

DESCRIPTION

METHODS OF SCREENING FOR COMPOUNDS THAT INHIBIT THE BIOSYNTHESIS OF
GPI IN MALARIA PARASITESTechnical Field

The present invention relates to methods of screening for compounds that inhibit the biosynthesis of GPI in malaria parasites.

Background Art

Malaria is the most common infectious human disease caused by parasitic protozoans. The disease is caused by infection with malaria parasites and is mediated by the mosquito, *Anopheles gambiae*. Every year there are estimated 500 million cases of malaria infection, including more than two million fatal cases (Gardner, et al., Nature 419:498-511, 2003). At present 40% of the world's population lives in malaria-epidemic areas, where it is said that one in every three infants dies from malaria.

Glycosylphosphatidylinositol (GPI) is known to play a key role in the growth and infectivity of parasites, including malaria parasites. There are many GPI-anchored proteins on the cell surface of these parasites. GPI-anchored proteins include proteins such as MSP-1 that function when the parasites invade red blood cells. GPI proteins act as parasitic antigens and initiate an immune response in the host. Thus, they have long been the subject of research aimed at vaccine development.

GPI not only functions as an anchor to tether proteins to the cell membrane, but is also an abundant glycolipid component of malaria parasite cell membranes. Recent studies have revealed that GPI is toxic and causes lethal symptoms. GPI induces the expression of inflammatory cytokines such as TNF- α , and of adhesion molecules. As a result, infected red blood cells adhere to capillaries, obstructing vessels (sequestration), brain blood vessels in particular. This induces further inflammatory reactions that are believed to lead to encephalopathy. Very recently, Schofield et al. reported that an anti-GPI antibody reduces lethality in an *in vivo* infection model

system using the murine malaria parasite *Plasmodium berghei*, and that *in vitro*, the antibody inhibits late inflammatory reactions caused by *Plasmodium falciparum* (Schofield L, et al., Nature 418:785-789, 2002). These findings suggest that GPI is a major factor in the lethality of malarial infections.

It has also been reported that the acylation of inositol is essential for binding mannose to GPI (Gerold, P. et al., Biochem. J. 344:731-738, 1999), and that the inhibition of inositol acylation, caused by excess glucosamine, inhibits the maturation of the malaria parasite *P. falciparum* (Naik, R. S. et al., J. Biol. Chem. 278:2036-2042, 2003). Thus, compounds that can selectively inhibit GPI biosynthesis, particularly the acylation of inositol, may be highly useful antimalarial drugs.

Disclosure of the Invention

An objective of the present invention is to provide antimalarial drugs that inhibit the biosynthesis of GPI. More specifically, the present invention provides the malaria parasite DNA that encodes the GWT1 protein, which is a protein involved in the biosynthesis of GPI (GPI synthase). The present invention also provides a method of using this DNA in methods of screening for antimalarial drugs. The present invention also provides degenerate mutant DNAs of the DNA that encodes the malaria parasite GPI biosynthesis protein. These degenerate mutant DNAs have a lower AT content than the original DNA. The present invention also provides a method of using the degenerate mutant DNAs in methods of screening for antimalarial drugs.

The GWT1 gene was originally found to encode a fungal GPI-anchored protein synthase (WO 02/04626), and is conserved in organisms ranging from yeasts to humans. The present inventors confirmed that GWT1 homologues (PfGWT1 for *P. falciparum* GWT1; PyGWT1 for *P. yoelii yoelii* GWT1) are included in the entire genomic sequences of *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium yoelii yoelii* (*P. yoelii yoelii*) (Gardner, et al., Nature 419:498-511, 2003; Carlton et al., Nature 419:512-519, 2003). The present inventors also found that the GWT1 gene product acts as a GlcN-PI acyltransferase in the GPI biosynthesis pathway. The PfGWT1 gene product is expected

to have similar activity, and thus compounds that inhibit this activity can be promising antimalarial drugs.

In WO 02/04626, the present inventors disclosed a group of compounds that inhibit the activity of the fungal GWT1 gene product. Compounds inhibiting the activity of the PfGWT1 gene product were expected to be antimalarial drugs.

In the present invention, the present inventors succeeded in isolating a region thought to be almost the full length of the PfGWT1. Using the GWT1 gene products of malaria parasites such as *P. falciparum*, antimalarial drugs can be screened through binding assays, glucosaminyl(acyl)phosphatidylinositol (PI-GlcN) acyltransferase assays, or using GPI-anchored protein detection systems. Compounds obtained from such screenings can be promising antimalarial drugs. Furthermore, the present inventors revealed that degenerate mutant DNAs (degenerate mutants of the DNA that encodes the malaria parasite GPI biosynthesis protein) having a lower AT content than the original DNA, complement the phenotype of the GWT1 gene-deficient fungus. Thus, it is possible to screen for compounds that inhibit the activity of proteins involved in GPI biosynthesis in malarial parasites by using, as an index, the phenotype of a GPI synthase gene-deficient fungus, into which a degenerate mutant DNA with a lower AT content (than the DNA encoding the GPI biosynthesis protein in malaria parasites) has been introduced.

Specifically, the present invention provides the following [1] to [25]:

[1] a DNA according to any one of (a) to (d), which encodes a protein of a malaria parasite having a GlcN-PI acyltransferase activity:

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 or 4,

(b) a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3,

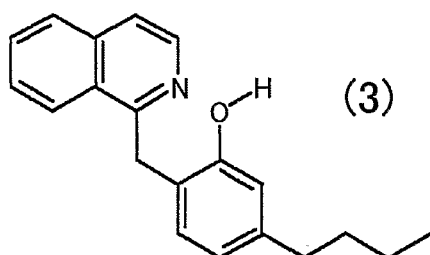
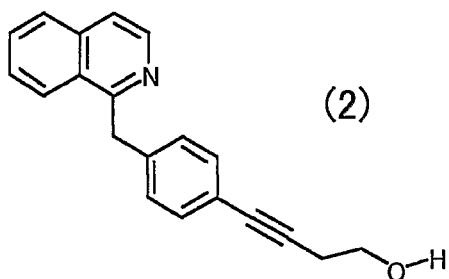
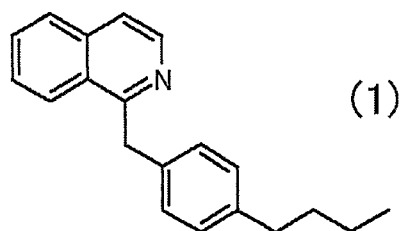
(c) a DNA hybridizing to a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3 under stringent conditions, and

