Pharmacogn J. 2022; 14(3): 571-575
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Azasterol Inhibition and Pharmacokinetic Effects on Thymidylate Synthase-Dihydrofolate Reductase from *T. gondii*: *In Silico* Study

Alan Dharmasaputra, Risma*, Annisa Ullya Rasyida

Alan Dharmasaputra, Risma*, Annisa Ullya Rasyida

Faculty of Medicine, Hang Tuah University, Surabaya, INDONESIA.

Correspondence
Risma
Faculty of Medicine, Hang Tuah University, Surabaya, INDONESIA.
Email: risma.parulian.evilinda@gmail.com

History
- Submission Date: 21-2-2022;
- Review completed: 18-3-2022;
- Accepted Date: 13-4-2022.

DOI : 10.5530/pj.2022.14.73

Article Available online
http://www.phcogj.com/v14/i3

ABSTRACT
Toxoplasmosis is a disease that causes health problems and can be found worldwide with a percentage of more than 60%, especially in developing countries such as Indonesia. Pyrimethamine-resistant strains of *T. gondii* have been found, and it may contribute to reducing therapeutic failure in the future. Azasterol is a synthetic analog of solacongestidine, which can potentially be used as a new anti-toxoplasma drug. Resistance to the anti-toxoplasma drug, Pyrimethamine, makes Azasterol a very profitable discovery as a new anti-toxoplasma drug. This study aimed to determine the inhibitory and pharmacokinetic effects of Azasterol compounds on the development of *T. gondii* based on *in silico* studies. This one-shot experimental study analyzed the predicted inhibitory effect of Azasterol on Thymidylate synthase-dihydrofolate reductase (TS-DHFR) from *T. gondii* to observe the pharmacokinetic prediction and toxicity test of the Azasterol compound. Besides, this one-shot experimental study utilized the *in silico* method. According to the results of molecular docking, Azasterol had an interaction with the TS-DHFR protein in the same binding area as the Pyrimethamine – TS-DHFR and Sulfadiazine – TS-DHFR complexes. Azasterol binding energy was higher than that of Pyrimethamine and Sulfadiazine. Azasterol had a good pharmacokinetic effect and had minimal toxic effects on the body.
Key words: *In silico*, Toxoplasmosis, Azasterol, TS-DHFR.

INTRODUCTION

Azasterol is a synthetic analog of solacongestidine, an alkaloid of the plant genus solanum.[1] Azasterol functions as an inhibitor of S-adenosyl-L-methionine: Δ24-sterol methyltransferase (24-SMT) in fungi, plants and some protozoan parasites.[2] A study on the anti-toxoplasmosis activity of Azasterol against *T. gondii in vitro* was conducted by Duarte in 2011. The morphological ultrastructural analysis results of this study showed that Azasterol caused mitochondrial swelling and breakdown of the plasma membrane of *Toxoplasma gondii* (*T. gondii*). However, the molecular target of Azasterol for *T. gondii* has not been found to date.[3]

T. gondii is an obligate intracellular parasite that causes toxoplasmosis and belongs to the phylum apicomplexa. *T. gondii* has the best survival strategy of all intracellular parasites by modifying its life cycle and eliminating dependence on the sexual cycle for transmission to new hosts. *T. gondii* can survive in a dormant phase for up to several years and can be transmitted to warm-blooded animals in various ways.[4]

Toxoplasmosis is a disease that causes health problems and can be found worldwide with a percentage of more than 60%, especially in developing countries such as Indonesia. The Centers for Disease Control and Prevention estimates that more than 40 million people in the United States are infected with Toxoplasma. Infection tends to be high in tropical and humid areas because oocysts can survive better in this particular environment.[5]

In Indonesia, the prevalence of Toxoplasma infection in humans has been reported as 43-88%. The prevalence of toxoplasmosis from several studies shows that toxoplasmosis cases are still relatively high in big cities in Indonesia.[6] The prevalence of toxoplasmosis tends to increase in patients with immunodeficiency. Out of 1,473 individuals with HIV/AIDS, 81% had toxoplasmosis.[7] The prevalence of toxoplasmosis in several areas in Central Java (positive seroprevalence) in 2016 was 62.5%.[8] According to medical record data from Arifin Achmad Hospital, Riau Province, Toxoplasmosis is included in the ten most common diseases in pregnancy. Toxoplasmosis cases increase every year, from 13 cases (1.2%) from 1,084 pregnancy visits in 2010 to 30 cases from 1,303 pregnancy visits in 2012.[6]

