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In Silico Screening of Novel Antimalarials Against Mutated DHFR and DHPS

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Abstract Malaria continues to be a profoundly impactful infectious disease responsible for a significant number of fatalities annually, primarily attributed to Plasmodium falciparum. The onset of antifolate drug resistance, notably characterized by mutations in Plasmodium falciparum dihydrofolate reductase (pfDHFR) and Plasmodium falciparum dihydropteroate synthase (PfDHPS), has significantly undermined the effectiveness of current therapeutic interventions, highlighting the urgent need for the development of new inhibitory agents. This research delineates a thorough in silico methodology that employs structure-based virtual screening, ADMET profiling, and molecular docking analyses to discern promising lead compounds targeting pfDHFR. A collection of 400 synthetic compounds was evaluated against the quadruple-mutant (N51I+C59R+S108N+I164L) pfDHFR and triple mutant PfDHPS crystal structures (PDB: 1J3K and 6JWZ). Drug-likeness criteria grounded in Lipinski's Rule of Five and ADMET assessments were utilized to refine the selection of candidates. Molecular docking analyses were conducted employing AutoDock Vina, and binding affinities were compared with those of the reference antifolate compounds pyrimethamine, cycloguanil, and sulfadoxine. Five lead compounds — ZINC000019331645, ZINC000426406087, ZINC000001154555, ZINC000001160009, and ZINC000013283483 — exhibited exceptional binding energies between −8.0 and −8.5 kcal/mol, indicating stronger predicted binding than the three reference drugs. (sulfadoxine, pyrimethamine, cycloguanil). The findings indicate that the identified scaffolds are promising candidates for additional experimental validation as novel antimalarial drugs, relevant for addressing drug-resistant malaria.

Keywords: *Malaria, Plasmodium falciparum, Molecular Docking, Virtual Screening, Antifolate Resistance, Drug Discovery, AutoDock Vina; ADMET, In Silico*

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1. Introduction

Malaria continues to represent one of the most significant global public health challenges of the 21st century with the World Health Organization (WHO) reporting an estimated 282 million malaria cases and 610, 000 deaths recorded in 2024, with the overwhelming burden concentrated in sub-Saharan Africa and Southeast Asia [1]. Of the five Plasmodium species known to infect humans, Plasmodium falciparum is responsible for the most severe and lethal form of the disease, accounting for over 95% of malaria-related mortalities worldwide [2].

The antifolate class of antimalarials, particularly pyrimethamine and its combination with sulfadoxine (SP), has historically served as a cornerstone of malaria treatment [3,4]. These compounds exert their therapeutic effect by inhibiting dihydrofolate reductase (DHFR), an enzyme critical for folate biosynthesis [5]. Specifically, pfDHFR catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), which is an indispensable cofactor in the de novo synthesis of thymidylate and purine nucleotides required for DNA replication and cell proliferation [6]. Given that P. falciparum depends exclusively on de novo folate biosynthesis, unlike humans who obtain folate from dietary sources, pfDHFR represents a highly selective and validated drug target [7].

Unfortunately, the widespread deployment of antifolate drugs has been undermined by the rapid emergence and dissemination of resistant Plasmodium falciparum strains harboring mutations in the pfDHFR and pfDHPS gene [8,9].

The pressing necessity to engineer next-generation pfDHFR and pfDHPS inhibitors proficient in surmounting resistance has driven significant interest within the realm of computational drug discovery methodologies [10,11]. Structure-based virtual screening and molecular docking constitute robust and economically viable methodologies that utilize the three-dimensional structural data of target proteins to discern and prioritize potential ligands predicated on anticipated binding affinity and interaction compatibility [12]. These methods enable rapid interrogation of large chemical libraries at a fraction of the time and cost of high-throughput experimental screening [13,14].

The structural diversity of natural products and their semi-synthetic analogues have drawn significant interest as potential scaffolds with reported antimalarial activity [15]. Despite the progress made in discovering such compounds, the ability to identify compounds that meet the requirements of high binding affinity to both WT and QM pfDHFR, good pharmacokinetics, and low toxicity presents great difficulties [16].