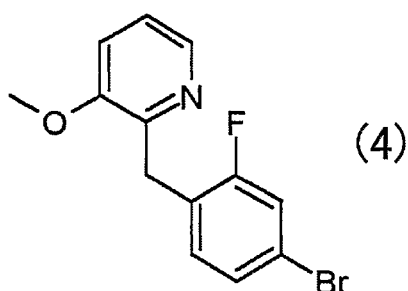
(d) a DNA encoding a protein which comprises the amino acid sequence of SEQ ID NO: 2 or 4, in which one or more amino acids have been added, deleted, substituted, and/or inserted;

[2] a protein encoded by the DNA according to [1];
[3] a vector into which the DNA according to [1] is inserted;
[4] a transformant which retains, in an expressible state, the DNA according to [1] or the vector according to [3] ;

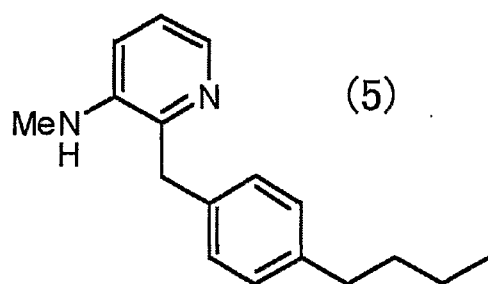
[5] an antimalarial drug which comprises as an active ingredient a compound that inhibits the activity of the protein according to [2];

[6] the antimalarial drug according to [5], wherein the compound that inhibits the activity of the protein according to [2] is at least one selected from the group consisting of the following compounds (1) to (5):





and,



[7] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting the protein according to [2] with a test sample and a labeled compound that has the activity of binding to the protein,
- (2) detecting the labeled compound that binds to the protein, and,
- (3) selecting a test sample that decreases the amount of labeled compound that binds to the protein;

[8] the method according to [7], wherein the labeled compound that has the activity of binding to the protein is produced by labeling at least one compound selected from the group consisting of the compounds (1) to (5) according to [6];

[9] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting a test sample with the protein according to [2],
- (2) detecting GlcN-(acyl)PI, and,
- (3) selecting a test compound that decreases the level of GlcN-(acyl)PI;

[10] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting a test sample with a cell overexpressing the protein

according to [2],

(2) determining the amount of GPI-anchored protein transported to the cell wall, and,

(3) selecting a test sample that decreases the amount of the GPI-anchored protein transported to the cell wall, as determined in step (2);

[11] a method for treating malaria, which comprises administering a compound that inhibits the activity of the protein according to [2];

[12] the method according to [11], wherein the compound that inhibits the activity of the protein according to [2] is the compound according to [5];

[13] a DNA encoding a protein that has the activity of complementing the phenotype of a GPI synthase gene-deficient yeast, which is a degenerate mutant of a DNA encoding a protein involved in GPI biosynthesis in malaria parasites, and that has a lower AT content than the original DNA;

[14] a DNA encoding a protein that has the activity of complementing the phenotype of a GPI synthase gene-deficient yeast, which is a degenerate mutant of a DNA encoding a protein involved in GPI biosynthesis in malaria parasites, and that has an AT content which is reduced by 70%;

[15] the DNA according to [13] or [14], which is selected from the group consisting of:

(a) a DNA encoding a protein that comprises any one of the amino acid sequences of SEQ ID NOs: 2 and 4, and odd sequence identification numbers in SEQ ID NOs: 6-47,

(b) a DNA comprising any one of the nucleotide sequences of SEQ ID NOs: 1 and 3, and even sequence identification numbers in - SEQ ID NOs: 6-47,

(c) a DNA hybridizing under stringent conditions to a DNA that comprises any one of the nucleotide sequences of SEQ ID NOs: 1 and 3, and even sequence identification numbers in SEQ ID NOs: 6-47, and,

(d) a DNA encoding a protein which comprises any one of the amino acid sequences of SEQ ID NOs: 2 and 4, and odd sequence identification numbers in SEQ ID NOs: 6-47, in which one or more amino acids have

been added, deleted, substituted, and/or inserted;

[16] a DNA comprising the nucleotide sequence of SEQ ID NO: 5;

[17] a vector in which a DNA according to any one of [13] to [16] is inserted;

[18] a transformant which retains, in an expressible state, the DNA according to any one of [13] to [16] or the vector according to [17];

[19] the transformant according to [18], which is a GPI synthase gene-deficient fungus;

[20] the transformant according to [18], which is a GPI synthase gene-deficient yeast;

[21] a method for producing a protein encoded by a DNA according to any one of [13] to [16], which comprises the steps of culturing the transformant according to any one of [18] to [20], and recovering the expressed protein from the transformant or the culture supernatant;

[22] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting a test sample with a GPI synthase gene-deficient fungus expressing the DNA according to any one of [13] to [16],
- (2) assaying the growth of that fungus, and,
- (3) selecting a test compound that inhibits the growth of that fungus;

[23] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting a test sample with a GPI synthase gene-deficient fungus expressing the DNA according to any one of [13] to [16],
- (2) determining the amount of a GPI-anchored protein transported to the fungal cell walls, and,
- (3) selecting a test sample that decreases the amount of the GPI-anchored protein transported to the cell wall, as determined in step (2);

[24] a method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) introducing the DNA according to any one of [13] to [16] into a GPI synthase gene-deficient fungus and expressing the protein

encoded by the DNA,

- (2) preparing the protein expressed in step (1),
- (3) contacting the prepared protein with a test sample and a labeled compound that has the activity of binding to the protein,
- (4) detecting the labeled compound that binds to the protein, and,
- (5) selecting a test sample that decreases the amount of labeled compound that binds to the protein; and,

[25] a method of screening for a compound having antimalarial activity, which comprises the steps of:

(1) introducing into a GWT1-deficient fungus, (i) a DNA encoding a protein that has the activity of complementing the phenotype of a GWT1-deficient yeast, wherein the DNA is a degenerate mutant of a DNA encoding a malaria parasite GWT1 protein that has a lower AT content than the original DNA, or (ii) a vector into which the degenerate mutant of DNA has been inserted, and expressing the protein encoded by the degenerate mutant DNA,

- (2) preparing the protein expressed in step (1),
- (3) contacting the prepared protein with a test sample,
- (4) detecting GlcN-(acyl)PI, and
- (5) selecting a test compound that decreases the level of GlcN-(acyl)PI.

