSD and B-factor was calculated using the CPPTRAJ module of AMBER software (Roe & Cheatham, 2013). The Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) tool was utilized to analyze hydrogen bond occupancy in each ternary complex. To evaluate ADME profiling of selected five hits, QikProp module of Schrödinger software was utilized.
Results and discussion

Various reports suggested that H-bond interactions with the Asp52 (negatively charged residue) is necessary to establish a molecule in the active site of the LdDHFR which requires a positively charged basic moiety so that it can strongly bind with Asp52 (a negatively charged amino acid) residue (Gilbert, 2002; Sharma et al., 2017; Zuccotto et al., 1998). Moreover, Phe91 a ring hydrophobic residue, along with the Glu43, Ser44, Met53 and Lys57 are responsible for the selective inhibition of LdDHFR (Gilbert, 2002; Khabnadideh et al., 2005; Zuccotto et al., 1998). Herein, attempts have been made to design molecules of highly basic nature (or possess higher proton affinity value) so that they can protonate easily at biological pH and thus interact with the negatively charged Asp52 residue at pteridine sub-site of the LdDHFR active site. Therefore, the basicity or proton affinity (Gas Phase Basicity) of the twenty scaffolds understudy were estimated using Guassian09 (Frisch et al., 2009; Kathuria et al., 2018) (see Supplementary material Table S1).

We started the study, by taking the natural substrate of DHFR i.e. dihydrofolate (DHF) which is having two sites for protonation i.e. N1 and N2. The calculated proton affinity values at N1 and N2 centers are 230.46 and 229.25 kcal/mol, respectively. The difference in proton affinity values for these two sites are not significant hence 230 kcal/mol was considered as a minimum reference proton affinity value for the scaffolds selectivity and hence, the head groups that show PA values ≥ 230 kcal/mol were considered for designing virtual combinatorial library. Thirteen scaffolds out of the twenty scaffolds (biguanide, benzpyrazole, guanopyrimidine, benzguanopyrimidine, guanopyridine, benzguanopyridine, 2,4-diaminopyrimidine, 2,4-diaminotriazine, 2-aminoteridin-4-one, 2,4-diaminoteridin, GTU, 4-aminoquinoline and 2,4-diaminoquinazoline) exhibit proton affinity values ≥ 230 kcal/mol and the same were utilized along with 20 different linkers and 32 tail groups in the design of a virtual combinatorial library (Supplementary material Table S1) of ~8320 molecules. All the ligands were considered to be in their mono-protonated form.