The high prevalence of toxoplasmosis also increases the mortality of toxoplasmosis patients.[9] The number of cases and deaths caused by toxoplasmosis shows that toxoplasmosis poses a very significant public health problem.[10] Toxoplasmosis is asymptomatic in most immunocompetent individuals, but it does cause symptoms in immunodeficiency patients. Clinical manifestations of toxoplasmosis in immunodeficiency patients include seizures, decreased vision, ocular toxoplasmosis, retinochoroiditis, Toxoplasmic encephalitis.[11] Severe clinical manifestations in pregnancy include miscarriage, congenital toxoplasmosis, hydrocephalus, mental retardation, epilepsy, blindness, low birth weight.[12]

The toxoplasmosis cases caused by *T. gondii* shall be treated with a combination of Sulfadiazine and Pyrimethamine drugs.[13] Pyrimethamine and Sulfadiazine work by inhibiting the Thymidylate synthase-dihydrofolate reductase protein in *T. gondii*. This protein is the primary regulator of folate metabolism and DNA synthesis in *T. gondii*. If Thymidylate synthase-dihydrofolate reductase can

Cite this article: Dharmasaputra A, Risma, Rasyida AU. Azasterol Inhibition and Pharmacokinetic Effects on Thymidylate Synthase-Dihydrofolate Reductase from *T. gondii*: *In Silico* Study. Pharmacogn J. 2022;14(3):571-575.

be inhibited, the parasite growth rate will also slow down. Therefore, this enzyme can serve as a candidate target for anti-toxoplasma drugs.[14]

In 2001, a Pyrimethamine-resistant strain of *T. gondii* was found. Pyrimethamine-resistant strains of *T. gondii* may contribute to reducing therapeutic failure in the future.[15] According to the above explanation, alternative treatment is needed to overcome anti-toxoplasma drug resistance, one of which is utilizing Azasterol.

Therefore, this study utilized an *in silico* method to observe in detail the interactions of Azasterol compounds with enzymes that may potentially be the targets, such as Thymidylate synthase-dihydrofolate reductase down to the molecular level.

This *in silico* study served as the first step to discover the primary drug and co-drug candidates for Toxoplasma infection before conducting *in vitro* or *in vivo* studies. Through the *in silico* study approach in this research, researchers can visualize the molecular docking structure and signify what compounds have the greatest potential to be used as references in further research.[16-20]

According to this phenomenon, Azasterol is expected to serve as an alternative therapy for Toxoplasma. To prove it, it is necessary to conduct *in silico* studies on the potential of Azasterol compounds against the Thymidylate synthase-dihydrofolate reductase enzyme.

METHOD

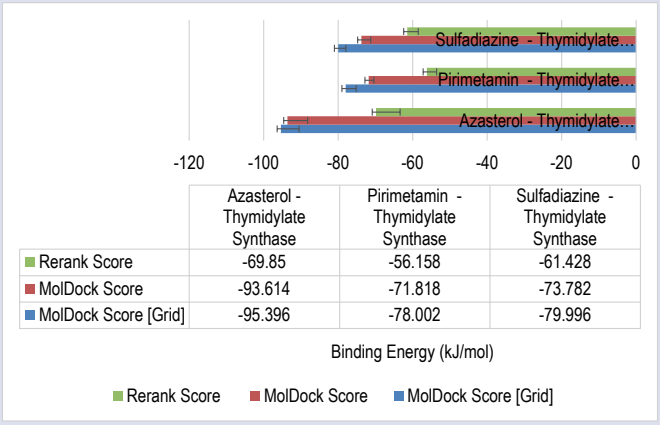
This One-Shot Experimental Study utilized the *in silico* method. The study was conducted at the Delta Science Laboratory, Lowokwaru, Malang, East Java, Indonesia, from April to August 2021. The independent variable in this study was the Azasterol compound, and the dependent variable in this study was the development of *T. gondii*.

