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

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**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%.6 The prevalence of toxoplasmosis from several studies shows that toxoplasmosis cases are still relatively high in big cities in Indonesia.8 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 2012.8

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
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**


**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 9049.6. 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/pkcsms/prediction](http://biosig.unimelb.edu.au/pkcsms/prediction).

**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, LEU329, PHE353, GLY354. Pyrimethamine and Sulfadiazine were identified to bind to the proteins at the same amino acid residues, namely LYS352 and CYS355.

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

The prediction of Azasterol ADME (absorption, distribution, metabolism, excretion) properties utilized the pkCSM webserver (http://biosig.unimelb.edu.au/pkcsmprediction). 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/pkcsmprediction). The toxicity prediction of Azasterol toxicity is presented in table 5.

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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 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**


**GRAPHICAL ABSTRACT**

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