In silico analysis of V48A dihydropteroate synthase mutation to dapsone on *Mycobacterium leprae* from Papua

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Abstrak

Latar belakang: *Lepra merupakan penyakit yang disebabkan oleh Mycobacterium leprae. Resistensi obat merupakan salah satu tantangan dalam pemberantasan kusta khususnya di Papua. Adanya mutasi pada gen folP1 penyandi dihydropteroate synthase (DHPS) merupakan dasar untuk deteksi molekuler resistensi dapson pada penyakit lepra. Tujuan penelitian ini adalah mendeteksi mutasi pada gen folP1 Mycobacterium leprae dari Papua, Indonesia dan menganalisis pengaruh mutasi tersebut terhadap dapson dengan metode in silico.*

Metode: *Identifikasi mutasi pada gen folp1 M. leprae dilakukan melalui proses Basic Local Alignment Search Tool (BLAST) di gene bank. Analisis efek mutasi dengan menggunakan server Have (y) Our Protein Explained (HOPE). Prediksi binding pocket menggunakan Computed Atlas of Surface Topography of proteins (CASTp). Homologi modeling struktur 3D DHPS menggunakan server Iterative Threading ASSEmbly Refinement (I-TASSER). Analisis docking dengan menggunakan AutoDock Vina yang terintegrasi dengan aplikasi Python Prescription (PyRx).*

Hasil: *Hasil sekuensing menunjukkan adanya variasi dalam gen folP1 M. leprae yaitu perubahan dari Timin (T) menjadi Sitosin (C) pada nukleotida 143. Residu yang bermutasi (V48A) terletak pada domain yang penting untuk aktivitas protein dan kontak dengan residu di domain lain. Ada kemungkinan bahwa interaksi ini penting untuk fungsi protein secara benar. Mutan V48A tidak banyak mempengaruhi stabilitas dari dihydropteroate synthase M. leprae.*

Kesimpulan: *Berdasarkan analisis molecular docking, mutasi V48A tidak mempengaruhi binding affinity dapson terhadap dihydropteroate synthase M. leprae. Hasil ini menunjukkan mutan V48A kemungkinan tetap rentan terhadap dapson. Dengan demikian perlu dilakukan uji in vivo untuk mengkofirmasi efek mutasi V48A.* ***(Health Science Journal of Indonesia 2020;11(2):70-6)***

Kata kunci: *Mycobacterium leprae*, *folP1 gene, dihydropteroate synthase, dapson*

Abstract

Background: Leprosy is a disease caused by *Mycobacterium leprae*. Drug resistance is one of the challenges in leprosy elimination especially in Papua. The presence of mutations in *folP1* gene that encode dihydropteroate synthase (DHPS) was considered as the exclusive basis for molecular detection of dapsone resistance in leprosy. The objective of this study was to detect mutations in the *folP1* gene of *Mycobacterium leprae* from Papua, Indonesia and to analyze the effect of these mutations on dapsone using the in-silico method.

Methods: Identification of mutations in the *folp1 M. leprae* gene is carried out through the Basic Local Alignment Search Tool (BLAST) process in the gene bank. The analysis of the effects of mutations using the Have (y)Our Protein Explained (HOPE) server. Bindings pocket prediction is done using the Computed Atlas of Surface Topography of proteins (CASTp). Homology modeling 3D structure of DHPS using the Iterative Threading ASSEmbly Refinement (I-TASSER) server. Docking analysis was performed using AutoDock Vina which is integrated with the Python Prescription (PyRx) application.

Results: The sequencing results showed a variation in the *folP1 M. leprae* gene, namely a change from thymine (T) to cytosine (C) in nucleotide 143. The mutated residue (V48A) is in a domain that is essential for the activity of the protein and in contact with residues in another domain. It is possible that this interaction is important for the correct function of the protein. V48A mutants did not significantly affect the stability of DHPS *M. leprae*.