Structure of proteins and ligands

The molecular structures of Azasterol (CID 11954297), Pyrimethamine (CID 4993) and Sulfadiazine (CID 5215) were obtained from the PubChem database https://pubchem.ncbi.nlm.nih.gov/compound/). https://pubchem.ncbi.nlm.nih.gov/compound/ The three-dimensional structure of Thymidylate synthase-dihydrofolate reductase was downloaded from the database of Protein Data Bank (https://www.rcsb.org/) with ID 4KY4.https://www.rcsb.org/

Molecular docking

Prediction on the interaction strength between the receptor and the ligand-based on the value of the binding energy can be obtained by docking—the more negative the value, the stronger the interaction between the receptor and the ligand. If the value of the tested active compound is close to or even lower than the control value, it can be predicted that the active compound has antagonistic activity against the target protein. The target protein in this docking was Thymidylate synthase-dihydrofolate reductase (TS-DHFR) with Pyrimethamine and Sulfadiazine as the control compounds. Domains A and B of Thymidylate Synthase protein were prepared with Molegro Virtual Docker 5 by predicting the cavity (active site of the protein) with Vander Waals force expansion parameters. Furthermore, the three compounds downloaded from the PubChem database were imported and docked with a cavity volume of 2946.56 with a surface of 9,049.6. Docking parameters with a MolDock Score Grid of 0.30; 10x running, a maximum RMSD of 2, a binding pose of 5. The interaction between the three compounds with Thymidylate Synthase protein was analyzed using Discovery Studio version 21.1.1. Azasterol was predicted to have ADMET (absorption, distribution, metabolism, toxicity) properties available online at http://biosig.unimelb.edu.au/pkcsm/prediction.



	Azasterol - Thymidylate Synthase	Pirimetamin - Thymidylate Synthase	Sulfadiazine - Thymidylate Synthase
Rerank Score	-69.85	-56.158	-61.428
MolDock Score	-93.614	-71.818	-73.782
MolDock Score [Grid]	-95.396	-78.002	-79.996

Table 1: The binding energy of Azasterol, Pyrimethamine, Sulfadiazine complexes with Thymidylate synthase-dihydrofolate reductase protein.

RESULTS AND DISCUSSION

The interaction between the three compounds (the active Azasterol, Pyrimethamine and Sulfadiazine compounds) and Thymidylate synthase-dihydrofolate reductase protein was analyzed using Discovery Studio version 21.1.1, the results of which can be seen in the table 1.

Prediction on the interaction strength between the receptor and the ligand based on the value of the binding energy can be obtained by molecular docking—the more negative the value, the stronger the interaction between the receptor and the ligand. Azasterol had the most negative binding energy value compared to the control compounds, namely Pyrimethamine and Sulfadiazine.

The interaction between Azasterol and Thymidylate synthase-dihydrofolate reductase (TS-DHFR) protein based on the overall 3D view showed the same binding area as the Pyrimethamine - Thymidylate synthase-dihydrofolate reductase and Sulfadiazine - Thymidylate synthase-dihydrofolate reductase complexes.

Azasterol bound to the Thymidylate synthase-dihydrofolate reductase protein at the amino acid residues GLN326, LEU330, CYS355, GLY354, ASP329, HIS322 and ARG320.

Pyrimethamine bound to the Thymidylate synthase-dihydrofolate reductase protein at the amino acid residues LYS352, CYS355, ASP329, LEU330, PHE353, GLY354.

Sulfadiazine bound to the Thymidylate synthase-dihydrofolate reductase protein at the amino acid residues PHE325, GLY321, ARG320, HIS322, THR356, ASP329, GLN326 and LEU330.

Azasterol, Pyrimethamine, Sulfadiazine were identified to bind to the proteins at the same amino acid residues, namely LEU330 and ASP329. Azasterol and Sulfadiazine were identified to bind to the proteins at the same amino acid residue, namely GLN326 and ARG320. In addition, Azasterol and Pyrimethamine were identified to bind to the proteins at the same amino acid residues, namely CYS355 and GLY354.

