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Exploiting *Salmonella* Typhi Dihydrofolate Reductase (DHFR) For The Discovery of Novel Antibacterial Agents: An *In-Silico* Study

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Abstract

Multidrug resistance trait acquired by *Salmonella* Typhi, the causative agent of typhoid fever in different parts of the world has exacerbated the treatment outcome of the disease creating more public health concern. DHFR is an essential enzyme involved in the folate metabolic pathway and plays an essential role in the biosynthesis of RNA, DNA and protein in living cells and there are no DHFR inhibitors in clinical use against infections caused by *Salmonella* Typhi currently. This study aimed to identify potential drug candidates against *S.* Typhi from existing DHFR inhibitors using computational techniques. DrugBank and PubChem databases were searched for DHFR inhibitors to create a library needed for molecular docking. Amino acid sequence of *Salmonella* Typhi DHFR was retrieved from NCBI database was modeled using SWISS-MODEL. Discovery Studio was then used for protein preparation and visualization, and PyRx was used for molecular docking. All the compounds were found to exhibit favorable binding score against *S.* Typhi DHFR. Top hit compounds identified were Talotrexin, Aminopterin, Methotrexate and Fanotaprim with binding energies of -10.7, -10.2, -10.0 and -10.0 Kcal/mol respectively. All the top hit compounds have many hydrogen bonds and hydrophobic interactions which can account for the favorable binding scores observed. The favorable binding scores translates into higher binding affinity, thus suggesting them as priority compounds for antibacterial validation. This finding suggest that the four top hits are promising drug candidates that can be experimentally validated for the discovery of new drugs against typhoid fever.

Key words: Typhoid fever; Drug discovery; *Salmonella* Typhi; Dihydrofolate reductase; Drug repurposing.

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Introduction

Typhoid fever is an infectious disease caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) acquired through feco-oral route, with its most common clinical manifestation as high body temperature, headache, body pains, diarrhea, abdominal discomfort, fatigue, cough, indigestion among others[1]. It is a disease of public health importance affecting population of all ages with developing nations bearing the highest burden due to poor hygiene system that facilitates transmission. It is the fourth-leading cause of death globally, with an estimated 128,000 to 161,000 fatalities annually and 11-18 million reported cases each year[2]. Chemotherapy with antibiotics remains the most effective treatment option for typhoid fever. However, various strains of this pathogen have developed resistance to the various antibiotic classes including first, second and third line antibiotics used for typhoid fever treatment [2] [1]. This compromises the efficacy of the treatment which worsenes the existing morbidity and mortality rate. Resistance has been reported against first, second, and third-line antimicrobials, including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, cephalosporins, and fluoroquinolones in different parts of the world including Nigeria. The World Health Organization (WHO) has classified antimicrobial resistance (AMR) as a critical global health threat, projecting that unchecked antimicrobial resistance could result in 10 million annual deaths by 2050, and *Salmonella spp* is listed among WHO's priority pathogens due to rise in its resistance against current antibiotic arsenal [2]. The growing antimicrobial resistance by *Salmonella* Typhi has been attributed to selective pressure due to antimicrobial abuse and misuse in both humans and animals, and the ease of acquisition of resistance genes by the pathogen through horizontal gene transfer with the help of mobile

genetic elements such as plasmids and transposons as well as spontaneous mutations. In a systematic review carried out by [3], forty two genes conferring antimicrobial resistance in *Salmonella* Typhi were reported with gyrA, parC, blaTEM, pare, sul1, catA1, gyrB, dfrA7, sul2 and qnrS1being the top ten must prevalent genes [3]. Another molecular screening study carried out by [4] in North central Nigeria detected blaTEM 42 (50.6%), floR 32 (38.6%), qnrA 24 (28.9%), tetB 20 (20.1%), tetA 10 (10.0%), and tetG 5 (6.0%) as the antibiotic resistance genes present in *Salmonella* isolates. These findings highlight the global nature of the growing antibiotic resistance by *Salmonella* Typhi.