Conclusion: Based on molecular docking analysis, this mutation does not affect binding affinity dapsone against *M. leprae* dihydropteroate synthase. These results indicate that the V48A mutant is likely to remain susceptible to dapsone. Thus, it is necessary to do an in vivo test to confirm the effect of the V48A mutation. ***(Health Science Journal of Indonesia 2020;11(2):70-6)***

Keywords: *Mycobacterium leprae*, *folP1* gene, dihydropteroate synthase, dapsone

Leprosy is a skin infection, membrane and prefers nerve disease.[1] Leprae is a neglected disease that still occurs in about 120 countries with more than 200,000 new cases reported annually. The regional proportions of all new cases in 2019 were: 71.3% (143 787) in South-East Asian Region (SEAR), 14.9% (29 936) in Americas Region (AMR), 9.9% (20 205) in African Region (AFR), 2.1% (4211) in Eastern Mediterranean Region (EMR), 1.9% (4004) in WPR and 42 in European Region (EUR).[2] This disease is still a significant disease in Jayapura City, Papua, furthermore, the burden disease of leprosy in Jayapura is considered as high.[3] Based on the Disability Number, Papua was the highest number in Indonesia in 2013 (26,88), followed by Aceh (18,62), and West Papua (17,72). The data from General Directorate of Disease Control and Health Environment in 2013 reveals that Case Detection Rate (CDR) of Papua was 35,64 in 100.000 people and declined to 30,43/100.000 on 2014.[4,5]

Since 1995, WHO has supplied Multi Drug Therapy (MDT) to all country with leprosy burden. The MDT consists of three antibiotics, contained rifampicin, clofazimine and dapsone for Multibacillary (MB) and rifampicin and dapsone combination for Paucibacillary (PB).[6] Unfortunately, MDT program in Papua and West Papua faces challenges such as the low awareness of patients, geographical barriers to access the health facility and other people's paradigm about drugs. DHPS is an enzyme that plays a role in the biosynthesis of folate in bacteria including *M. leprae*, which targets dapsone by inhibiting p-aminobenzoic acid (PABA).[7] The presence of point mutations in *folP1* gene that encodes dihydropteroate synthase (DHPS) was considered as the exclusive basis for molecular detection of dapsone resistance in leprosy.[8] Dapsone-resistant *M. leprae* isolates have shown mutation at codon 53 or 55 in the *folP1* gene. [9,10,11] The most frequently detected mutation associated with dapsone resistance in *M. leprae* is CCC→CTC in codon 55 of *folP1* resulting in the substitution of leucine for a proline residue (Pro55Leu) in the DHPS.[12] Predictively, the mutation will decrease the effectiveness of dapsone therapy.

The effect of drug resistance due to the point mutation on the amino acid residues of the targeted protein can be studied by bioinformatics simulation (in-silico). This method is relatively accurate, rapid and cost-effective compared to in vitro and in vivo method. Therefore, computational studies can be performed to study drug resistance.[13,14] Many of the molecular docking is successful in predicting the binding form of the ligand in the receptor binding sides.[15] The objectives of this study were to detect mutations in the *folP1* gene of *M. leprae* from Papua Island, Indonesia and to analyze the effect of these mutations on dapsone using the *in silico* method.

METHODS

This study was a cross section. This research was ethically approved by Ethics Committee of National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia number LB.02.01/5.2/KE.065/2016. The samples in this study were the result of an incision in one or both ears skin of leprosy patients. Sampling was carried out at Hamadi Public Health Centers, Jayapura and Bintuni Regency by making direct visits to patients' homes or to patients who were conducting control at health facilities. The total samples were 100 leprosy patients.