The resulting bond energies of the compounds were as follows: Azasterol (-93,614), Pyrimethamine (-71,818), and Sulfadiazine (-73,782). The more negative the value of the binding energy, the stronger the interaction between the receptor and the ligand, increasing the inhibitory potential of the target protein. In this study, Azasterol had a very negative value, even lower than the value of the two control compounds. Therefore, the inhibitory potential of Azasterol was significantly high against the TS-DHFR protein. This was possible because the amino acid residues bound by Azasterol were stronger than the two control compounds. The low binding energy was

also supported by the type of bond between the ligand and protein. The hydrophobic bonds found in Azasterol compounds had an Alkyl bond type, classified as a covalent bond, while the hydrophobic bonds in Pyrimethamine and Sulfadiazine belong to the Pi-Alkyl and Amide Pi-stack types, classified as non-covalent interactions. Among the three compounds, only Azasterol indicated covalent interactions, which were much stronger than non-covalent interactions and supported the bond energy results. The inhibition of TS-DHFR resulted in dTMP deficiency and led to the inhibition of DNA synthesis and cell replication.[21-23]

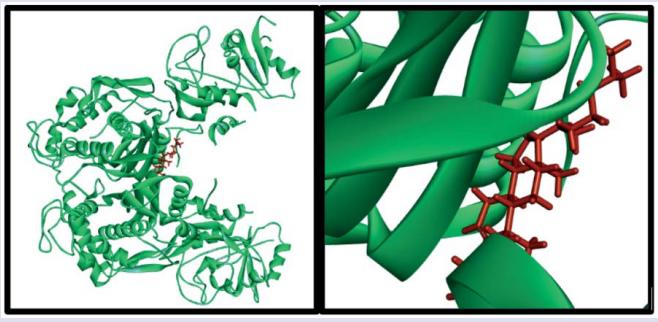


Figure 1: Visualization of the overall display of Thymidylate synthase-dihydrofolate reductase – Azasterol docking results.

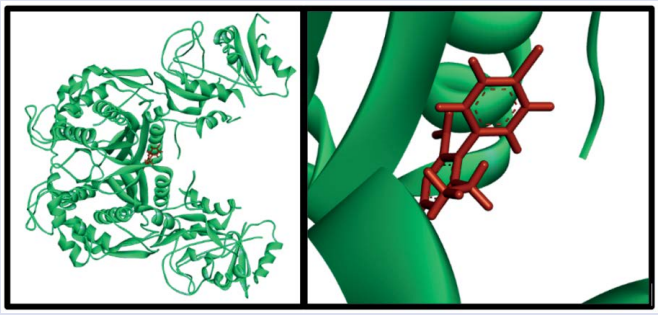


Figure 2: Visualization of the overall display of Thymidylate synthase-dihydrofolate reductase – Pyrimethamine docking results.

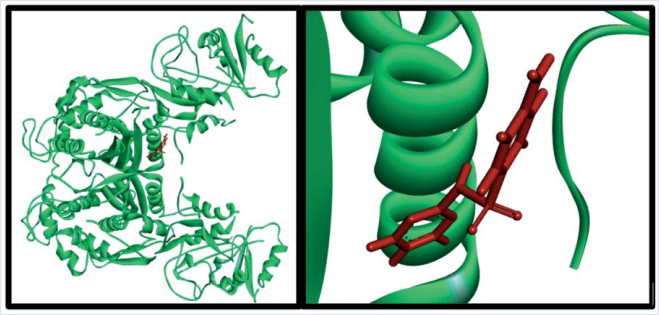


Figure 3: Visualization of the overall display of Thymidylate synthase-dihydrofolate reductase - Sulfadiazine docking results.

Table 2: Interaction between Azasterol, Pyrimethamine, and Sulfadiazine on Thymidylate synthase-dihydrofolate reductase protein.

Compounds	Hydrophobic Bond	Hydrogen Bond
Pyrimethamine (control)	LYS352, **LEU330**	**CYS355**, **ASP329**, PHE353, GLY354
Sulfadiazine (control)	**LEU330**, GLN326	GLY321, **ARG320**, **HIS322**, THR356, **ASP329**, PHE325
Azasterol	**LEU330**, **CYS355**, ARG358	GLN326, GLY354, **HIS322**, **ARG320**, **ASP329**.

Note: The same amino acid residues between control and comparison ligands are in bold.

Table 3: Prediction results of Azasterol ADME properties.