The DNA encoding the GWT1 protein of *Plasmodium falciparum* (PfGWT1) was isolated for the first time in the present invention. The nucleotide sequence of the DNA encoding the PfGWT1 protein is shown in SEQ ID NO: 1, and the amino acid sequence of the PfGWT1 protein is set forth in SEQ ID NO: 2. In addition, the nucleotide sequence of the DNA encoding the GWT1 protein of *Plasmodium vivax* (PvGWT1) is shown in SEQ ID NO: 3, and the amino acid sequence of the PvGWT1 protein is set forth in SEQ ID NO: 4.

The GWT1 protein is involved in the biosynthesis of glycosylphosphatidylinositol (GPI), which is essential for the growth and infectivity of malaria parasites. Thus, compounds that inhibit the activity of the malaria parasite GWT1 protein can be used as antimalarial drugs. Such antimalarial drugs can be screened using this malaria parasite GWT1 protein.

The present invention provides DNAs encoding the malaria parasite GWT1 protein. Such DNAs include DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 or 4, and DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3.

The present invention also provides DNAs encoding proteins that are functionally equivalent to the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4. Herein, the expression "functionally equivalent" refers to having biological properties equivalent to those of the protein of interest, comprising the amino acid sequence of SEQ ID NO: 2 or 4 (the PfGWT1 or PvGWT1 proteins). The biological properties of the PfGWT1 and PvGWT1 proteins include GlcN-PI acyltransferase activity. The GlcN-PI acyltransferase activity can be measured by the method reported by Costello and Orlean (J. Biol. Chem. (1992) 267:8599-8603), or Franzot and Doering (Biochem. J. (1999) 340:25-32).

DNAs encoding proteins functionally equivalent to the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4 include: DNAs that hybridize under stringent conditions to the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3, and DNA encoding a protein which comprises the amino acid sequence of SEQ ID NO: 2 or 4, in which one or more amino acids have been added, deleted, substituted, and/or inserted.

The DNAs of the present invention can be isolated by methods well known to those skilled in the art. Examples of such methods include the use of hybridization (Southern E.M., J. Mol. Biol. 98: 503-517, 1975) and the polymerase chain reaction (PCR) (Saiki R.K. et al., Science 230: 1350-1354, 1985; Saiki R.K. et al., Science 239: 487-491, 1988). More specifically, it would be routine experimentation for those skilled in the art to isolate, from malaria parasites, a DNA highly homologous to DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3, using the DNA of SEQ ID NO: 1 or 3 or portions thereof as a probe, or by using as a primer a DNA which specifically hybridizes to the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3. Furthermore, DNAs that can be isolated by hybridization or PCR techniques, and that hybridize with the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3, are also comprised in the DNAs of the

present invention. Such DNAs include DNA encoding a malaria parasite homologue of the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4. The malaria parasite homologue includes those of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, which comprise the amino acid sequence of SEQ ID NO: 2 or 4.

Preferably, a DNA described above is isolated using hybridization reactions under stringent hybridization conditions. As used herein, the expression "stringent hybridization conditions" refers to, for example, hybridization in 4x SSC at 65°C followed by washing in 0.1x SSC at 65°C for one hour. Alternative stringent conditions are hybridization in 4x SSC containing 50% formamide at 42°C. Further alternative stringent conditions are hybridization in PerfectHyb™ (TOYOBO) solution at 65°C for 2.5 hours, followed by washing: (1) in 2x SSC containing 0.05% SDS at 25°C for five minutes; (2) in 2x SSC containing 0.05% SDS at 25°C for 15 minutes; and (3) in 0.1x SSC containing 0.1% SDS at 50°C for 20 minutes. The DNA thus isolated is expected to encode a polypeptide with a high homology at the amino acid level to the amino acid sequence of SEQ ID NO: 2 or 4. Herein, "high homology" means a sequence identity of at least 70% or more, preferably 80% or more, more preferably 90% or more, and most preferably 95% or more, in the whole amino acid sequence.

The degree of identity at the amino acid sequence level or nucleotide sequence level can be determined using the BLAST algorithm of Karlin and Altschul (Karlin S. and Altschul S.F, Proc. Natl. Acad. Sci. USA. 87: 2264-2268, 1990; Karlin S. and Altschul S.F, Proc. Natl. Acad. Sci. USA. 90: 5873-5877, 1993). BLAST algorithm-based programs, called BLASTN and BLASTX, have been developed (Altschul S.F. et al., J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is analyzed using BLASTN, the parameters are set, for example, at score= 100 and word length= 12. On the other hand, when an amino acid sequence is analyzed using BLASTX, the parameters are set, for example, at score= 50 and word length= 3. When the BLAST and Gapped BLAST programs are used, the default parameters for each program are used. Specific procedures for such analysis are known (please see the web site of the National Institute of Biotechnology Information

<http://www.ncbi.nlm.nih.gov>).

DNAs of the present invention comprise genomic DNAs, cDNAs, and chemically synthesized DNAs. A Genomic DNA or cDNA can be prepared according to conventional methods known to those skilled in the art. For example, a genomic DNA can be prepared as follows: (i) extracting a genomic DNA from malaria parasites; (ii) constructing a genomic library (using, for example, a plasmid, phage, cosmid, BAC, or PAC, as a vector); (iii) spreading the library; and then (iv) conducting colony hybridization or plaque hybridization using probes prepared based on a DNA which encodes the malaria parasite GWT1 protein of the present invention (e.g., SEQ ID NO: 1 or 3). Alternatively, genomic DNA can be prepared by PCR, using primers specific to a DNA which encodes the malaria parasite GWT1 protein of the present invention (e.g., SEQ ID NO: 1 or 3). On the other hand, cDNA can be prepared, for example, as follows: (i) synthesizing cDNA based on mRNA extracted from malaria parasites; (ii) constructing a cDNA library by inserting the synthesized cDNA into vectors such as λ ZAP; (iii) spreading the cDNA library; and (iv) conducting colony hybridization or plaque hybridization as described above. Alternatively, the cDNA can also be prepared using PCR.