This study aims to address this challenge through a systematic in silico pipeline integrating: (i) homology-validated target preparation of QM pfDHFR and pfDHPS structures; (ii) compound library curation and drug-likeness filtering; (iii) ADMET profiling; and (iv) comparative molecular docking analysis [17]. The outcomes of this study provide a prioritized set of lead compounds with the structural and energetic attributes required to advance toward experimental hit validation against drug-resistant malaria [18].

2. Materials and Methods

2.1.1. Target Protein Retrieval and Preparation

The three-dimensional crystal structures of the Quadruple mutant (N51I+C59R+S108N+I164L) Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) complexed with WR99210, NADPH, and Dump and pfDHPS were retrieved from the RCSB Protein Data Bank (PDB) using accession codes 1J3K and 6JWZ (QM, resolution: 2.21 Å). [19] Both structures were co-crystallized with the inhibitor WR99210, providing well-defined active site coordinates [20]. Protein preparation was performed using Chimera. The preparation workflow encompassed: (i) removal of water molecules and co-crystallized ligands; (ii) addition of missing hydrogen atoms and Gasteiger partial charges; (iii) merging of non-polar hydrogen atoms; (iv) assignment of atom types according to the AutoDock force field. The active site was identified based on the co-crystallized ligand binding pose. [21]

2.1.2. Compound Library Curation and Drug-Likeness Filtering

A virtual compound library was assembled by querying the SwissSimilarity web server (https://www.molecular-modelling.ch/swiss-drug-design.html) using three reference antimalarial drugs—sulfadoxine, pyrimethamine, and cycloguanil—as separate templates. [22] For each reference drug, the top 400 structurally analogous compounds were retrieved using commercial database and drug-like filter. Structural data for all 1,200 compounds (3 references × 400 compounds) were processed for format standardization, duplicate removal, and energy minimization [23]. Molecular similarity to each respective reference drug was calculated, and compounds were ranked based on their Tanimoto similarity coefficients [24]. The top 60 highest-scoring compounds for each reference drug (sulfadoxine, pyrimethamine, and cycloguanil) were selected for further analysis, yielding a total of 180 prioritized compounds.

Drug-likeness evaluation was implemented on the 180 selected compounds using multi-parametric filtering based on Lipinski's Rule of Five (MW ≤ 500 Da, cLogP ≤ 5, HBD ≤5, HBA≤10) using SWISSADME software (https://www.swissadme.ch/) [25,26]. PAINS filters were applied to eliminate promiscuous chemical scaffolds prone to assay interference. [27]

2.1.3. ADMET Profiling

The Pharmacokinetics (ADME) and toxicity parameters were predicted using SwissADME and pkCSM (https://biosig.lab.uq.edu.au/pkcsm/) respectively [25,28]. The evaluated parameters included gastrointestinal absorption, blood-brain barrier (BBB) permeability, P-glycoprotein (P-gp) substrate/inhibitor potential, cytochrome P450 (CYP) enzyme inhibition profiles (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4), aqueous solubility, plasma protein binding, and predictions for hepatotoxicity and cardiotoxicity (hERG channel inhibition). The CYP3A4 received the highest attention during analysis due to it being the most abundant hepatic isoform being responsible for the biotransformation of over 50% of drugs [29]. Only compounds that matched both favorable ADMET profiles and satisfactory drug-likeness criteria were chosen for molecular docking. [30]

Table 1. Docking scores, binding interactions, and predicted ADME parameters of cycloguanyl

COMPOUND	% SIMILARITY	DOCKING SCORE	BBB PERMEA-BILITY	LIPINSKI	GI ABSOR-PTON	PGP SUBST-RATE	CYP ENZYMES	LD50	HEPATO-TOXICITY	HERG 1 INHIBI-TION	HERG 2 INHIBI-TION	AMES TOXICITY
CYCLOGUANIL	-	-6.7	No	0	High	No	1A2	2.78	No	No	No	Yes
ZINC000001666573	0.933	-7.3	No	0	High	No	1A2	3.201	No	No	No	No
ZINC000000071371	0.921	-7.6	No	0	High	No	1A2	2.979	No	No	No	No
ZINC000005051752	0.908	-7.3	No	0	High	No	No	2.461	No	No	No	No
ZINC000016981852	0.902	-6.9	No	0	High	No	1A2	2.989	No	No	No	No
ZINC000016981838	0.897	-7.3	No	0	High	No	1A2, 2C19	3.036	No	No	No	No
ZINC000000183513	0.854	-6.8	No	0	High	No	1A2	3.154	No	No	No	No
ZINC000002464231	0.848	-7.6	No	0	High	No	No	2.987	No	No	No	No
ZINC000019331645	0.815	-8.5	No	0	High	No	No	2.697	No	No	No	No