a. Identification of mutation of *folP1* gene *M. leprae* from Papua islands

The molecular examination began with DNA extraction process using QIAamp DNA Mini Kit (REF:51306, Qiagen, German), followed by Polymerase Chain Reaction (PCR) process. The primers used to amplify the *folP1* gene *M. leprae* in this study were WHOF15'-GCAGGTTATTGGGGTTTTGA-3' as a forward primer and WHOF2 5'-CCACCAGACACATCGTTGAC-3' as reverse primers. The reagents for PCR are GoTaq® Green Master Mix (REF:M7122, Promega, USA). A touchdown PCR method was performed, preheating was done at 98 °C for 2 minutes, followed by 5 cycles of 98 °C for 20 seconds, 60 °C to 56 °C with decrement 1 °C per cycle for 30 seconds, and 72 °C for 20 seconds. The further cycle was done at 98 °C for 20 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds for 40 times, with a final extension at 72 °C for 5 minutes. The resulting PCR product was 312 bp which is a partial part of the *folP1* gene. The PCR product was purified by Applied Biosystems™ CleanSweep™ PCR Purification Reagent (Thermo Fisher Scientific, USA) with a ratio of 2:5 The purified PCR product proceeded to Sanger Sequencing procedure. The sequencing cycle uses BigDye™ Terminator v3.1 Cycle Sequencing Kit (REF:4336917, Thermo Fisher Scientific, USA) as 4 µL, BigDye Terminator buffer v1.1 / v3.1 5X buffer (REF:4336917, Thermo Fisher Scientific, USA) 4 µL, 1 µL molded DNA, and nuclease free water 7 µL. DNA pGEM -3Zf was used as a positive control and Primary control -21 M13 as a positive control primer. The primers for sequencing are the same as the primers for PCR. The reaction of the sequencing

cycle was carried out under conditions: 96°C 1 min, 96°C 10 sec, 50°C 5 sec, 60 °C 4 min. The cycle was repeated 25 times later the result of the sequencing cycle is purified by XTerminator Solution and SAM solution (REF:4376486, Thermo Fisher Scientific, USA)10:45. The sample volume used is 10 µL. The tube containing the premix and the vortexed sample for 30 minutes then in the centrifuge for 1 minute. The supernatant was inserted into a 20 µL wellbore slab and read by using 3500 Genetic Analyzer. The sequencing results were then processed in the gene bank to identify the presence of mutations in the *folP1* gene *M. leprae*.

b. Variation analysis and dihydropteroate synthase *M. leprae*

DNA sequence from our clinical samples was edited by referring it with reference sequence of *folP1* gene (NC_002677.1) using Bioedit. The edited sequence was aligned to the corresponding sequences in the database using Basic Local Alignment Sequence Tool (BLAST) in NCBI website.

c. Mutation effect of V48A, T53A, P55L on *folP1* gene *M. leprae*

The amino acid sequence of the DHPS compiler was downloaded from Uniprot (P0C0X1). The analysis of structural effects of point mutation in a protein sequence was performed using HOPE web service (http://www.cmbi.ru.nl/hope/). The prediction of binding pocket (wide and volume) was performed using CASTp server (http://sts.bioe.uic.edu/castp).

d. Homology modelling of dihydropteroate synthase *M. leprae*

Homology of 3D dihydropteroate synthase structure was analyzed by using I-TASSER[16] (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Variants in T53A, V48A, and P55L were constructed using the fold-X[17] based on the wildtype structure (P0C0X1).

e. Preparation of drug molecules

The 3D structure of dapsone (CID:2955) was downloaded from Pubchem database, available at https://pubchem.ncbi.nlm.nih.gov/. Dapsone molecule was downloaded in SDF format. Molecule energy was reduced and converted pdbqt using Open Babel software.

f. Molecular docking

Docking analysis was performed by using AutoDock Vina[18] which is integrated in PyRx application.[19] Docking results were visualized using PyMol 1.8.6 and Discovery Studio 2017.