The conventional drug discovery process is a very long, tedious and expensive process which makes discovery of new drug time consuming and resource demanding [5]. This does not allow the discovery of new drugs particularly antimicrobials to keep up with the rate of development of antimicrobial resistance that continuously deplete the existing antimicrobial arsenal. Discovery of new drugs usually consists of five main stages that include: Target identification stage in which basic research is carried out to understand the mechanisms leading to diseases and propose possible targets (e.g., proteins); hit-lead identification stage, during which scientists search for molecules (two main large families, small molecules and biologics) or other therapeutic strategies that interfere or cure the investigated disease or at least alleviate the symptoms; the preclinical development stage, which focuses on clarifying the mode of action of the drug candidates, investigates potential toxicity, validates efficacy on various *in vitro* and *in vivo* models, and starts evaluate formulation; the clinical stage that investigates the drug candidate in humans; the reviewing, approval and post-market monitoring stage during which the drug is approved or not [6]. This process takes about twelve to seventeen years to be completed with tendencies of attrition at the tail end[7]. To circumvent these challenges, a new approach termed drug repurposing has been developed. This approach is also referred to as drug repositioning, drug re-tasking, drug reprofiling, drug rescuing, drug recycling, drug redirection, or therapeutic switching. It has been developed to bring new drugs to patients faster and at significantly lower cost [8]. The approach implies researching new indications for already approved drugs or advancing previously studied but unapproved drugs and remains a core approach in drug development. It involves exploring the potential therapeutic uses of drugs that have already been discovered or developed regardless of whether they are approved, discontinued, abandoned, or still in the experimental phase [9]. The advantage of drug repurposing lies in the fact that existing drugs have already undergone preclinical and/or clinical testing for safety and efficacy, which reduces the time and cost associated with traditional drug development. By repurposing drugs, researchers have higher chances of finding new treatments for various diseases more efficiently and rapidly [6]. Drug repositioning can be approached through two alternative and complementary methods: the experiment based approach and the *in silico*-based approach. According to literature, about 30-40 % of drugs and biologics approved by the US Food and Drug Administration (FDA) are classified as repositioned drugs [9] and about 30% of repurposing efforts are successful and result in a product approved for marketing, in comparison to about 10% for new drug applications more generally [7]. Some repositioned drugs already developed include Amphotericin B (AMB) for fungal infections but is now indicated in Leishmaniasis, Aspirin whose original indication was pain and inflammation but now indicated in prostate cancer, Ibudilast which was originally indicated in asthma is now indicated in neuropathic pain among many [9].

Dihydrofolate reductase (DHFR) is a crucial enzyme present in both prokaryotic and eukaryotic cells that catalyzes the NADPH dependent conversion of dihydrofolate to tetrahydrofolate, involved in subsequent metabolic reactions such as thymidylate, purine nucleotide and amino acid biosynthesis [10]. DHFR has long been exploited as a target for anti-bacterial, anti-parasitic, anti-fungal, and anti-cancer drugs [11]. The reason for this can be attributed to the critical role of this enzyme to the pathogens such that inhibition of its activity leads to arrest of DNA synthesis and consequently cell death [12]. Several competitive inhibitors of dihydrofolate reductase have been discovered and used as chemotherapeutic agents (e.g., methotrexate), as antibiotics (e.g., trimethoprim) or as antimalarials (pyrimethamine) [13].

Currently, DHFR inhibitors are not used in the treatment of typhoid fever. Therefore, this study employed computational techniques (*in silico*) with the aim of discovering new antibacterial drug candidates against *Salmonella* Typhi from existing DHFR inhibitors to counter the growing resistance of the pathogen to the existing antimicrobial arsenal.

Materials and Methods

Generation of DHFR inhibitors (ligands) library

All DHFR inhibitors that are currently at different levels of studies, withdrawn or currently in clinical use were identified through searching of DrugBank (www.drugbank.ca) and PubChem (www.pubchem.ncbi.nlm.nih.gov) data bases. List of drugs/compounds identified were recorded and their 3D structures were downloaded in SDF format from PubChem database.

Ligand preparation

The ligands were imported in to OpenBabel embedded in PyRx software version 0.9.8 for preparation and molecular docking. Energy minimization was carried out for all the ligands and then converted to pdbqt format ready for molecular docking.