Molecular docking and prime-MMGBSA analysis

The designed ~8300 molecules from a combinatorial library were docked in the active site of LdDHFR followed by prime-MMGBSA analysis. The best 100 molecules were identified based on binding score (Glide Gscore ≤ −6 kcal/mol) as well as molecular recognizable interactions with key active site residues of LdDHFR. To ensure the selectivity toward LdDHFR, these 100 molecules were re-docked in the active site of HsDHFR followed by prime-MMGBSA analysis. On the basis of binding score and binding free energy value in LdDHFR, these 100 molecules were categorized into three major groups (Group 1–3). Group 1 (20 hits) molecules showed less binding score (Glide Gscore) toward LdDHFR but they possess binding free energy (Prime-MMGBSA) equal or greater than HsDHFR (highlighted with orange rows in Supplementary material Table S2). Group 2 (15 molecules) molecules showed binding free energy and Glide Gscore greater than or equal to HsDHFR (highlighted with yellow rows in Supplementary material Table S2). Likewise, Group 3 molecules (14 hits, highlighted with green rows in Supplementary material Table S2) 24DIAMPYL5T14CC1, BGNL15T17, BGNL15T23 (B23), BGNL19T16, BGNL2T24, BGNL2T28 (B28), BGNL5T17, BGNL7T14CC2, BGNL7T30, BGNL8T26, GTUL13T27 (G27), GTUL15T16 (G16), GTUL6T28, PYRIMIDINYLGUAN13T32 (P32) showed greater binding affinities (Glide Gscore) and found to be energetically more favorable for the LdDHFR. The molecules with ≥ −10 kcal/mol binding free energy difference between LdDHFR and HsDHFR in comparison to HsDHFR are considered under Group 3). The remaining 51 molecules have not shown better Glide Gscore as well as prime-MMGBSA score in comparison to HsDHFR. All the 14 hits showed multiple H-bond interactions with the key residue Asp52 which is crucial to establish a ligand in the active site of LdDHFR.
Among the 14 hits, 5 molecules (leads, see Figure 2) B23, B28, G16, G27 and P32 have been found to be best and their molecular interactions with the LdDHFR have been discussed below. In B23, biguanide amine group showed H-bond interactions with the main chain ‘O’ atom of Val30 and multiple H-bonds with the side chain ‘O’ atoms of Asp52. H-bond interaction with the main chain ‘N’ atom of Val49 and ‘O’ atom of alkyl methoxy tail group were also observed. Similarly, multiple H-bond interactions between amine group of biguanide moiety of B28 and Asp52 was observed. Apart from that, ‘O’ atom of carboxylic tail group of B28 showed multiple H-bond interactions with the side chain ‘N’ atom of Arg97 along with H-bond interaction between side chain ‘N’ atoms of Thr61 and the carboxylic group of B28 at glutamate pocket of active site were observed. This additional H-bond interaction between Thr61 and carboxylic group helps in establishment of B28 molecule in LdDHFR active site along with the π–π hydrophobic interaction between Phe91 and chlorobenzene ring at glutamate sub-site which was absent in HsDHFR active site. In G16, ‘N’ atoms of GTU moiety showed multiple H-bonds with main chain ‘O’ atom of Val30 and side chain ‘O’ atom of Asp52. Similarly, multiple H-bond interactions between carboxylic tail groups of G16 and side chain ‘N’ atoms of Arg48, Arg97, respectively along with side chain ‘O’ atom of Thr61 were also observed. Likewise, multiple H-bond interactions between side chain ‘O’ atom of Asp52 and ‘N’ atoms of GTU moiety, ‘O’ atoms of carboxylic tail group and side chain ‘N’ atoms of Arg97, respectively were observed in G27. Apart from that, H-bond interactions between carboxylic tail groups and Lys57 and Arg97 along with Val156 were also observed in G27 molecule. In P32, ‘N’ atoms of Guanabenz moiety formed multiple H-bonds with the side chain ‘O’ atoms of Asp52. H-bond interactions between main chain ‘O’ atom of Val31 and ‘N’ atom of Guanabenz and between ‘CI’ atom of tail group and side chain ‘N’ atom of Arg97 also exist. Apart from this, π–π hydrophobic interaction between pyrimidine ring of P32 and Phe56 residue which help P32 molecule to accommodate in the active site of LdDHFR was also observed in P32 molecule (see Figures 2 and 3 and Supplementary material Tables S2 and S3).

**MD simulation analysis**

Best five hits B23, B28, G16, G27 and P32 obtained from molecular docking followed by prime-MMGBSA analysis were chosen for MD simulation study to understand the stability, interaction profiling as well as to estimate the binding affinities of the selected five molecules under dynamic conditions. The purpose of current MD simulations is to estimate binding affinity between the ligand and target macromolecule and to further compare with that of standard molecule. For this purpose, reliable starting structures for MD simulations are most important and essential. The same was obtained using molecular docking analysis. The suitability of pose was assessed based on the pharmacophoric features of known ligands and the known interactions with the macro-molecules (Kuca et al., 2018). First, ternary complexes for all
the five hits were prepared (LdDHFR-NADPH-Ligands) and subjected to MD simulations for 50 ns. The stability of all the five ternary complexes was analyzed in terms of RMSD from the starting structure. The whole protein and backbone RMSD values suggested that all the five complexes get stabilized after 35 ns. The last 10 ns simulation runs (40 ns to 50 ns) data suggested that all the five complexes were maintained their stability and RMSD was in the range of acceptability i.e. < 2 Å. Similarly, RMSD values of all the hits in ternary complexes suggested that all the ligands achieved sufficient stability during the simulations run and RMSD values were consistently stable in the last 10 ns simulations run except G16 (Figure 4, Supplementary material Figure S1). In order to further confirm the stability of compound G16 in the ternary complex, LdDHFR-NADPH-G16 complex was subjected to MD simulations for additional 20 ns (a total of 70 ns). Analysis of MD simulations data such as protein backbone RMSD, ligand RMSD, B-factor and Per-residue decomposition for LdDHFR-NADPH-G16 ternary complex (depicted in Supplementary material Figure S3) for last 20 ns (50 ns to 70 ns) reassured that G16 molecule maintains sufficient stability in the ternary complex during the simulations run.