Parameters	Values
Absorption	
Intestinal absorption	87.14 %
Distribution	
VDss (human)	0,831 Log L/kg
BBB Permeability	0.703 log BB
Metabolism	
CYP3A4 substrate	Yes
Excretion	
Total Clearance	0.658 log ml/min/kg

Table 4: Molecular characteristics of the Azasterol compound.

Parameters	Values
Molecular weight	461.562
LogP	5.4
Rotatable Bonds	5
Acceptors	3
Donor	1
Surface area	197.405

Table 5: Prediction results of Azasterol toxicity.

Toxicity	
AMES toxicity	No
Hepatotoxicity	Yes
T.pyriformis toxicity	0.373 log ug/L
Minnow toxicity	0.17 log mM

The prediction of Azasterol ADME (absorption, distribution, metabolism, excretion) properties utilized the pkCSM webserver (http://biosig.unimelb.edu.au/pkcsm/prediction).http://biosig.unimelb.edu.au/pkcsm/prediction. The prediction results of Azasterol ADME properties are presented in table 3.

A compound is said to be poorly absorbed if the absorption value by the intestine is below 30%. Azasterol has an intestinal absorption value of 87.14%, indicating that it can be orally absorbed with ease. The higher the absorption value, the higher the bioavailability and therapeutic effect of a drug. A volume of distribution is the volume required by a substance to have the same concentration value in all blood plasma. The higher the VDss value of a substance, the more the concentration of a drug in the tissue than in blood plasma. Azasterol has a VDss value of 0.831 Log L/kg, where VDss is said to be high if it exceeds a Log BB of 0.3. Azasterol also has a high BBB Permeability with a Log BB number of 0.703. Thus, Azasterol is very suitable for the treatment of toxoplasmosis, especially toxoplasmosis in immunocompromised patients who have clinical manifestations of encephalitis and meningoencephalitis. Azasterol metabolism occurs in the liver *via* cytochrome p450 substrate CYP3A4. Therefore, it should be administered carefully and dose adjustment is necessary for patients with liver disorders.

Azasterol has a total clearance of 0.658 Log ml/min/kg, which is the combination of hepatic and renal clearance. It is important to determine the required dose to achieve a stable therapeutic concentration.[24]

The toxicity prediction of Azasterol was conducted utilizing pkCSM webserver (http://biosig.unimelb.edu.au/pkcsm/prediction).http://

biosig.unimelb.edu.au/pkcsm/prediction Prediction results of Azasterol toxicity can be seen in table 5.

Azasterol toxicity was tested with AMES toxicity, *T. pyriformis* toxicity, and minnow toxicity with negative results on AMES toxicity, proving that Azasterol is not mutagenic and carcinogenic when consumed by humans. In addition, Azasterol has a *T. pyriformis* toxicity value of 0.373 log ug/L. Therefore, Azasterol is non-toxic to humans. Azasterol has hepatotoxic properties, requiring careful administration in patients with liver disorders. Minow toxicity is a toxicity test to determine the lethal concentration of 50% of flathead minnow fish. Azasterol has a minnow toxicity value of 0.17 log mM, which is non-toxic, making its administration safe for humans.[24]