Salmonella Typhi DHFR (receptor) modelling

Salmonella Typhi DHFR was selected as the target molecule for the docking in this study. The reason for this is rooted in the critical role of this enzyme to the bacterium's nucleic acid and amino acid metabolism. This enzyme catalyzes the NADPH dependent conversion of dihydrofolate to tetrahydrofolate involved in subsequent metabolic reactions such as thymidylate, purine nucleotide and amino acid biosynthesis. Thus, inhibition of activity of this enzyme can lead to the arrest of DNA synthesis and consequently cell death. The three-dimensional (3D) structure of DHFR of *S.* Typhi is not available. As such, the amino acid sequence of DHFR was retrieved from NCBI (accession number– AGK65722.1) in FASTA format, and the 3D structure was predicted using SWISS-MODEL (www.swissmodel.expasy.org) which is a web tool for homology modeling. Briefly, the amino acid sequence was copied and pasted in the "sequence" window in the web tool environment and the search for template option was clicked. Series of templates were displayed and the template with the highest similarity with no substrate in the active site was selected. The "build model" option was selected to build the 3D model of protein. PDB format of the model generated was then downloaded and saved for further analysis.

Receptor preparation

The molded receptor in PDB format was imported into Discovery Studio software version 2024 for preparation. Water molecules were removed and polar hydrogen added. It was then screened for heteroatoms and saved in same PDB format.

Molecular docking

The prepared receptor was imported into PyRx and converted to PDBQT format ready for docking. Blind docking was conducted and the following grid parameters were generated. Grid center generated for docking analysis were X = 4.60, Y = 0.87, Z = -3.22, and the dimensions of the grid box were X = 90.00 x Y = 90.00 x Z = 90.00Å having a spacing of 0.50Å between the grids points. The known binding site of *S.* Typhi DHFR may not be the actual binding site of the repurposed drugs. Thus, blind docking was conducted to exploit the entire receptor protein so as to identify the best binding site for the repurposed drugs (ligands). All the ligands were selected and molecular docking was executed at exhaustiveness of 8 to allow the algorithm spend a moderate level of computational effort searching for optimal binding conformation which strikes the balance between speed and accuracy of the docking process. The docking result was then generated and saved in CSV format. The best pose of the top four hit compounds (compounds with highest binding affinity) were saved in PDB format for further analysis.

Visualization

Each of the poses of the top hit compounds and the PDB format of the receptor were imported into Discovery Studio. Ligand-receptor complex of each of the ligand was generated by opening hierarchy in the view option in the window, copying a ligand and pasting it in hierarchy of the receptor one for each. This leads to the generation of 3D and 2D structure of the ligand-receptor complexes and the resulting 3D and 2D structures for each ligand-receptor complex were saved as image. Different interactions between the ligands and receptor were also generated and saved as CSV.

Results

The binding scores of the various ligands to DHFR are presented in Table 1. It shows the binding affinity of various DHFR inhibitors with *S.* Typhi DHFR arranged in decreasing order of binding affinity. All the ligands exhibited favorable binding score against the enzyme with Talotrexin and Aminopterin, having a binding score of -10.7 and -10.2 Kcal/mol respectively, and each of Methotrexate and Fanotaprim having binding score of -10.0 Kcal/mol.