H-bond occupancy data for the selected five hits were retrieved through the VMD tool. H-bond occupancy data suggested that compound B23 showed greater propensity to form H-bond interactions with the Asp52 (93.20% and 6.40%) in comparison to the other four ligands (see Figure 5, Supplementary material Figure S2). Compound B23 showed very weak H-bond occupancy with Val30 (4.20%) and Val156 (15.00%). In B28, Asp52 (71.40%), Thr61 (67.80%) and Arg97 (81.40%) exhibit strong H-bond contribution while, Val30 (17.20%), Val31 (29.20%) and Lys57 (38.80) showed weak to moderate H-bond occupancy in the ligand-protein ternary complex. Likewise for G16, Arg48 (103.80%) was the strongest contributor in H-bonding with multiple bond formation. Asp52 (47.20%), Arg97 (34.40%) and Val156 (60.60%) also showed moderate to strong impact on H-bonding in G16 while Lys57 (12.00%) possessed very weak impact on H-bond formation.

Compound G27 showed greater propensity to form H-bond interactions with the Thr61 (64.80%) and Arg97 (78.60%). Asp52 (27.80%) showed moderate impact on H-bond formation for compound G27 along with very weak propensity with Val156 (10.60%). Apart from that, very weak H-bond occupancy of G27 with Ser86 (2.00%) and Val30 (5.20%) was also observed. Compound P32 showed greater propensity to form H-bond interactions with the Asp52 (43.20%) with very weak contribution from Phe56 (1.20%) and Ser86 (4.00%).

B-factor and atomic fluctuation data of all the five ternary complexes revealed that there were no major fluctuations in the 3D structures of the complexes. Major atomic displacements were observed for the loop regions especially for the starting N-terminal loop (amino acids 5–20). Minor fluctuations at the atomic level were also recorded for the amino acid regions 60–75, 115–120, 190–200 and 210–220 which corresponded to loop regions and far from the core active site residues (Figure 5).

Per-residue decomposition analysis of the selected hits indicated that amino acids Asp52, Met53, Phe56 and Arg97 have major contribution in the ligand-binding pocket in the LdDHFR active site. In the case of B23, major contributors for binding are Val30, Asp52, Phe56 and Val87 while Arg97...
showed a very small negative impact on binding. Similarly, Val30, Val31, Asp52, Met53, Phe56, Lys57, Thr61 and Arg97 were the major interacting residues for binding of B28 with LdDHFR. Asp52, Met53, Phe56, Arg97 and Val156 showed major binding contribution for compound G16 in the LdDHFR active site. Small binding energy contribution of Lys57, Val87, Phe91 and Leu94 were also observed for the compound G16. In compound G27, Asp52, Met53, Phe56, Thr61, Val87, Leu94, Arg97 and Val156 were the major energy contributors in the ligand binding domain. Apart from that, mild energy contributions of Ile45, Lys57 and Phe91 also observed for G27. Likewise, Asp52 and Phe56 showed greater binding energy contribution for compound P32 while amino acids Val31, Ser86 and Val87 moderately contributed to ligand binding in the LdDHFR active site.

MMGBSA analysis of last 5 ns trajectories was carried out for selected five hits which reveals that out of five hits four molecules B23, B28, G16 and P32 possess greater binding affinity toward LdDHFR (−65.06, −80.09, −67.54 and −52.37 kcal/mol, respectively) in comparison to the previously reported lead molecule (−58.65 kcal/mol) (Sharma & Bharatam, 2021). Compound G27 shows more selectivity toward HsDHFR due to van der Waals contribution. Among all, compound B28 showed the highest binding affinity (−80.09 kcal/mol) toward LdDHFR enzyme which is even higher than the natural substrate i.e. protonated dihydrofolate (DHFP, −75.99 kcal/mol) (Sharma & Bharatam, 2021).