RESULTS

From 100 clinical samples, 53 PCR positive result samples were obtained and qualified to be proceed to sequencing. The BLAST result in NCBI shows that variation was identified in *M. leprae* Papua strain compared with TN strain in *folP1* gene. The type of mutation is missense where Thymine (T) was replaced by Cytosine (C) on nucleotide 143 (Figure 1). This mutation gave rise to the replacement of amino acid Valine become Alanine on DHPS (Figure 2b). The wild type and mutant amino acids differ in size. Alanine is smaller than Valine. The mutation will cause an empty space in the core of the protein. Among the 53 samples that were successfully amplified, there were six samples that show the mutation.

Mutation in the 143 nucleotides *folP1* gene of *M. leprae* was identified as a single peak (Figure 2b) and in a double peak (Figure 2c). This mutation caused a change from the Thymine (Figure 2a) to Cytosine. In some samples this mutation was found in a mixed allele (Figure 2c). As a comparison, we used the P55L, T53A variants which were confirmed as mutations that cause resistance to dapsone.[10,11]

Figure 1. Alignments folP1 gene of M. leprae Papua strain comparing with M. leprae strain TN (NC_002677.1) Variation was identified in folp1 gene of M. leprae Papua strain

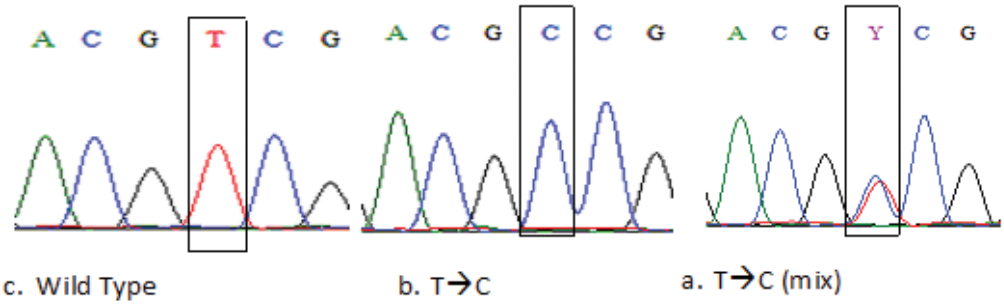


Figure 2. Comparison of electropherogram between wild type and mutant (a), Thymine mutation becomes Cytosine with single pic (b) and mix between mutant and wildtype (c).

Figure 3. Overview of the protein in ribbon-presentation. The protein is colored grey, the side chain of the mutated residue is colored magenta and shown as small balls.

Protein structure analysis of DHPS conformation on mutant of *M. leprae* at V48A, P55L, T53A, showed that each mutation influences the protein function (Figure 3). The mutation on V48A is located within a domain, annotated in UniProt as Pterin-binding. The mutation introduces an amino acid with different properties, which can disturb this domain and abolish its function[20] (Figure 3a). The wild-type residue on 55 DHPS is a proline. Prolines are known to be very rigid and therefore induce a special backbone conformation which might be required at this position. The mutation P55R/L can disturb this unique conformation[20] (Figure 3b dan 3c). The wild-type residue on 53 DHPS is a threonine. The mutation T53A introduces an amino acid with different properties, which can disturb this domain and abolish its function[20] (Figure 3).

The wide and the volume of binding pocket DHPS were predicted using CASTp servers.[21] Binding pocket from DHPS is in the red area (Figure 4). The V48A mutant showed the identical area and volume as wildtype while the binding pocket of the T53A and P55L mutants showed the increasing area and volume (Table 1).