Table 1. Binding scores of the respective ligands to DHFR

DHFR inhibitors	Binding Affinity (Kcal/mol)	FDA status	Clinical use
Talotrexin	-10.7	Withdrawn	-
Aminopterin	-10.2	Clinical trial	-
Methotrexate	-10.0	Approved	Cancer and autoimmune disease
Fanotaprim	-10.0	Clinical trial	-
Ketotrexate	-9.8	Clinical trial	-
Ethanesulfonic acid	-9.7	Preclinical	-
Edatrexate	-9.6	Approved	Cancer
Dichlorometho -trexate	-9.4	Approved	Cancer
Pralatrexate	-9.0	Approved	Cancer
Iclaprim	-9.0	Withdrawn from clinical trial	-
Piritrexim	-8.9	Withdrawn from clinical trial	-
Trimetrexate	-8.2	Approved	Pneumocystis carnii pneumonia
Epiroprim	-8.1	Clinical trial	-
Proguanil Hydrochloride	-7.8	Approved	Malaria, anemia and sickle cell
Cycloguanil	-7.7	Clinical trial	-
Ormetoprim	-7.6	Approved	Antibacterial
Cycloguanil Pamoate	-7.6	Clinical trial	-
Aditoprime	-7.6	Clinical trial	-
Brodimoprim	-7.6	Withdrawn from clinical trial	-
Tetroxoprim	-7.5	Clinical trial	-
Metoprine	-7.3	Clinical trial	-
Etoprine	-7.2	Clinical trial	-
Trimethoprim hydrochloride	-7.1	Approved	Osteomyelitis , antibacterial
Pyrimethamin e	-7.0	Approved	Malaria and toxoplasmosi s
Trimetrexate Glucuronate	-5.3	Approved	Antibiotic

The 3D structure of DHFR-Talotrexin complex interaction is shown in Figure 1. The structures of interacting amino acids in the binding pocket of *S.* Typhi DHFR are shown and labeled with three letter convention. Bonds formed with talotrexin are shown in various colors with their respective bond distances labeled. More details about the interaction of the complex can be observed in the 2D DHFR-Talotrexin complex below.

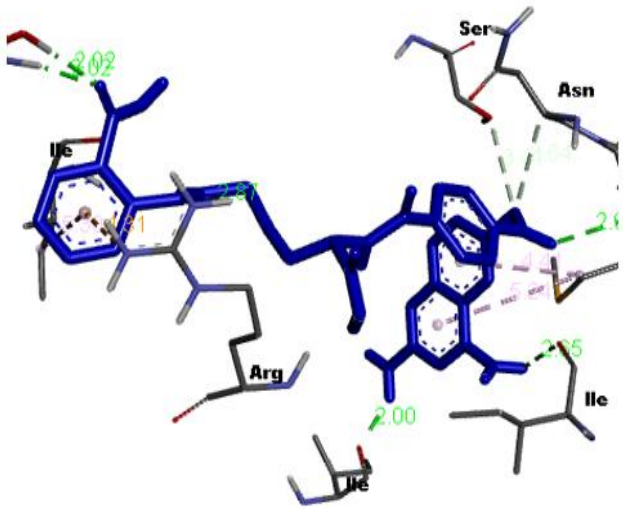

Figure 1. 3D structure of DHFR-Talotrexin complex

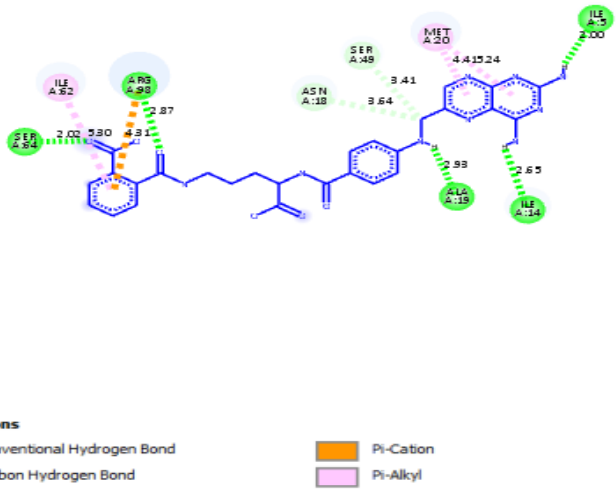

Figure 2. 2D structure of DHFR-Talotrexin complex

The 2D interaction between DHFR-Talotrexin are shown in figure 2. Nine amino acids in the binding pocket are seen to interact with the inhibitor through hydrogen bond, hydrophobic and electrostatic interactions at different distances with ARG A98 participating in both hydrogen bonding and electrostatic interaction with the ligand.

The 3D structure of DHFR- Aminopterin complex interaction is shown in figure 3 with the structures of interacting amino acids in the binding pocket of *S.* Typhi DHFR shown and labeled with the three letter naming convention. Bonds formed with Aminopterin are shown in various colors with their respective bond distances labeled accordingly. More details about the interaction of the complex and participating amino acids can be observed in the 2D DHFR- Aminopterin complex structure below.