**ADME analysis**

In an attempt to find potential hits with good pharmacokinetic properties we evaluated the ADME properties of all the five hits using computational ADME/Toxicity prediction tools. The pharmacokinetic profile of molecules was carried out using QikProp module of Schrödinger software (QikProp 4.3, Schrödinger LLC, 2015). The pharmacokinetic profiles of these molecules suggest it is worthy consideration these molecules for further study. Table S5 (Supplementary material) depicting the physically relevant descriptors of ADME have been provided in the supporting information.

**Conclusions**

The aim of the current work is to design selective novel inhibitors for LdDHFR. In an attempt to do so, pharmacoinformatic approaches like quantum medicinal chemistry methods (DFT) for the calculation of scaffolds basicity (proton...
affinity), virtual combinatorial library design, molecular docking, and MD simulations were utilized. On the basis of proton affinity, 13 functional groups were selected as head groups. Previously reported 20 linkers and 32 tail groups along with 13 scaffolds were utilized to design a virtual combinatorial library of ~8300 molecules.

On the basis of pharmacoinformatic strategies, 14 hits were finalized which had the greater binding affinity and energetically more favorable for the LdDHFR enzyme. Most of the selected hits showed H-bond interaction with the key binding residue i.e. Asp52 along with the π-π or π-cation hydrophobic interaction with the Phe56/Phe91. Five selected hits B23, B28, G16, G27 and P32 were further forwarded to MD simulation of 50 ns run. Based on the stable interactions and binding free energies contribution toward LdDHFR, four designed selective compounds namely BGNL15T23 (B23), BGNL2T28 (B28), GTUL15T16 (G16) and PYRIMIDINYL-GUANL13T32 (P32) have been identified as new leads toward LdDHFR enzyme inhibition. All the four hits showed significant molecular binding affinity toward LdDHFR with respect to HsDHFR enzyme and thus suggested to carry forward for further biological evaluation and optimization.

Acknowledgements

VKS thanks NIPER S.A.S. Nagar for providing research facilities. PVB thanks Department of Biotechnology (DBT) for financial assistance. DK acknowledges financial support from the INSPIRE program of Department of Science and Technology (DST), New Delhi.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Vishnu Kumar Sharma http://orcid.org/0000-0003-3182-3379

References

Adane, L., Bhagat, S., Arfeen, M., Bhatia, S., Sirawaraporn, R., Sirawaraporn, W., Chakraborti, A. K., & Bharatam, P. V. (2014). Design and synthesis of guanithiourea derivatives as potential inhibitors of Plasmodium falciparum dihydrofolate reductase enzyme. Bioorganic & Medicinal Chemistry Letters, 24, 613–617. https://doi.org/10.1016/j.bmcl.2013.12.009

Abbat, S., Bhagat, S., & Bharatam, P. V. (2015). PdHFR enzyme inhibitors: Rational design using pharmacoinformatic tools. Frontiers in Medicinal Chemistry, 7, 228–273.

Abbat, S., & Bharatam, P. V. (2016). Electronic structure and conformational analysis of P218: An antimalarial drug candidate. International Journal of Quantum Chemistry, 116, 1362–1369. https://doi.org/10.1002/qua.25189

Adane, L., & Bharatam, P. V. (2008). Modelling and informatics in the analysis of P. falciparum DHFR enzyme inhibitors. Current Medicinal Chemistry, 15, 1552–1569. https://doi.org/10.2174/092986708784911551

Adane, L., & Bharatam, P. V. (2011). Computer-aided molecular design of 1H-imidazole-2, 4-diamine derivatives as potential inhibitors of Plasmodium falciparum DHFR enzyme. Journal of Molecular Modeling, 17, 657–667.

Bartolotti, L., & Fluchick, K. (1996). In K. B Lipkowitz & D. B. Boyd (Eds.), Reviews in computational chemistry (Vol. 7, p. 187). VCH Publishers.