COMPOUND	% SIMILARITY	DOCKING SCORE	BBB PERMEA-BILITY	LIPIN SKI	GI ABSOR-PTON	PGP SUBST-RATE	CYP ENZYMES	LD50	HEPATO-TOXICITY	HERG 1 INHIBI-TION	HERG 2 INHIBI-TION	AMES TOXICITY
ZINC000000063598	0.814	-7.4	No	0	High	Yes	1A2, 2C19	2.758	No	No	No	No
ZINC000013467525	0.781	-7.0	No	0	High	No	No	2.659	No	No	No	No

Table 2. Molecular docking energetics and pharmacokinetic profiling of sulfadoxine

COMPOUND	% SIMILARITY	DOCKING SCORE	BBB PERME-ABILITY	LIPI-NSKI	GI ABSOPTION	Pgp	CYP METABOLISM	LD50	HEPATO TOXICITY	Herg 1	Herg 2	AMES TOXICITY
SULFADOXINE	-	-6.6	No	0	High	No	No	1.746	Yes	No	No	No
ZINC000000002094	1.000	-6.8	No	0	High	No	No	1.782	Yes	No	No	No
ZINC000000049137	0.998	-7.0	No	0	High	No	No	1.94	Yes	No	No	No
ZINC000019877626	0.995	-7.0	No	0	Low	No	No	1.985	Yes	No	No	No
ZINC000086071788	0.988	-6.9	No	0	High	No	No	2.646	Yes	No	No	No
ZINC000071618084	0.987	-6.8	No	0	High	No	No	1.916	Yes	No	No	No
ZINC000095444306	0.984	-6.9	No	0	High	No	CYP 1A2, 2C19	2.8	Yes	No	No	No
ZINC000075166482	0.980	-7.3	No	0	High	No	No	2.781	Yes	No	No	No
ZINC000000311155	0.979	-6.8	No	0	High	No	No	2.555	Yes	No	No	No
ZINC000011615790	0.978	-7.5	No	0	Low	No	No	2.522	Yes	No	No	No
ZINC000001056190	0.976	-7.4	No	0	High	No	No	2.154	Yes	No	No	No
ZINC000000002100	0.973	-7.8	No	0	High	No	No	1.77	Yes	No	No	No
ZINC000000390734	0.973	-6.8	No	0	High	No	No	1.879	Yes	No	No	No
ZINC000075166481	0.971	-7.0	No	0	High	No	CYP 1A2	2.848	Yes	No	No	No
ZINC000075166486	0.970	-6.9	No	0	High	No	CYP 1A2, 2C19	3.000	Yes	No	No	No
ZINC000077100990	0.969	-6.9	No	0	High	No	CYP 1A2, 2C19, 2C9	2.959	Yes	No	No	No
ZINC000328594119	0.965	-7.0	No	0	Low	No	CYP 2C19, 2C9	2.26	Yes	No	No	No
ZINC000004543159	0.961	-7.1	No	0	High	No	No	2.165	Yes	No	No	No
ZINC000001772111	0.960	-7.4	No	0	High	No	CYP 2C9, 3A4	2.16	Yes	No	No	No
ZINC000000671377	0.953	-6.7	No	0	High	No	CYP 2C19, 2C9, 3A4	2.19	Yes	No	No	No
ZINC000000826341	0.953	-7.3	No	0	High	No	No	2.589	Yes	No	No	No
ZINC000000049142	0.952	-7.1	No	0	High	No	No	1.887	Yes	No	No	No
ZINC000000852704	0.951	-7.6	No	0	High	No	CYP 2C9, 3A4	2.738	Yes	No	No	No
ZINC000000392447	0.938	-7.3	No	0	High	No	No	1.933	Yes	No	No	No
ZINC000001160009	0.930	-8.0	No	0	High	No	CYP 1A2, 2C19, 2C9, 3A4	2.174	Yes	No	yes	No
ZINC000001154555	0.929	-8.4	No	0	High	No	CYP 1A2, 2C19, 2C9, 3A4	2.243	Yes	No	Yes	No