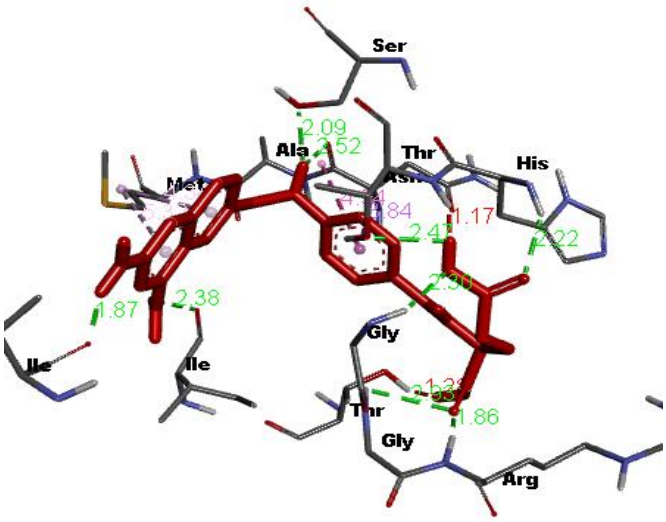

Figure 3: 3D structure of DHFR-Aminopterin complex

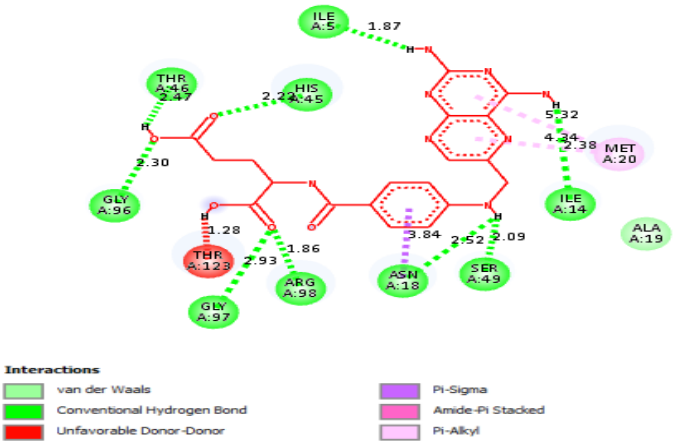

Figure 4. 2D structure of DHFR-Aminopterin complex

The 2D interaction between DHFR- Aminopterin complex is shown figure 4 with twelve amino acids in the binding pocket interacting with Aminopterin through hydrogen bond and hydrophobic interactions at different distances can be observed with the aid of the color keys. ASN A18 can be seen interacting with different atoms of the ligand through Pi-sigma and hydrogen bonds, whereas MET A20 interacts with the ligand through different atoms. Details of the different types of bonds and interactions are shown in Table 2.

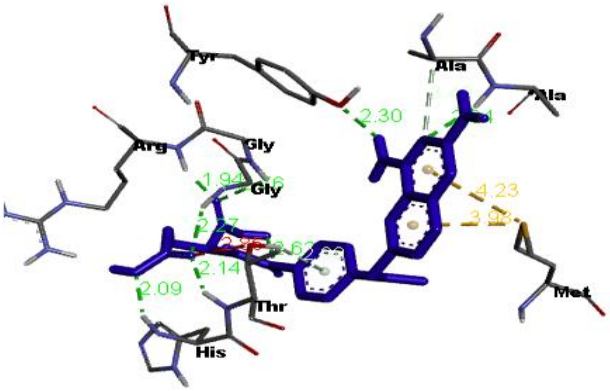

Figure 5: 3D structure of DHFR- Methotrexate complex

The 3D structure of DHFR- Methotrexate complex is shown in figure 5. The ligand interacted with various amino acids in the binding pocket of *S.* Typhi DHFR with the amino acids labeled with the three letter naming convention. Bonds formed with Methotrexate are shown in various colors with their respective bond distances labeled accordingly. More details about the interaction of the complex and participating amino acids can be observed in the 2D DHFR- Methotrexate complex below.