The present invention also provides DNAs encoding proteins structurally similar to the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4. Such DNAs include those which comprise nucleotide sequences encoding proteins comprising amino acid sequences in which one or more amino acid residues are substituted, deleted, inserted, and/or added. There is no limitation on the number and site of the amino acid mutation in proteins mentioned above, so long as the mutated protein retains functions of the original protein such as those described in Mark, D. F. *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) 81, 5662-5666; Zoller, M. J. & Smith, M., *Nucleic Acids Research* (1982) 10, 6487-6500; Wang, A. *et al.*, *Science* 224, 1431-1433; Dalbadie-McFarland, G. *et al.*, *Proc. Natl. Acad. Sci. USA* (1982) 79, 6409-6413. The percentage of mutated amino acids is typically 10% or less, preferably 5% or less, and more preferably 1% or less of the total amino acid residues. In addition, the number of mutated amino acids is usually 30 amino acids or less, preferably 15 amino

acids or less, more preferably five amino acids or less, still more preferably three amino acids or less, even more preferably two amino acids or less.

It is preferable that the mutant amino acid residue be one that retains the properties of the side-chain after its mutation (a process known as conservative amino acid substitution). Examples of amino acid side chain properties are hydrophobicity (A, I, L, M, F, P, W, Y, V) and hydrophilicity (R, D, N, C, E, Q, G, H, K, S, T). Side chains include: aliphatic side-chains (G, A, V, L, I, P); side chains containing an hydroxyl group (S, T, Y); side chains containing a sulfur atom (C, M); side chains containing a carboxylic acid and an amide (D, N, E, Q); basic side-chains (R, K, H); and aromatic side-chains (H, F, Y, W).

A fusion protein comprising the malaria parasite GWT1 protein is an example of a protein to which one or more amino acids residues have been added. Fusion proteins can be made by techniques well known to a person skilled in the art. For example, and without limitation to this particular technique, the DNA encoding the malaria parasite GWT1 protein of the present invention can be combined with DNA encoding another peptide or protein such that their reading frames match. A protein of the present invention can form a fusion protein with a number of known peptides. Such peptides include FLAG (Hopp, T. P. *et al.*, Biotechnology (1988) 6, 1204-1210), 6x His, 10x His, Influenza agglutinin (HA), human c-myc fragment, VSP-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40T antigen fragment, lck tag, α -tubulin fragment, B-tag, and Protein C fragment. Examples of proteins that may be fused to a protein of the present invention include glutathione-S-transferase (GST), HA, immunoglobulin constant region, β -galactosidase, and maltose-binding protein (MBP).

In addition to using the above-mentioned hybridization and PCR techniques, those skilled in the art could prepare the above-described DNA by methods including, for example, site-directed mutagenesis to introduce mutations in that DNA (Kramer W. and Fritz H-J., Methods Enzymol. 154: 350, 1987). A protein's amino acid sequence may also be mutated in nature due to mutation of the nucleotide sequence which encodes the protein. In addition, degenerate mutant DNAs, in which

nucleotide mutations do not result in amino acid mutations in the proteins (degeneracy mutants), are also comprised in the present invention. Furthermore, the present invention also comprises proteins encoded by the above-described DNAs of this invention.

The present invention provides vectors containing the DNAs of the present invention, transformants retaining the DNAs or vectors of the present invention, and methods for producing proteins of the present invention which utilize these transformants.

A vector of the present invention is not limited so long as the DNA inserted into the vector is stably retained. For example, pBluescript® vector (Stratagene) is preferable as a cloning vector when using *E. coli* as a host. An expression vector is particularly useful when using a vector to produce a protein of the present invention. The expression vector is not specifically limited, so long as it expresses proteins *in vitro*, in *E. coli*, in cultured cells, and *in vivo*. Preferable examples of expression vectors include the pBEST vector (Promega Corporation) for *in vitro* expression, the pET vector (Novagen) for expression in *E. coli*, the pME18S-FL3 vector (GenBank Accession No. AB009864) for expression in cultured cells, and the pME18S vector (Mol. Cell Biol. 8: 466-472, 1988) for *in vivo* expression. The insertion of a DNA of the present invention into a vector can be carried out by conventional methods, for example, by a ligase reaction using restriction enzyme sites (Current Protocols in Molecular Biology, ed. by Ausubel et al., John Wiley & Sons, Inc. 1987, Section 11.4-11.11).

The host cell into which the vector of the present invention is introduced is not specifically limited, and various host cells can be used according to the objectives of this invention. For example, cells that can be used to express the proteins include, but are not limited to, bacterial cells (e.g., *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, *Bacillus subtilis*), fungal cells (e.g., yeast, *Aspergillus*), insect cells (e.g., *Drosophila* S2, *Spodoptera* SF9), animal cells (e.g., CHO, COS, HeLa, C127, 3T3, BHK, HEK293, Bowes melanoma cell), and plant cells. The transfection of a vector to a host cell can be carried out by conventional methods such as calcium phosphate precipitation, electroporation (Current protocols in

Molecular Biology, ed. by Ausubel et al., John Wiley & Sons, Inc. 1987, Section 9.1-9.9), the Lipofectamine method (GIBCO-BRL), and microinjection.

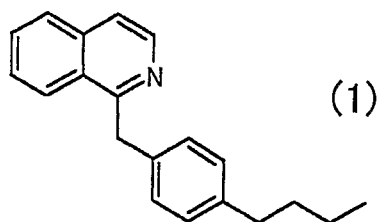
By incorporating an appropriate secretion signal into the protein of interest, the protein expressed in host cells can be secreted into the lumen of the endoplasmic reticulum, into cavities around the cells, or into the extracellular environment. These signals may be endogenous or exogenous to the protein of interest.

When a protein of the present invention is secreted into the culture medium, it is collected from that medium. If a protein of the present invention is produced intracellularly, the cells are lysed and then the protein is collected.

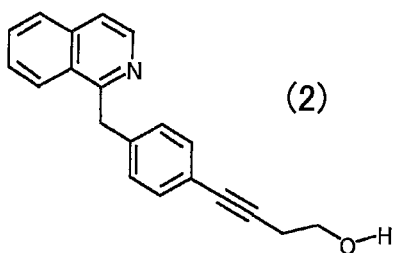
A protein of the present invention can be collected and purified from a recombinant cell culture using methods known in the art, including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anionic or cationic exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography.