Table 3. Docking-based binding mode comparison and absorption–metabolism profile of pyrimethamine

CPD	% SIMILARITY	DOCKING SCORE	BBB PERMEABILITY	LIPINSKI	GI ABSOP	Pgp	CYP METABOLISM	LD50 MOL/KG	HEPATOTOXICITY	Herg 1	Herg 2	AMES Toxicity
PYRIMETHAMINE	-	-7.0	Yes	0	High	No	No	2.912	No	No	No	No
ZINC000013282257	0.999	-7.1	No	0	High	No	CYP 1A2	2.863	No	No	No	No
ZINC000013283483	0.993	-7.7	No	0	High	Yes	1A2, 2C19, 2D6, 3A4	2.82	Yes	No	Yes	No
ZINC000026466001	0.985	-7.1	No	0	High	No	1A2	2.73	Yes	No	No	No
ZINC000013212481	0.968	-7.5	No	0	High	Yes	1A2	2.909	Yes	No	Yes	No
ZINC000211018172	0.955	-7.3	No	0	High	No	1A2, 2C19, 2C9	2.553	Yes	No	No	No
ZINC000426406087	0.926	-8.1	No	0	High	Yes	1A2, 2D6, 3A4	3.043	Yes	No	No	No
ZINC000075273399	0.919	-7.7	No	0	High	No	1A2, 2C19, 2C9	2.553	Yes	No	No	No
ZINC000085388629	0.898	-7.9	No	0	High	Yes	1A2, 2C19	2.392	Yes	No	No	No
ZINC000013726676	0.876	-7.4	No	0	High	No	No Enzyme interactions	2.199	Yes	No	No	No

2.1.4. Ligand Preparation

Compounds with satisfying ADMET properties were selected and drawn on PubChem Sketcher tool. [31] The molfile was downloaded and converted to their respective 3-D structures by Avogadro software. [32] By using the Avogadro software, the 3-D structures were optimised to the most stable conformation by using MMFF94s as the force field. With the help of Chimera software, hydrogen atoms and charge were added to the stable conformations of the selected compounds.

2.1.5. Docking

Molecular docking studies were carried out using AutoDock Vina in UCSF Chimera, which involves docking of the ligand and the target protein as Mol.2 files. It applies an iterated local search algorithm together with a hybrid scoring function to predict ligand–protein interactions. [33] An exhaustiveness value of 32 was selected to ensure extensive conformational sampling, thereby improving docking reliability. [34] For each compound, the ten best binding poses were generated and ranked based on their predicted binding free energies (ΔG, kcal/mol). [35] The ligand-protein interaction was then visualized using Biovia Studio software in 2D dimension to analyze the critical amino acid interaction. [36]

3. Results and Discussion

Of the 400 structurally analogous compounds retrieved for each reference drug (cycloguanil, pyrimethamine, and sulfadoxine), percentage similarity cutoffs were applied as follows based on the distribution of similarity scores: 0.9 for sulfadoxine, 0.78 for cycloguanil, and 0.85 for pyrimethamine. This filtering process yielded 60 compounds per reference drug, which were subsequently subjected to ADMET analysis.

To determine drug-likeness and safety, the overall ADMET profiles of the reference medications (pyrimethamine, sulfadoxine, and cycloguanil) and their corresponding ZINC counterparts were examined. Cycloguanil showed high GI absorption, was not a Pgp substrate, and had no predicted hepatotoxicity or Herg inhibition. However, it lacked BBB permeability and tested positive for Ames toxicity. It is encouraging to note that all of the Cycloguanil analogues maintained the favorable characteristics of the parent drug (high GI absorption, no Pgp efflux, zero Lipinski violations, and no cardiotoxicity) while significantly improving safety by reducing Ames toxicity from "Yes" to "No". Sulfadoxine showed a low LD50, positive hepatotoxicity, strong GI absorption, and no Pgp efflux. With a few notable exceptions, such as ZINC000001160009 and ZINC000001154555, which produced improved docking scores but introduced several CYP interactions and hERG2 inhibition, raising safety concerns. However, most other sulfadoxine analogues shared the parent drug's overall profile.