Becke, A. (1993). Density-functional thermochemistry. III. The role of exact exchange. Journal of Chemical Physics, 98, 5648. https://doi.org/10.1063/1.464913

Bhagat, S., Arfeen, M., Adane, L., Singh, S., Singh, P. P., Chakraborti, A. K., & Bharatam, P. V. (2017). Guanithiourea derivatives as potential antimalarial agents: Synthesis, in vivo and molecular modelling studies. European Journal of Medicinal Chemistry, 135, 339–348.

Bhagat, S., Arfeen, M., Das, G., Ramkumar, M., Khan, S. I., Tekwani, B. L., & Bharatam, P. V. (2019). Design, synthesis and biological evaluation of 4-aminoquinoline-guanithiourea derivatives as antimalarial agents. Bioorganic & Medicinal Chemistry, 91, 103094.

Case, D. A., Darden, T. A., Cheatham III, T. L., Simmerling, C. L., Wang, J., Duke, R. E., Luo, R., Walker, R. C., Zhang, W., & Merz, K. M. (2012). AMBER 12; University of California.

Cocco, L., Groff, J. P., Temple, C. J., Montgomery, J. A., London, R. E., Matwiyoff, N., & Blakley, R. (1981). Carbon-13 nuclear magnetic resonance study of protonation of methotrexate and aminopterin bound to dihydrofolate reductase. Biochemistry, 20, 3972–3978. https://doi.org/10.1021/bi00517a005

Cocco, L., Roth, B., Temple, C., Montgomery, J. A., London, R. E., & Blakley, R. L. (1983). Protonated state of methotrexate, trimethoprim, and pyrimethamine bound to dihydrofolate reductase. Archives of Biochemistry and Biophysics, 226, 567–577.

de Lima, W. E., Pereira, A. F., de Castro, A. A., da Cunha, E. F., & Ramalho, T. C. (2016). Flexibility in the molecular design of acetylicholinesterase reactivators: Probing representative conformations by chemometric techniques and docking/QM calculations. Letters in Drug Design & Discovery, 13, 360–371.

DeLano, W. L. (2002). Pyml: An open-source molecular graphics tool. CCP4 Newsletter On Protein Crystallography, 40, 82–92.

Eldridge, M. D., Murray, C. W., Auton, T. R., Paolini, G. V., & Mee, R. P. (1997). Empirical scoring functions: I. The development of a fast empirical scoring function to estimate the binding affinity of ligands in receptor complexes. Journal of Computer-Aided Molecular Design, 11, 425–445.

Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, E. A., Dillner, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., & Perry, J. K. (2004). Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. Journal of Medicinal Chemistry, 47, 1739–1749.

Frisch, M., Trucks, G., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., & Petersson, G. (2009). Gaussian 09, Revision d. 01. Gaussian, Inc.

Genheden, S., & Ryde, U. (2015). The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. Expert Opinion on drug discovery, 10, 449–461. https://doi.org/10.1517/17460441.2015.102936

Gilbert, I. H. (2002). Inhibitors of dihydrofolate reductase in Leishmania and trypanosomes. Biochimica et Biophysica Acta, Molecular Basis of Disease, 1587, 249–257.

Glide 6.7. (2015). Schrödinger, LLC.

Hou, T., Wang, J., Li, Y., & Wang, W. (2011). Assessing the performance of the MM/PBSA and MM/GBSA methods. Journal of Chemical Information and Modeling, 51, 69–82. https://doi.org/10.1021/ci100275a

https://www.ebi.ac.uk/chembl

Huc, I., & Lehnh, J.-M. (1997). Virtual combinatorial libraries: Dynamic generation of molecular and supramolecular diversity by self-assembly. Proceedings of the National Academy of Sciences, 94, 2106–2110. https://doi.org/10.1073/pnas.94.6.2106

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. Journal of Molecular Graphics, 14, 33–38. https://doi.org/10.1016/0263-7855(96)00018-5

Jaladanki, C. K., Gahlawat, A., Rathod, G., Sandhu, H., Jahan, K., & Bharatam, P. V. (2020). Mechanistic studies on the drug metabolism and toxicity originating from cytochromes P450. Drug Metabolism Reviews, 52, 366–394. https://doi.org/10.1080/03602532.2020.1765792
Kathuria, D., Bankar, A. A., & Bharatam, P. V. (2018). “What’s in a structure?” The story of biguanides. Journal of Molecular Structure, 1152, 61–78. https://doi.org/10.1016/j.molstruc.2017.08.100

Khabnadideh, S., Pez, D., Musso, A., Brun, R., Pérez, L. M. R., González-Pacanowska, D., & Gilbert, I. H. (2005). Design, synthesis and evaluation of 2, 4-diamoquinazolines as inhibitors of trypanosomal and leishmanial dihydrofolate reductase. Bioorganic & Medicinal Chemistry, 13, 2637–2649.