Compounds including DNAs of the present invention are isolated compounds. Herein, the term "isolated" refers to being separated from the original environment (for example, the natural environment if it is naturally-occurring). A compound in a sample where the compound of interest is substantially abundant, and/or in a sample where the compound of interest has been partially or substantially purified, is an "isolated" compound. The term "substantially purified", as used herein, refers to a state where the compound has been separated from the original environment, and from which at least 60%, preferably 75%, and most preferably 90% of other coexisting natural components have been removed.

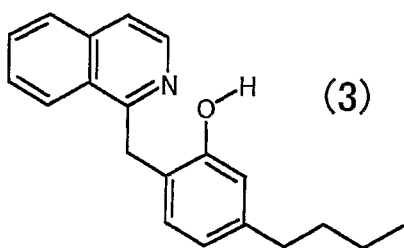
The present invention provides an antimalarial drug that inhibits the activity of the GWT1 gene product of malaria parasites. A preferred compound inhibiting the activity of the GWT1 gene product of malaria parasites is the compound described in WO 02/04626, and includes the compounds (1) to (5):



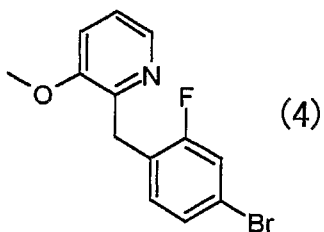
compound (1): 1-(4-butyl benzyl) isoquinoline



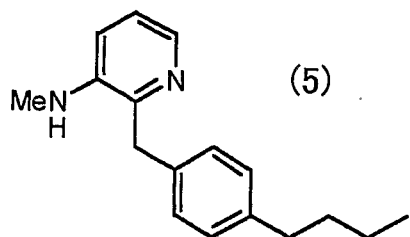
compound (2): 4-[4-(1-isoquinolyl methyl) phenyl]-3-butyne-1-ol



compound (3): 5-butyl-2-(1-isoquinolyl methyl) phenol



compound (4): 2-(4-bromo-2-fluorobenzyl)-3-methoxypyridine



compound (5): N-[2-(4-butyl benzyl) -3-pyridyl]-N-methylamine

A Compound that inhibits the activity of the malaria parasite GWT1 gene product, or a salt thereof, or a hydrate thereof, can be administered as it is to mammals (preferably humans). It can also be formulated by a conventional method into a tablet, powder, fine granule, granule, coated tablet, capsule, syrup, troche, inhalant, suppository, injection, ointment, eye ointment, eye drop, nasal drop, ear drop, cataplasm, lotion, and such, and then administered.

For formulation of a pharmaceutical, auxiliary agents ordinarily used in pharmaceutical formulations (for example, fillers, binders, lubricants, coloring agents, flavoring agents, and as necessary, stabilizers, emulsifiers, absorbefacient, surfactants, pH regulators, antiseptics, and antioxidants) can be used. A pharmaceutical formulation can be prepared using an ordinary method combining components that are generally used as ingredients for pharmaceutical preparations.

For example, oral formulations can be produced by combining a compound of the present invention or a pharmaceutically acceptable salt thereof with a filler, and as necessary, a binder, disintegrator, lubricant, coloring agent, flavoring agent, and such, and then formulating the mixture into a powder, fine granule, granule, tablet, coated tablet, capsule, and such by usual methods.

Examples of these components include: animal fat and vegetable oils such as soybean oil, beef tallow, and synthetic glyceride; hydrocarbons such as liquid paraffin, squalene, and solid paraffin; ester oils such as octyldodecyl myristate and isopropyl myristate; higher alcohols such as cetostearyl alcohol and behenyl alcohol; silicone resin; silicone oil; surfactants such as polyoxyethylene fatty acid ester, sorbitan fatty acid ester, glycerol fatty acid ester, polyoxyethylene sorbitan fatty acid ester, polyoxyethylene hardened castor oil, and polyoxyethylene polyoxypropylene block copolymer; water-soluble macromolecules such as hydroxyethyl cellulose, polyacrylic acid, carboxyvinyl polymer, polyethylene glycol, polyvinyl pyrrolidone, and methyl cellulose; lower alcohols such as ethanol and isopropanol; polyhydric alcohols such as glycerol, propylene glycol, dipropylene glycol, and sorbitol; sugars such as glucose and sucrose; inorganic powder such as silicic acid anhydride,

magnesium aluminum silicate, and aluminum silicate; and purified water. Examples of fillers include lactose, corn starch, refined white sugar, glucose, mannitol, sorbitol, crystalline cellulose, and silicon dioxide. Binders are polyvinyl alcohol, polyvinyl ether, methyl cellulose, ethyl cellulose, gum arabic, tragacanth, gelatin, shellac, hydroxypropylmethyl cellulose, hydroxypropyl cellulose, polyvinyl pyrrolidone, polypropyleneglycol polyoxyethylene block polymer, meglumine, and such. Examples of disintegrators include starch, agar, powdered gelatin, crystalline cellulose, calcium carbonate, sodium hydrogencarbonate, calcium citrate, dextrin, pectin, and calcium carboxymethylcellulose. Lubricants are magnesium stearate, talc, polyethyleneglycol, silica, hardened vegetable oil, and such. Examples of coloring agents are those accepted for addition to pharmaceuticals. Flavoring agents are cocoa powder, *l*-menthol, aromatic dispersant, mint oil, borneol, cinnamon powder, and such. The use of sugar coating and other appropriate coating as necessary is of course permissible for these tablets and granules.

Furthermore, liquid formulations such as syrups and injections can be prepared using conventional methods. In such methods, pH regulators, solubilizers, isotonizing agents, and such, and as necessary solubilizing adjuvants, stabilizers, and so on, are added to the compounds of this invention or pharmaceutically acceptable salts thereof.