Pyrimethamine was notable because it showed no hepatotoxicity, no Ames toxicity, high GI absorption, and BBB permeability. All pyrimethamine analogues, however, lost BBB permeability, suggesting reduced potential for central nervous system (CNS) penetration. This could be advantageous if CNS-related side effects are a concern for the parent drug. Sadly, several analogues added hepatotoxicity and hERG1 inhibition, which were not present in the parent drug. Docking scores (kcal/mol) served as a proxyfor binding affinity. All of the analogues surpassed cycloguanil (reference: -6.7), with

ZINC000019331645 having the highest affinity at -8.5, followed by ZINC000000071371 (-7.6) and ZINC000000063598 (-7.4), showing steadily improved binding across the series. Sulfadoxine (reference: -6.6) demonstrated even greater improvements; ZINC000001154555 (-8.4) and ZINC000001160009 (-8.0) showed the strongest binding, while ZINC000000002100 (-7.8) and ZINC000000852704 (-7.6) also significantly outperformed the parent. Several analogues outperformed pyrimethamine (reference: -7.0), including ZINC000426406087 (-8.1) and ZINC000085388629 (-7.9), while ZINC000013283483 and ZINC000075273399 both scored -7.7. All three series' projected binding affinities were generally increased by structural changes, with the sulfadoxine analogues exhibiting the biggest absolute improvements. Based on the docking scores and 2D diagrams, analysis of important amino acid interactions identifies likely structural factors of increased affinity. The parent compound in the cycloguanil series mostly forms hydrogen bonds and hydrophobic interactions with conserved DHFR residues like Ile14, Leu46, and Phe58. Extended aromatic character probably facilitates more π-π stacking or halogen bonding with Phe58 or Tyr170, which accounts for ZINC000019331645's higher affinity (-8.5). The top-scoring analogues (e.g., ZINC000001154555) of sulfadoxine, a sulfonamide that interacts with the DHPS pterin-binding site via hydrogen bonds with Asn115, Gln217, and Lys221, likely form additional electrostatic contacts or hydrophobic packing due to substituted aromatic rings; but these modifications also introduced potential metabolic soft spots, such as interactions with CYP2C9 and 3A4.

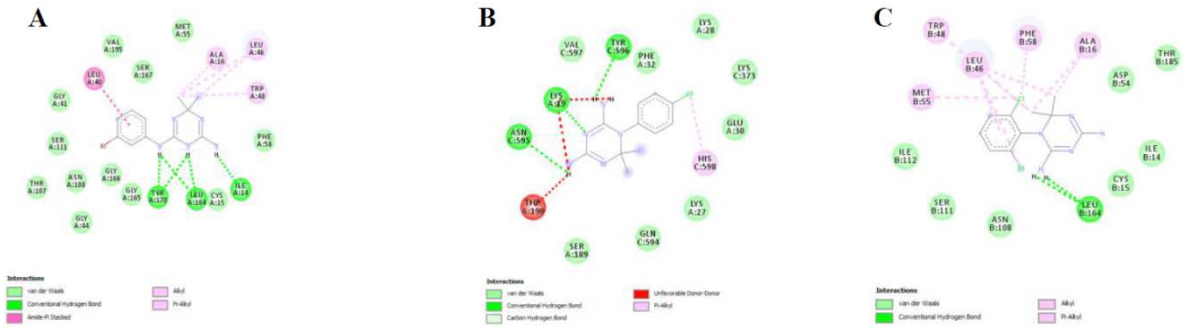

Figure 1. 2D interaction diagram of cycloguanil (A), ZINC000000071371 (B), ZINC000019331645 (C) bound to Plasmodium falciparum dihydrofolate reductase (pfDHFR)