Table 1. The active binding and volume of the *M. leprae* DHPS binding pocket

WT and mutant DHPS	Active binding pocket (Å^2)	Volume of the Binding Pocket (Å^3)
WT	931.323	697.065
V48A	931.323	697.065
T53A	949.217	712.906
P55L	1005.725	803.004

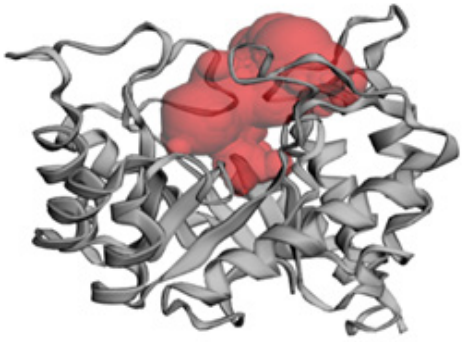


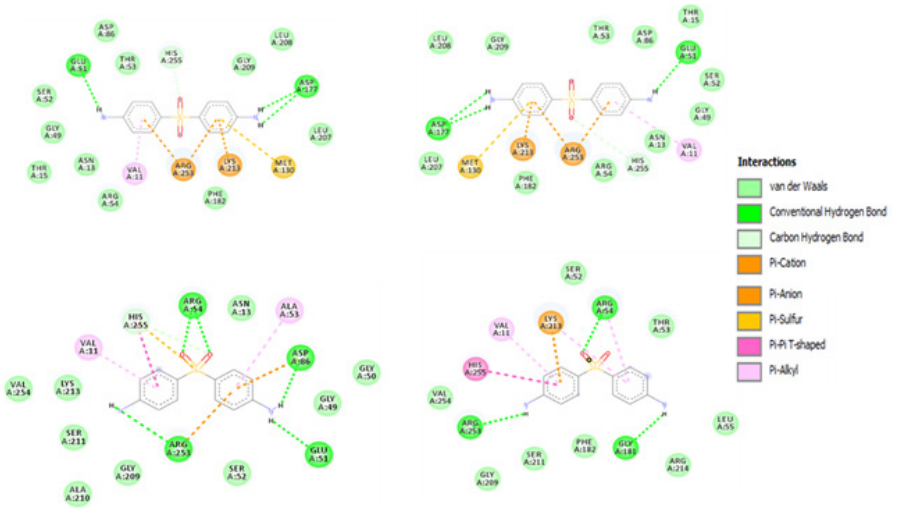
Figure 4. The prediction of the binding pocket of DHPS of M. leprae was labelled by red color zone

The wildtype DHPS (P0C0X1) modeling was performed using I-TASSER, while the mutant DHPS protein structure was derived using fold-X by emphasizing the mutations in the amino acids 48, 53 and 55. The model used in this study was the model with the highest confidence level based on the C- score. [16,22] Mutations in all three amino acids did not significantly alter the stability of the energy of the molecules (Table 2). Molecular docking between dapsone and DHPS showed the ability of dapsone to interact with wild type DHPS and mutant DHPS. Binding affinity between dapsone was higher on different sides of wildtype, specifically on variants T53A and P55L, whereas the binding affinity between dapsone and wild type and V48A of DHPS were equal (Table 2).

Table 2. Stability energy dihydropteroate synthase dan its binding energy to dapsone

Receptor	Binding Energy (kcal/mol)	Stability Energy (kcal/mol)
Wildtype	-6.7	170.39
V48A	-6.7	174.23
T53A	-7.2	167.86
P55L	-6.5	169.91

Interaction between dapsone and DHPS consisted of hydrogen bond, unfavorable donor-donor, Pi-cation, Pi-sulfur, and Pi-Pi T-shaped (Figure 5).



DISCUSSIONS

We found mutations in the *folP1* gene of *M. leprae* from Papua in nucleotide no 143 where there has been a change of amino acids from Thymine (T) to Cytosine (C) (Figure 1). This mutation caused changes in the amino acid Valine to Alanine in the DHPS enzyme (Figure 2b). These mutations exist in the region of Drug Resistance-Determining Regions (DRDR) of the *folP1* gene. The *folP1* gene is the gene that encodes the formation of the DHPS enzyme. This enzyme is the target of dapsone in the treatment of leprosy.[9] WHO has recommended this area for surveillance of drug resistance using PCR-direct sequencing. [12] This mutation was found in a single allele (Figure 2a) as well as multiple alleles (Figure 2a). Confirmation of mixed alleles in the *folP1*, *gyrA,* and *rpoB* genes of *M. leprae* has been reported previously.[23,24] Mutation V48A has been detected by Nakata et al in their clinical samples.[25]