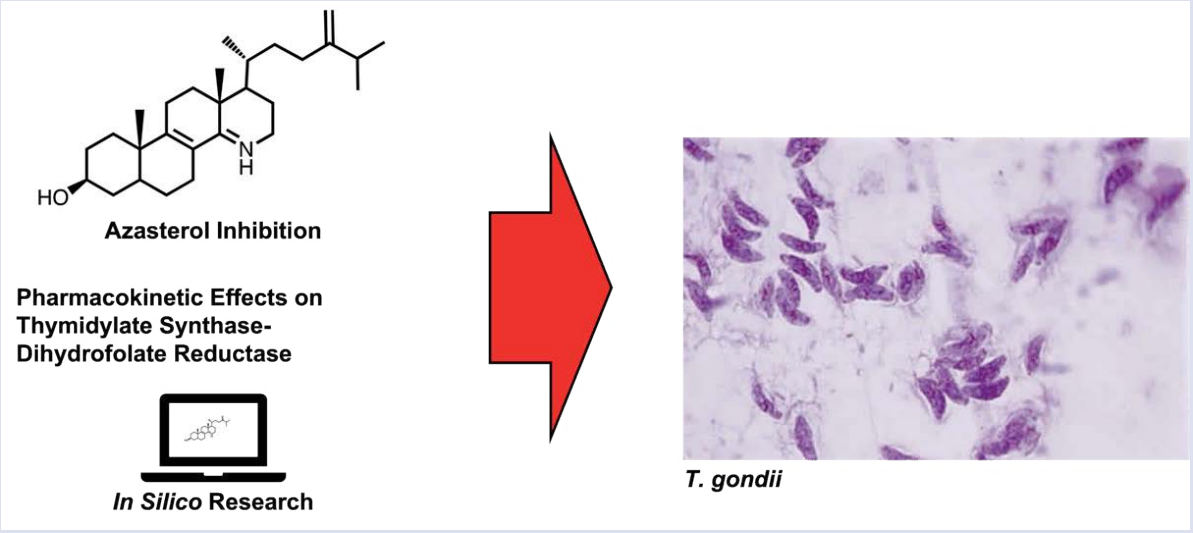
CONCLUSIONS

Azasterol compound was predicted *in silico* to have an inhibitory effect on TS-DHFR from *T. gondii*. Based on the inhibition mechanism of Azasterol, it had the same function as Pyrimethin and Sulfadiazine as the control compounds. The ADMET prediction also explained that Azasterol has good pharmacokinetic effects (absorption, distribution, metabolism and excretion) in the body. It should be carefully administered in patients with liver disorders.