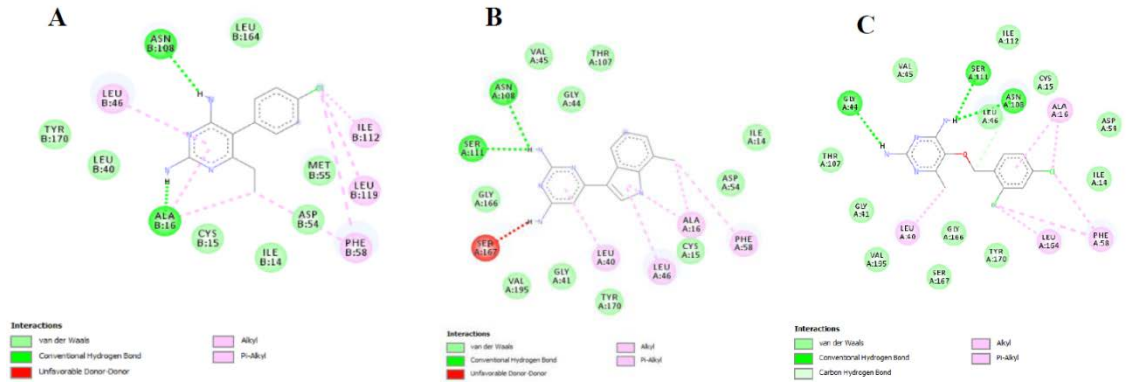

Figure 2. 2D binding diagram of pyrimethamine (A), ZINC000426406087 (B), ZINC000085388629 (C) in the active site of P.falciparum dihydrofolate reductase (pfDHFR)

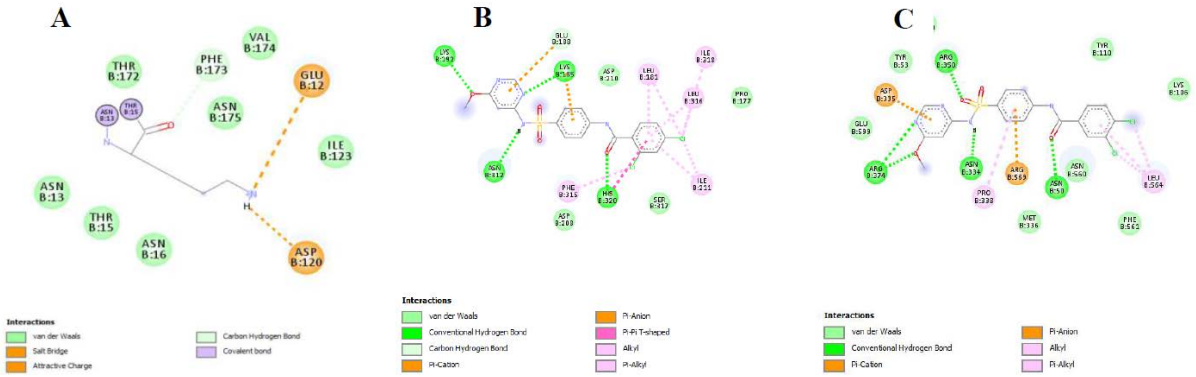

Figure 3. 2D binding diagram of Sulfadoxine (A), ZINC000001160009 (B), ZINC000001154555 (C) in the active site of P.falciparum dihydropteroate synthase (pfDHPS)

The parent in the pyrimethamine series binds DHFR by stacking with Phe58 and forming hydrogen bonds with Asp54 and Ile164. Although the in silico Pgp substrate prediction is still negative, the universal loss of BBB permeability across all analogues suggests that modifications increased polarity or introduced unrecognized Pgp recognition motifs. The strong affinity of ZINC000426406087 (-8.1) may be due to additional interactions with the hydrophobic subpocket (Leu46, Pro113). In this study, we have discovered several ZINC analogues with better binding affinities and advantageous or improved ADMET profiles. Analogues of cycloguanil, such as ZINC000019331645 (best docking: -8.5) and ZINC000000071371 (-7.6), effectively eliminated Ames toxicity while maintaining high GI absorption and low overall toxicity, making them viable options for additional enzymatic and cellular tests. While ZINC000001154555 and ZINC000001160009 had better docking scores (-8.4 and -8.0, respectively) for sulfadoxine, their broad CYP inhibition and hERG2 risk are concerning; ZINC000000002100 (-7.8) and ZINC0000000852704 (-7.6) offer a better balance between affinity and safety (no hERG, no Ames, minimal CYP), though hepatotoxicity is still a class-wide concern. ZINC000426406087 (-8.1) and ZINC000085388629 (-7.9) are strong binders for pyrimethamine, but they also added hepatotoxicity, even though analogues often lost the desired BBB permeability. Therefore, while these analogues may be suitable for peripheral indications, pyrimethamine might retain an advantage if CNS penetration is desired.