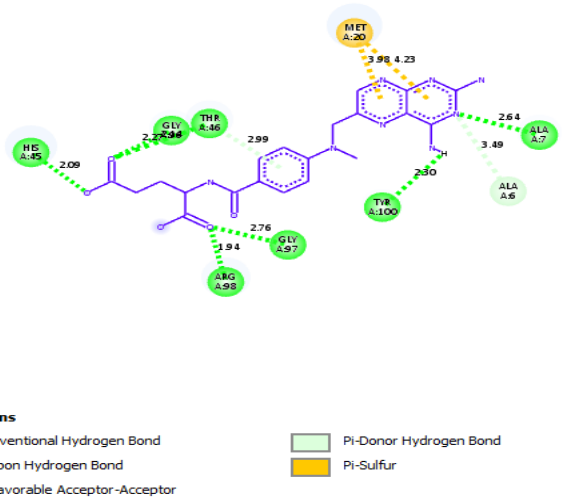

Figure 6. 2D structure of DHFR- Methotrexate complex

The 2D interaction between DHFR- Methotrexate complex is shown in figure 6. Nine amino acids in the binding pocket interacting with Methotrexate through hydrogen bond and hydrophobic interactions at different distances can be observed with the help of the keys. Details of the different types of bonds and interactions are shown in Table 2. MET A20 can be seen interacting with the ligand through two sulphur bridges, and GLY A97 and ARG A98 interacting with same atom through hydrogen bonding.

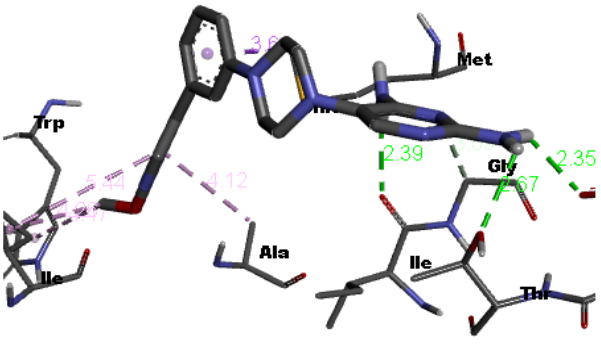

Figure 7: 3D structure of DHFR- Fanotaprim complex

The 3D structure of DHFR- Fanotaprim complex is shown in figure 7 with various structures of interacting amino acids in the binding pocket of *S.* Typhi DHFR displayed and labeled with the three-letter naming convention. Bonds formed with Fanotaprim are shown in

Table 2. Detailed interaction of the four top hit DHFR inhibitors and *S.* Typhi DHFR

Inhibitors	Bindingenergy (Kcal/mol)	Hydrogen bond		Hydrophobic interactions		Electrostatic attraction	
		Number of bonds	Participating amino acid residues	Number of interactions	Participating amino acid residue	Number of interactions	Participating amino acid residue
Talotrexin	-10.7	8	SER64, ARG98, ALA19, ILE5, ILE14, ASN18, SER49	3	ILE62, MET20,	1	ARG98
Aminopterin	-10.2	9	HIS45, GLY96, GLY97, RG98, ASN18, SER49, THR46, ILE5, ILE14	4	ASN18, ALA1, MET20,	0	-
Methotrexate	-10.0	9	ALA7, HIS45, THR46, LY96, GLY97, RG98, TYR100, ALA6, THR46	2	MET20,	0	-
Fanotaprim	-10.0	4	THR123, ASP124, ILE14, GLY15	5	MET20, ILE5, TRP30, ALA7	0	-

various colors with their respective bond distances labeled accordingly. More details about the interaction of the complex and participating amino acids can be observed in the 2D DHFR- Fanotaprim complex below.