REFERENCES

1. Shehzad A, Masud A, Fatima T, Khan FM, Rehman S, Effendi MH, *et al*. Seroprevalence of Toxoplasma gondii and associated alterations in hematology and serum biochemistry of one-humped camels (*Camelus dromedarius*) in Pakistan, Veterinary World. 2022;15(1):110-118.
2. Nurdianto AR, Aryati A, Suryokusumo MG, Mufasirin M, Suwanti LT, Sunarjog, *et al*. Effect of hyperbaric oxygen therapy on icam-1 expression in artery spiralis of pregnant *Rattus norvegicus* infected by tachyzoite from *Toxoplasma gondii*. EurAsian Journal of BioSciences. 2020;14(1):1757-1762.
3. Afililla Z, Suwanti LT, Puspitasari H, Suyono EA, Budiman A, Koerniawan MD, *et al*. Inability of polysaccharides of *Spirulina platensis* to protect hepatocyte cells line on *Toxoplasma gondii* infection in vitro. Journal of Global Pharma Technology. 2020;12(1):654-659.
4. Kortagere S. Screening for small molecule inhibitors of *Toxoplasma gondii*. Expert Opinion on Drug Discovery. 2012;7(12):1193-1206.
5. Nurdianto AR, Aryati A, Suryokusumo MG, Mufasirin M, Suwanti LT, Sunarjog, *et al*. Effects of hyperbaric oxygen therapy on IL-17, fetal body weight and total fetus in pregnant *Rattus norvegicus* infected with tachyzoite *Toxoplasma gondii*. Systematic Reviews in Pharmacy. 2020;11(3):628-634.
6. Yudhana A, Praja RN. Toxoplasmosis occurrence in stray cat from Banyuwangi City, Indonesia. Indian Veterinary Journal. 2019;96(7):14-15.
7. Suwanti LT, Mufasirin. Increased apoptosis skull of pups born to *Toxoplasma gondii*-infected mice associated with increased expression of interferon gamma, but not tumor necrosis factor alfa. African Journal of Infectious Diseases. 2018;12(1):68-71.
8. Retmanasari A. Prevalence and Risk Factors for Toxoplasmosis in Middle Java, Indonesia. EcoHealth. 2017;14(1):162-170.
9. Mboera LEG. Mortality patterns of toxoplasmosis and its comorbidities in Tanzania: A 10-year retrospective hospital-based survey. Frontiers in Public Health. 2019;7:25.
10. Sobanski V. Severe toxoplasmosis in immunocompetent hosts: Be aware of atypical strains. American Journal of Respiratory and Critical Care Medicine. 2013;187(10):1143-1145.
11. Furtado JM. Toxoplasmosis: A global threat. Journal of Global Infectious Diseases. 2011;3(3):281-284.
12. Ahmed M, Sood A, Gupta J. Toxoplasmosis in pregnancy, European Journal of Obstetrics and Gynecology and Reproductive Biology. 2020;255:44-50.
13. Yuliawati I, Nasronudin. Pathogenesis, Diagnostic and Management of Indonesian Journal of Tropical and Infectious Disease. 2015;5(4):100-106.
14. Puspitasari H, Suwanti LT, Mufasirin. Addition of anti-*Toxoplasma gondii* membrane immunoglobulin y to reduce necrotic index in mice's liver. African Journal of Infectious Diseases. 2018;12(1):72-75.
15. Montazeri M. Drug resistance in *Toxoplasma gondii*. Frontiers in Microbiology. 2018;9:2587.
16. Kharisma VD, Ansori ANM. Construction of epitope-based peptide vaccine against SARS-CoV-2: Immunoinformatics study. Journal of Pure and Applied Microbiology. 14:999-1005.
17. Kharisma VD, Agatha A, Ansori ANM, Widyananda MH, Rizky WC, Dings TGA, *et al*. Herbal combination from *Moringa oleifera* Lam. and *Curcuma longa* L. as SARS-CoV-2 antiviral *via* dual inhibitor pathway: A viroinformatics approach. Journal of Pharmacy and Pharmacognosy Research. 2022;10(1):138.
18. Widyananda MH, Pratama SK, Samoedra RS, Sari FN, Kharisma VD, Ansori ANM, *et al*. Molecular docking study of sea urchin (*Arbacia lixula*) peptides as multi-target inhibitor for non-small cell lung cancer (NSCLC) associated proteins. Journal of Pharmacy and Pharmacognosy Research. 2021;9(4):484-496.
19. Kharisma VD, Probojati RT, Murtadlo AAA, Ansori ANM, Antonius Y, Tamam MB. Revealing potency of bioactive compounds as inhibitor of dengue virus (Denv) ns2b/ns3 protease from sweet potato (*Ipomoea batatas* L.) leaves. Indian Journal of Forensic Medicine and Toxicology. 2021;15:1627-1632.
20. Ansori ANM, Kharisma VD, Parikesit AA, Dian FA, Probojati RT, Rebezov M, *et al*. Bioactive compounds from mangosteen (*Garcinia mangostana* L.) as an antiviral agent via dual inhibitor mechanism against SARS-CoV-2: An *in silico* approach. Pharmacognosy Journal. 2022;14:85-90.
21. Sharma M, Chauhan P. Dihydrofolate reductase as a therapeutic target for infectious diseases: opportunities and challenges. Future Med Chem. 2012;4(10):1335-1365.
22. Landau MJ, Sharma H, Anderson KS. Selective peptide inhibitors of bifunctional Thymidylate synthase-dihydrofolate reductase from *T. gondii* provide insights into domain-domain communication and allosteric regulation. Protein Science. 2013;22(9):1161-1173.
23. Zaware N. Discovery of potent and selective inhibitors of *T. gondii* thymidylate synthase for opportunistic infections. ACS Medicinal Chemistry Letters. 2013;4(12):1148-1151.
24. Pires DEV, Blundell TL, Ascher DB. pkCSM: Predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. Journal of Medicinal Chemistry. 2015;58(9):4066-4072.

GRAPHICAL ABSTRACT



ABOUT AUTHORS

Alan Dharmasaputra, S.Ked is graduated from Hang Tuah University Surabaya with bachelor degree in Medicine.

Risma, dr., M.Si. is graduated from Airlangga University with a Master's Degree in Basic Medical Sciences and a senior lecturer at the Department of Parasitology, Faculty of Medicine, Hang Tuah University, Surabaya.

Dr. Annisa Ullya Rasyida, dr., M.Si. is graduated from Airlangga University with a doctorate in Health Sciences and a senior lecturer at the Department of Public Health Sciences, Faculty of Medicine, Hang Tuah University, Surabaya.

Cite this article: Dharmasaputra A, Risma, Rasyida AU. Azasterol Inhibition and Pharmacokinetic Effects on Thymidylate Synthase-Dihydrofolate Reductase from *T. gondii*: *In Silico* Study. Pharmacogn J. 2022;14(3):571-575.