In this study, we analyze V48A mutant using in silico method. Former studies show mutations in the *folP1* gene were missense mutations located at codon 53 (Thr53Ile, Thr53Arg and Thr53Ala) or codon 55 (Pro55Arg, Pro55Leu).[11] Nakata *et al*, found all mutations that cause amino acid substitutions at codon 55 resulted in dapsone resistance and mutations at codon 53 also gave rise to dapsone resistance except for the T53S substitution, which resulted in less resistance to dapsone than the wild-type sequence.[25] Mutation on V48A is located within a domain, annotated in UniProt as Pterin-binding which can disturb this domain and abolish its function.[20] The wild-type residue is very conserved, but a few other residue types have been observed at this position too. The mutant residue was not among the other residue types observed at this position in other, homologous proteins. However, residues that have some properties in common with your mutated residue were observed. This means that in some rare cases mutation V48A might occur without damaging the protein. The mutant residue is located near a highly conserved position important for the activity of the protein and in contact with residues

in another domain. It is possible that this interaction is important for the correct function of the protein (Figure 3). This mutation does not affect the volume binding pocket (Table 1) and protein stability (Table 2). However, the mutation may affect this interaction and as such affect protein function.[20]

On P55L mutant, the mutated residue is located in a domain that is important for the activity of the protein and in contact with residues in another domain. The mutant increased the volume binding pocket of DHPS (Table 1). It is possible that this interaction is important for the correct function of the protein. The mutation can affect this interaction and as such affect protein function.[20] The mutated residue of T53A mutant is located in a domain that is important for the activity of the protein and in contact with another domain that is also important for the activity. The interaction between these domains could be disturbed by the mutation, which might affect the function of the protein.[20]

On wildtype, the binding position of dapsone mutants V48A, T53A and P55L present in the binding pocket. The binding affinity between dapsone in T53A DHPS was stronger than wildtype, but preferably bond to a site that was different from wildtype. Dapsone linked with the T53A mutant through four hydrogen bonds on the residues Q51, E54, D86 and R253 (Figure 5). Dapsone is bound to the mutant P55L with three hydrogen bonds, R54, G181, and R253. Binding affinity formed was lower than that of wildtype, but also on sites that were different from wildtypes (Table 1). This indicated that the variants of P55L and T53A caused Dapsone to experience orientation changes in binding to DHPS. So that dapsone activity in inhibiting the performance of DHPS in both variants had decreased, or resistance to dapsone. T53A mutations, P55L in the *folP1* gene have also been confirmed to cause resistance to dapsone.[10,11] Chaitanya also found the greatest decrease in free energy bindings was present in the T53I and T55V mutants.[8] The energy and changes in the bonding patterns revealed the structural and mechanistic effects of these mutations on inducing dapsone resistance in leprosy.[8]

The binding affinity of V48A mutant was similar to wildtype (Figure 5). Dapson was bound with V48A through three covalent bonds of hidrogen namely 2 hidrogen bonds on residual E51 and 1 on D177 residual. This interaction was similar to the dapsone interaction on the wildtype (Gambar 5). This shows that the V48A mutant might still possess the ability to interact with dapsone. The testing of the effect of mutant 448A based on MIC values has been conducted before and it is known that the V48A mutation effect might give rise to low-level resistance to dapsone in *M. leprae* based on MIC value.[25] Thus it is necessary to do an in vivo test to confirm the effect of the V48A mutation.

In conclusion, based on molecular docking analysis, this mutation does not affect binding affinity dapsone against *M. leprae* dihydropteroate synthase. These results indicate that the V48A mutant is likely to remain susceptible to dapsone. Thus it is necessary to do an in vivo test to confirm the effect of the V48A mutation.[26]

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