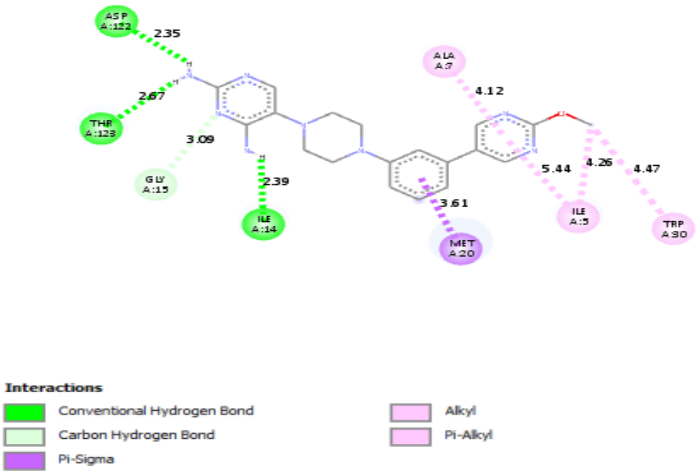

Figure 8: 2D structure of DHFR- Fanotaprim complex

The 2D interaction between DHFR- Fanotaprim complex is shown in figure 8 with eight amino acids in the binding pocket interacting with Fanotaprim through hydrogen bond and hydrophobic interactions at different distances which can be observed with the help of the color keys. Details of the different types of bonds and interactions are shown in Table 2.

Details of the interactions between the top four hits and *S.* Typhi DHFR are shown in Table 2. The table enumerated all the interacting amino acids for each of the complexes, provided the detailed types of hydrogen, hydrophobic or electrostatic interactions which are very vital in understanding the binding affinity of the ligands and the receptor.

Discussion

Drugs and compounds described as DHFR inhibitors in DrugBank and PubChem databases regardless of their FDA status were used to generate ligand library comprising of twenty five (25) candidates used for molecular docking. Pharmacological activity reports of the drug/compounds were investigated in those databases and it was revealed that they are indicated forvarious ailments that include antibacterial, anti-cancer, antimalarial, antiprotozoal and antifungal, whereas others are still under different levels of trial studies. The fact that some of these drugs are already in use as medications in humans through selective inhibition of DHFR will make discovering new drug that can be used for the treatment of typhoid fever easier, cheaper and faster, whereas those at clinical trials holds the potential of providing newer antibacterials. Many drugs have been reported in literature to have been discovered through this approach. Remdesivir, a drug candidate for the treatment of Ebola developed by Gilead Sciences, is currently being assessed for treating SARS-CoV-2 infection and has also received approval for emergency use in some regions of the world [14]. Others include Atomoxetine previously used as anti-depressant but has been repurposed in the treatment of hyperactivity disorder, Aspirin previously indicated in pain and inflammation has also been indicated in prostate cancer among many [5].

Pathogen proteins serve as key molecular targets in in antimicrobial drug discovery [15]. Many pathogens have specific proteins critical for their survival, making them ideal targets for docking studies aimed at identifying potential inhibitors. These proteins exhibit diverse