Methods for producing external formulations is not restricted and can be a conventional method. That is, base materials used for formulation can be selected from various materials ordinarily used for medicaments, quasi-drugs, cosmetics, and such. Specifically, the base materials to be used are, for example, animal fat and vegetable oils, mineral oils, ester oils, waxes, higher alcohols, fatty acids, silicone oils, surfactants, phospholipids, alcohols, polyhydric alcohols, water soluble macromolecules, clay minerals, and purified water. As necessary, pH regulators, antioxidants, chelating agents, antiseptic and antifungal agents, coloring matters, fragrances, and such may also be added. However the base materials of the external formulations of the present invention are not limited thereto. Furthermore, as necessary, components such as those that

have a differentiation-inducing effect, blood flow accelerants, fungicides, antiphlogistic agents, cell activators, vitamins, amino acids, humectants, and keratolytic agents can be combined. The above-mentioned base materials are added in an amount that leads to the concentration usually used for external formulations.

The term "salt" as described in the present invention preferably includes, for example, a salt with an inorganic or organic acid, a salt with an inorganic or inorganic base, or a salt with an acidic or basic amino acid. In particular, a pharmaceutically acceptable salt is preferable. Acids and bases form salts at an appropriate ratio of 0.1 to 5 molecules of acid or base to one molecule of the compound.

Preferable examples of a salt with an inorganic acid are a salt with hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, and phosphoric acid. Preferably, a salt with an organic acid includes a salt with acetic acid, succinic acid, fumaric acid, maleic acid, tartaric acid, citric acid, lactic acid, stearic acid, benzoic acid, methanesulfonic acid, and p-toluenesulfonic acid.

Preferable examples of a salt with an inorganic base are: an alkali metal salt such as a sodium salt and a potassium salt; an alkaline earth metal salt such as a calcium salt and a magnesium salt; an aluminum salt, and an ammonium salt. Preferably, a salt with an organic base includes a salt with diethylamine, diethanolamine, meglumine, and N,N'-dibenzylethylenediamine.

Preferable examples of a salt with an acidic amino acid are a salt with aspartic acid and glutamic acid, and preferably, a salt with a basic amino acid includes a salt with arginine, lysine, and ornithine.

The compounds of the present invention or salts thereof, or hydrates thereof can be administered orally or parenterally by a conventional method without limitation as to their form. They can be formulated into dosage forms such as tablets, powders, fine granules, capsules, syrups, troches, inhalants, suppositories, injections, ointments, eye ointments, eye drops, nasal drops, ear drops, cataplasms, and lotions. The dose of the pharmaceutical compositions of this invention can be selected appropriately

depending on the degree of the symptoms, the patient's age, sex and weight, the dosage form, the type of salt, the specific type of disease, and such.

Compounds of the present invention are administered to a patient in a therapeutically effective dose. Herein, "therapeutically effective dose" refers to the amount of pharmaceutical agent that yields the desired pharmacological result and is effective in the recovery or relief from the symptoms of the patient to be treated. The dose differs markedly depending on the type of disease, the degree of symptoms, the patient's weight, age, sex, sensitivity to the agent. However, the normal adult dosage for one day is about 0.03 mg to 1000 mg, preferably 0.1 mg to 500 mg, more preferably 0.1 mg to 100 mg, when administered from once to several times a day, or from once to several times over several days. The dose for injections is normally, about 1 to 3000 $\mu\text{g/kg}$, and is preferably about 3 to 1000 $\mu\text{g/kg}$.

In addition, the present invention relates to a method of screening for antimalarial drugs using the malaria parasite GWT1 gene product. Such a screening method includes, but is not limited to: [1] A binding assay which screens for compounds that compete with a labeled compound to bind with the malaria parasite GWT1 gene product; [2] A GlcN-PI acyltransferase assay system to screen for compounds that inhibit the GlcN-PI acyltransferase activity of the malaria parasite GWT1 gene product; and [3] A GPI-anchored protein detection system in which the malaria parasite GWT1 gene product is expressed in cells, preferably fungal cells, and then the GPI-anchored proteins on the cell surface are detected. The present invention is not limited to these methods, and comprises any method of screening for antimalarial drugs using the malaria parasite GWT1 gene product. The methods [1] to [3] listed above are described below in detail.

[1] A binding assay to screen for compounds that compete with a labeled compound to bind with the malaria parasite GWT1 gene product

The two methods according to the present invention are disclosed below, namely (1) a method for preparing the malaria parasite GWT1 gene product (hereinafter referred to as the malaria parasite GWT1

protein) and (2) a method for a binding experiment involving a labeled compound (hereinafter referred to as a binding assay).

(1) Method for preparing the malaria parasite GWT1 protein

The malaria parasite GWT1 protein is prepared from a cell membrane fraction, preferably from fungal cells, more preferably from cells of *S. cerevisiae* into which the DNA encoding the malaria parasite GWT1 protein of SEQ ID NO: 2 has been introduced. It is preferable to introduce such a DNA into GWT1 gene-deficient cells. In the binding assay, the prepared membrane fraction may be used without any further treatment, or can be further purified before use. The procedure using *S. cerevisiae* is described below in detail.

(a) Introduction of the malaria parasite GWT1 gene

The malaria parasite GWT1 gene used in the present invention can be a naturally-occurring gene, or preferably, it can be synthesized based on the amino acid sequence of SEQ ID NO: 2 or 4. The malaria parasite GWT1 gene is very rich in adenine and thymine. Thus, it was predictable that the gene will be difficult to manipulate with ordinary gene recombination techniques, and that gene expression in yeast, cells, and such will be inefficient. Therefore, it is preferable to design a nucleotide sequence in which codons corresponding to each of the corresponding amino acids have been replaced with those that are thought to express efficiently in yeast, cells, and such, and conduct DNA synthesis based on this designed sequence to create an artificial malaria parasite GWT1 gene, which is then used in the experiments described below.

An expression plasmid for the malaria parasite GWT1 is prepared by inserting the malaria parasite GWT1 gene into an *S. cerevisiae* expression vector, for example, an expression vector prepared by inserting a suitable promoter and terminator, such as the pKT10-derived GAPDH promoter and GAPDH terminator, into the expression vector YEp352's multi-cloning site (Tanaka et al., Mol. Cell Biol., 10:4303-4313, 1990). *S. cerevisiae* (e.g., G2-10 strain) is cultured in an appropriate medium (e.g., YPD medium (Yeast extract-Polypeptone-Dextrose medium)) while shaking at an appropriate temperature (e.g., 30°C), and the cells are harvested during the late logarithmic growth phase. After washing, the

GWT1-expression plasmid is introduced into *S. cerevisiae* cells using, for example, the lithium-acetate method. This method is described in the User Manual of YEAST MAKER™ Yeast Transformation System (BD Biosciences Clontech). A malaria parasite GWT1-overexpressing strain and a strain carrying a negative control vector can be obtained by culturing the transformed cells on SD (ura-) medium at 30°C for two days.