Lastly, we suggest that ZINC000019331645 (Cycloguanil series) and ZINC000000002100 (Sulfadoxine series) be given priority for experimental validation because they combine improved binding with better or comparable safety profiles in comparison to their respective reference drugs, whereas pyrimethamine analogues need to be further optimized to restore BBB permeability without sacrificing potency.

4. Conclusion

Here we present a systematic in silico pipeline to identify novel antifolate inhibitors against quadruple-mutant (N51I+C59R+S108N+I164L) Plasmodium falciparum dihydrofolate reductase (pfDHFR), a clinically validated target against which resistance to the first line antimalarials pyrimethamine, cycloguanil and sulfadoxine has developed. An integrated workflow of structure-based virtual screening, Lipinski drug-likeness filtering, extensive ADMET profiling and AutoDock Vina molecular docking prioritized five lead compounds, ZINC000019331645, ZINC000426406087, ZINC000001154555, ZINC000001160009 and ZINC000013283483 as the most promising candidates.These compounds showed improved binding free energies of -8.0 to -8.5 kcal/mol against the QM pfDHFR crystal structure (PDB: 1J3K) compared to the three reference antifolate drugs. Interaction analysis revealed that the increased binding affinities of the lead compounds are attributed to favorable hydrophobic packing, extended π–π stacking interactions with conserved active site residues (Phe58, Leu46), and additional hydrogen bond contacts with Asp54, Ile164, and associated catalytic residues. ADMET profiling confirmed that ZINC000019331645 (cycloguanil series) and ZINC000000002100 (sulfadoxine series) had particularly attractive safety margins, with improved or comparable toxicity profiles to their parent drugs, including removal of Ames mutagenicity and absence of hERG-mediated cardiotoxicity. However, some potent binding analogs, such as ZINC000001154555 and ZINC000001160009, presented broad CYP inhibition and hERG2 liability that require further structural optimization before moving forward.

In sum, the present results validate the utility of the employed computational framework for the fast and inexpensive discovery of structurally diverse scaffolds with the ability to bind the resistant pfDHFR active site with high affinity. The compounds identified provide a promising chemical foundation for the development of next-generation antifolates against drug-resistant malaria.

Recommendations

In light of the findings of the current investigation, the following recommendations are proposed to advance the identified lead compounds: 1. **Molecular Dynamics (MD) Simulation**: The static docking poses produced in this study depict a singular energetic snapshot of ligand–protein interaction, neglecting the conformational flexibility of the pfDHFR active site under physiological settings. We highly advocate for the comprehensive molecular dynamics simulations of all five lead compounds, utilizing known MD platforms such as GROMACS, AMBER, or NAMD, with a minimum trajectory duration of 100 ns. MD analysis will facilitate the evaluation of binding stability, conformational flexibility of the protein-ligand complex, and the longevity of dynamic interactions across time. Additional free-energy perturbation (FEP) or MM-PBSA/GBSA computations are required to get more precise and thermodynamically robust binding affinity predictions and a more refined lead ranking compared to static docking. 2. **Experimental Hit Validation**: The prioritizing of lead drugs driven by molecular dynamics should be substantiated using in vitro enzymatic inhibition experiments utilizing recombinant QM pfDHFR, followed by parasite growth inhibition studies against drug-resistant P. falciparum strains. This will validate the anticipated binding affinities and assist in identifying the most physiologically active scaffolds. 3. **Structure Optimization of Flagged Compounds**: Compounds exhibiting robust binding yet deficient ADMET properties, particularly those with extensive CYP enzyme inhibition (e.g., CYP2C9, CYP3A4) and hERG2 channel inhibition, necessitate optimization through medicinal chemistry to mitigate metabolic liabilities while maintaining potency. Two-dimensional interaction diagrams can be utilized to inform structure-activity relationship (SAR) investigations and to pinpoint locations amenable to substitution modifications.

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