structural features, providing a broad spectrum for evaluating ligand binding affinities [16]. Thus, exploiting *S.* Typhi DHFR as target becomes justifiable. Molecular docking of the DHFR inhibitors against *S.* Typhi DHFR revealed that all the compounds have a favorable binding score against the enzyme, with binding energies ranging from -10.7 to -5.3Kcal/mol. This suggest that the studied compounds are potential antibacterial compounds that can be investigated as drug against *S.* Typhi. The binding affinities of these inhibitors were deduced from the binding energy obtained from the docking results (Table 1) with Talotrexin, Aminopterin, Methotrexate and Fanotaprim becoming the top four hits identified with binding energies of -10.7, -10.2, -10.0 and -10.0 respectively. The high binding affinities exhibited by these compounds can be attributed to the abundance of hydrogen bonds and hydrophobic interactions between them and the amino acid residues in the binding pocket of the enzyme. This agrees with previous reports which mentioned that high binding affinity in ligand-receptor interactions are attributed to abundance of these non-covalent interactions formed in their complexes [16]. High binding affinity in ligand-receptor interactions is indicative of the stability of the complex and specificity of the binding. This is indicative of the therapeutic potential of drug candidate [17] in providing potent drugs against diseases. The variety and number of interactions in each of the ligand-receptor complexes are described in Table 2. It can be observed that the number and variety of the interactions decreases with decreasing binding affinity, thus affirming the role of non-covalent interactions in determining binding score. Variation in the number and types of interaction between ligands and the receptor binding pocket can be attributed to the differences in functional groups present in the ligand molecules. Ligands with abundant polar groups like hydroxyl(-OH), amino(-NH_2), carbonyl(C=O), carboxyl(-COOH) etc forms many hydrogen bonds with polar amino acid residues present in active site of receptor molecules [18]. The high number of hydrogen bonds formed by top hits talotrexin, aminopterin and Methotrexate can be attributed to the interactions of the polar groups of the respective ligands and polar amino acids that include SER, ASN, ARG, HIS, THR and TYR present in the binding pocket of *S. Typhi* DHFR (Table 2). Additionally, some hydrophobic bonds were formed between the ligands non-polar groups and non-polar amino acids (ALA, ILE, TRP and MET) in the receptor binding pocket (Table 2). Ultimately, these interactions account for the affinity and strength of the ligand-receptor interaction observed between these compounds and the receptor molecule and underscores the significance of functional groups in pharmacological activity. Thus, pharmacophore modification which is the alteration of molecular structure of bioactive compound through addition or removal of functional groups [19] can be employed in lead optimization of these compounds to enhance their pharmacological effects. Bond distance significantly influence the binding affinity of receptor-ligand complex and have been reported to exhibit see-saw relationship with binding affinity [18]. The sorter the bond the stronger the affinity. Short bond distances have been observed particularly in hydrogen bonds of all the three top hit-receptor complexes (figure 2, 4, 6 and 8). This adds to the strength of the interaction and hence the binding affinity. Overall, the abundant interactions and short bond distance in the complexes accounts for the high affinity observed, hence, the potency of the compounds. However, it is imperative to understand that blind docking employed in this study is important in the discovery of novel binding or allosteric sites, but also has the demerit of ligands binding to non druggable pockets of receptor molecules. This makes proper protein preparation and docking validation imperative in blind docking.

The crucial role of DHFR to the survival of every living organism *S.* Typhi inclusive underscores its importance as drug targets. The ability of the top hit compounds to bind with such high affinity highlight their potential to inhibit *S.* Typhi DHFR. Inhibition of *S.* Typhi dihydrofolate reductase (DHFR) will be lethal *S.* Typhi as it will prevent the synthesis of tetrahydrofolate, which plays a important role as a methyl donor in the synthesis of thymidylate from uridylate, purine nucleotides, methionine, serine, and glycine required for DNA, RNA, and protein synthesis [21]. Inhibition of this enzyme leads to the arrest of DNA synthesis causing cell death and forms the basis of clinical use of many drugs such as methotrexate used in the cancer therapy, trimethoprim used as antibacterial and pyrimethamine used as antimalarial drugs [5]**.** Thus, these candidates can be channeled into the drug discovery pipeline for the development of novel drugs against typhoid fever. Additionally, some of these drugs such as edatrexate, dichloromethotrexate, and pralatrexate have been indicated in the treatment of cancer; proguanil and pyrimethamine are used for the treatment of malaria [20], methotrexate indicated in leukemia, breast cancer, rheumatoid arthritis, severe asthma [21] etc. This diminishes the chances of attrition in the developmental processes and reduces the development time of the drug. Many other compounds including tetroxoprim metoprine, etoprinein etc reported in this study are at

various stages of clinical trial for various ailments. Therefore, they are promising candidates for the discovery of new drugs against *S*. Typhi.

Conclusion

This research discovered Talotrexin, Aminopterin, Methotrexate and Fanotaprim as the top hit inhibitors of *S*. Typhi DHFR through molecular docking with binding energies of -10.7, -10.2, -10.0 and -10.0 Kcal/mol respectively. This finding highlights that the top hit compounds are potential candidates for experimental validation for the discovery of potential antibacterial drug candidates against *S*. Typhi particularly in the context of antimicrobial resistance. Further studies particularly *in vitro* and *in vivo* validation are recommended to validate the activities reported in the present study.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's declaration

The authors declare that the work presented in this article is their original work and that no part of this work was presented nor submitted for consideration anywhere.

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