Expression vectors and gene transfer methods for fungal species other than *S. cerevisiae* have been reported as follows: expression vectors such as pCL for *Schizosaccharomyces pombe* (*S. pombe*) and their transfer methods are described by Igarashi et al. (Nature 353:80-83, 1991); expression vectors such as pRM10 for *C. albicans* and their transfer methods are described by Pla J. et al. (Yeast, 12: 1677-1702, 1996); expression vectors such as pAN7-1 for *A. fumigatus* and their transfer methods are described by Punt P.J. et al. (GENE, 56: 117-124, 1987); and expression vectors such as pPM8 for *C. neoformans* and their transfer methods are described by Monden P. et al. (FEMS Microbiol. Lett., 187: 41-45, 2000).

(b) Method for preparing membrane fractions

S. cerevisiae cells in which the malaria parasite GWT1 gene has been introduced are cultured in an appropriate medium (e.g., SD (ura-) liquid medium) while being shaken at an appropriate temperature (e.g., 30°C). The fungal cells are harvested during the mid-logarithmic growth phase, washed, and then suspended in an appropriate amount (e.g., three times the volume of fungal cells) of homogenization buffer (e.g., 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, Complete™ (Roche)). An appropriate amount of glass beads (e.g., four times the volume of fungal cells) is added to the suspension. The mixture is vortexed and then allowed to stand on ice. This operation is repeated several times to crush fungal cells.

One milliliter of the homogenization buffer is added to the resulting lysate. The mixture is centrifuged, for example at 2,500 rpm for five minutes, to precipitate the glass beads and uncrushed fungal cells. The supernatant is transferred to another tube. The tube is centrifuged, for example at 13,500 rpm for ten minutes, to

precipitate a membrane fraction (total membrane fraction) comprising organelles. The precipitate is suspended in 1 ml of binding buffer (e.g., 0.1 M Phosphate buffer, pH 7.0, 0.05% Tween 20, Complete™ (Roche)), and then centrifuged, for example, at 2,500 rpm for one minute to remove unsuspended material. The supernatant is then centrifuged, for example at 15,000 rpm for five minutes. The precipitate is resuspended in 150 to 650 µl of binding buffer to prepare a membrane fraction.

Membrane fractions can be prepared from fungal species other than *S. cerevisiae* using the method of Yoko-o et al. for *S. pombe* (Eur. J. Biochem. 257:630-637, 1998); the method of Sentandreu M et al. for *C. albicans* (J. Bacteriol., 180: 282-289, 1998); the method of Mouyna I et al. for *A. fumigatus* (J. Biol. Chem., 275: 14882-14889, 2000); and the method of Thompson JR et al. for *C. neoformans* (J. Bacteriol., 181: 444-453, 1999).

Alternatively, the malaria parasite GWT1 protein can be prepared by expressing an *E. coli*, insect and mammalian cell or the like in non-fungal cells.

When mammalian cells are used, the malaria parasite GWT1 gene is ligated with an over-expression vector containing, for example, the CMV promotor, and then introduced into the mammalian cells. Membrane fractions can then be prepared according to the method of Petaja-Repo et al. (J. Biol. Chem., 276:4416-23, 2001).

Insect cells expressing the malaria parasite GWT1 gene (e.g., Sf9 cells) can be prepared using, for example, a baculovirus expression kit such as the BAC-TO-BAC® Baculovirus Expression system (Invitrogen). Membrane fractions can then be prepared according to the method of Okamoto et al. (J. Biol. Chem., 276:742-751, 2001).

The malaria parasite GWT1 protein can be prepared from *E. coli* by, for example, ligating the malaria parasite GWT1 gene into an *E. coli* expression vector such as the pGEX vector (Pfizer Inc.), and introducing the construct into *E. coli* such as BL21.

(2) Binding assay methods

(a) Synthesis of labeled compound

The labeled compound is prepared from a compound that has been

confirmed to bind to GWT1 proteins. Any compound which can bind to GWT1 proteins can be used. The labeled compound is preferably prepared from the compound described in WO 02/04626, more preferably from compounds according to (1) to (5) described above.

Any labeling method can be used. Preferably, the compound is labeled with a radioisotope, more preferably with ^3H . The radiolabeled compound can be prepared by typical production methods using a radioactive compound as a starting material. Alternatively, ^3H labeling can be achieved using an ^3H exchange reaction.

(b) Confirmation of specific binding

The labeled compound is added to the prepared membrane fraction and the mixture is allowed to stand on ice for an appropriate time, for example, one to two hours, while the binding reaction between the labeled compound and the membrane fraction takes place. The membrane fraction is precipitated by centrifuging the mixture, for example at 15,000 rpm for three minutes. The precipitate is resuspended in binding buffer, and the suspension is centrifuged. This is repeated appropriately (twice) to remove any unbound labeled compound. The precipitate is again suspended in binding buffer. The resulting suspension is transferred into a scintillation vial, and a scintillator is added. Radioactivity is measured using a liquid scintillation counter.

The specific binding of the labeled compound to the GWT1 protein can be confirmed by assessing whether binding of the labeled compound is inhibited by adding a large excess of unlabeled compound (ten times or more), and whether the compound binds negligibly to membrane fractions prepared from fungal cells which do not express the GWT1 protein.

(c) Binding inhibition of a labeled compound by a test sample

A test sample and the labeled compound are added to the prepared membrane fraction, and the mixture is allowed to stand on ice for an appropriate period of time, for example, one to two hours, while the binding reaction to the membrane fraction takes place. Test compounds used in the present invention's screening method include:

a simple naturally-occurring compound, an organic compound, an inorganic compound, a protein, or a peptide, as well as a compound library, an expression product of a genetic library, a cell extract, a cell culture supernatant, a product from fermentative bacteria, an extract of a marine organism, a plant extract, and the like.