Knighton, D. R., Kan, C.-C., Howland, E., Janson, C. A., Hostomska, Z., Lee, C., Yang, W., & Parr, R. G. (1988). Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. Physical Review B, 37, 186–194. https://doi.org/10.1002/jcc.24105

Lehn, J. M. (1999). Dynamic combinatorial chemistry and virtual combinatorial libraries. Chemistry – A European Journal, 5, 2455–2463. https://doi.org/10.1002/(SICI)1521-3765(19990903)5:9<2455::AID-CHEM2455>3.0.CO;2-H

LigPrep. (2015). Version 3.5, Schrödinger LLC.

Lindorff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J. L., Dror, R. O., & Shaw, D. E. (2010). Improved side-chain torsion potentials for the Amber ff99SB protein force field. Proteins: Structure, Function, and Bioinformatics, 78, 1950–1958.

Mark, P., & Nilsson, L. (2001). Structure and dynamics of the TIP3P, SPC, and SPC/E water models at 298 K. The Journal of Physical Chemistry A, 105, 9954–9960.

Parr, R., & Yang, W. (1989). Density-functional theory of atoms and molecules. Oxford University Press.

Prime. (2015). Version 4.0, Schrödinger LLC.

QikProp. (2015). Version 4.3, Schrödinger LLC.

Ramstrom, O., & Lehn, J.-M. (2002). Drug discovery by dynamic combinatorial libraries. Nature Reviews Drug Discovery, 1, 26. https://doi.org/10.1038/nrd704

Roe, D. R., & Cheatham, T. E., III. (2013). PTRAJ and CPPTRAJ: Software for processing and analysis of molecular dynamics trajectory data. Journal of Chemical Theory and Computation, 9, 3084–3095. https://doi.org/10.1021/ct400341p

Schnell, J. R., Dyson, H. J., & Wright, P. E. (2004). Structure, dynamics, and catalytic function of dihydrofolate reductase. Annual Review of Biophysics and Biomolecular Structure, 33, 119–140.

Sharma, V. K., Abbat, S., & Bharatam, P. (2017). Pharmacoinformatic study on the selective inhibition of the protozoan dihydrofolate reductase enzymes. Molecular Informatics, 36, 1600156. https://doi.org/10.1002/minf.201600156

Sharma, V. K., & Bharatam, P. V. (2021). Identification of selective inhibitors of LdDHFR enzyme using pharmacoinformatic methods. Journal of Computational Biology, 28, 43–59. https://doi.org/10.1089/cmb.2019.0332

Tawari, N. R., Bag, S., & Degani, M. S. (2011). A review of molecular modelling studies of dihydrofolate reductase inhibitors against opportunistic microorganisms and comprehensive evaluation of new models. Current Pharmaceutical Design, 17, 712–751. https://doi.org/10.2174/138161211795428966

Tjong, E., Dimri, M., & Mohiuddin, S. S. (2019). Biochemistry, tetrahydrofolate. StatPearls Publishing.

Vickers, T. J., & Beverley, S. M. (2011). Folate metabolic pathways in Leishmania. Essays in Biochemistry, 51, 63–80. https://doi.org/10.1042/bs510063

Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., & Case, D. A. (2004). Development and testing of a general amber force field. Journal of Computational Chemistry, 25, 1157–1174. https://doi.org/10.1002/jcc.20035

Zuccotto, F., Martin, A. C. R., Laskowski, R. A., Thornton, J. M., & Gilbert, I. H. (1998). Dihydrofolate reductase: A potential drug target in trypanosomes and leishmania. Journal of Computer-Aided Molecular Design, 12, 241–257. https://doi.org/10.1021/AC101608S005275