The mixture is centrifuged, for example at 15,000 rpm for three minutes to precipitate the membrane fraction. The precipitate is resuspended in binding buffer and the suspension is centrifuged. This is repeated appropriately (twice) to remove any unbound labeled compound. The precipitate is suspended in the binding buffer. The suspension is transferred into a scintillation vial, and scintillator is added thereto. The radioactivity is measured using a liquid scintillation counter.

When the binding of the labeled compound to the membrane fraction is inhibited in the presence of a test sample, the test sample is judged to have the activity of binding to the malaria parasite GWT1 protein.

[2] The GlcN-PI acyltransferase assay system for screening compounds that inhibit the GlcN-PI acyltransferase activity of the malaria parasite GWT1 protein

The transfer of an acyl group to GPI can be detected by the method reported by Costello L.C and Orlean P., J. Biol. Chem. (1992) 267:8599-8603; or Franzot S.P and Doering T.L., Biochem. J. (1999) 340:25-32. A specific example of the method is described below. The following experimental conditions are preferably optimized for each malaria parasite GWT1 protein to be used.

The malaria parasite GWT1 protein is prepared according to the procedure described in Section 1. A membrane fraction comprising the malaria parasite GWT1 protein is added to a buffer which comprises an appropriate metal ion (Mg^{2+} , Mn^{2+}), ATP, Coenzyme A, and preferably an inhibitor that prevents the consumption of UDP-GlcNAc in other reactions, for example, nikkomycin Z as an inhibitor of chitin synthesis, or tunicamycin as an inhibitor of asparagine-linked glycosylation. A test sample is then added to the mixture and the resulting mixture is incubated at an appropriate temperature for an

appropriate period of time (for example, at 24°C for 15 min).

A GlcN-(acyl)PI precursor (for example UDP-GlcNAc, Acyl-Coenzyme A, and preferably UDP-[¹⁴C]GlcNAc) which has been appropriately labeled, and preferably radiolabeled, is added to the mixture. The resulting mixture is incubated for an appropriate period of time (for example, at 24°C for one hour). A mixture of chloroform and methanol (1:2) is added, the resulting mixture is stirred to halt the reaction, and the lipids are extracted. The extracted reaction product is dissolved in an appropriate solvent, preferably butanol. Then, GlcN-(acyl)PI produced in the reaction is separated by a method such as HPLC or thin layer chromatography (TLC), preferably TLC. When TLC is used, the developer can be selected appropriately from, for example, CHCl₃/CH₃OH/H₂O (65:25:4), CHCl₃/CH₃OH/1M NH₄OH (10:10:3), and CHCl₃/pyridine/HCOOH (35:30:7). A preferred developer is CHCl₃/CH₃OH/H₂O (65:25:4). The separated GlcN-(acyl)PI is quantified using a method appropriate for the label used. When labeled with a radioisotope, the separated GlcN-(acyl)PI can be quantified based on its radioactivity.

When the amount of GlcN-(acyl)PI produced is reduced in the presence of a test sample, the test sample is judged to have the activity of inhibiting acyl group transfer by the malaria parasite GWT1 protein.

[3] A GPI-anchored protein detection system which comprises expressing the malaria parasite GWT1 protein in cells and detecting the GPI-anchored protein on the cell surface

The ability of a test sample to inhibit the activity of the malaria parasite GWT1 protein can be determined using a GPI-anchored protein detection system that comprises expressing the GWT1 protein in cells, preferably fungal cells, and then detecting the GPI-anchored protein on the cell surface. The fungi of the present invention are those belonging to Zygomycota, Ascomycota, Basidiomycota, and Deuteromycete, and preferably pathogenic fungi, *Mucor*, *Saccharomyces*, *Candida*, *Cryptococcus*, *Trichosporon*, *Malassezia*, *Aspergillus*, *Trichophyton*, *Microsporum*, *Sporothrix*, *Blastomyces*, *Coccidioides*, *Paracoccidioides*, *Penicillium*, and *Fusarium*, more preferably *C.*

albicans, *C. glabrata*, *C. neoformans*, and *A. fumigatus*, and even more preferably, yeast. Such yeasts include *S. cerevisiae* and *S. pombe*. The method for introducing into the above-described fungal cells an expression vector containing inserted DNA encoding the malaria parasite GWT1 protein is known to those skilled in the art.

When the malaria parasite GWT1 protein is expressed in fungal cells, the amount of GPI-anchored protein transported to the fungal cell wall can be determined by the following methods: (1) by using a reporter enzyme; (2) by using an antibody that reacts with the surface glycoprotein of fungal cell walls; (3) by using the protein's ability to adhere to animal cells; or (4) by observing fungal cells under a light microscope or electron microscope.

The methods of (1) to (4) have been disclosed in WO 02/04626, which is described specifically in Examples of this invention. The methods (1) to (4), and preferably a combination of these methods (1) to (4), can determine whether a test sample inhibits the transport of the GPI-anchored protein onto the cell wall, or the expression of the GPI-anchored protein on the fungal cell surface.

Hereinafter, the methods of (1) to (4) will be described.

(1) A method using a reporter enzyme

The process that transports GPI-anchored proteins to the cell wall can be quantified using a tracer experiment such as one where a GPI-anchored protein is labeled with a radioactive isotope, the fungal cell wall fraction is obtained, and immunoprecipitated using an antibody against the GPI-anchored protein. Alternatively, quantification can be more readily performed as follows: the C-terminal sequence, which is considered to function as a transport signal and is commonly observed among GPI-anchored proteins, can be expressed as a fusion protein with an easily measurable enzyme (reporter enzyme), the fungal cell wall fraction can be obtained,; and a reporter system that measures the enzyme activity of each fraction can be used (Van Berkel MAA et al., FEBS Letters, 349: 135-138, 1994). Hereinafter, a method which uses a reporter enzyme will be described, but in the present invention such methods are not to be construed as being limited thereto.

First, the reporter gene is constructed and introduced into

fungi. The reporter gene is constructed by linking a promoter sequence that functions in fungi with DNAs that respectively encode a signal sequence, a reporter enzyme, and a GPI-anchored protein C-terminal sequence in such a way that the reading frames match. Examples of the promoter sequence are GAL10 and ENO1. Examples of the signal sequence include α -factor, invertase, and lysozyme. Examples of reporter enzymes are β -lactamase, lysozyme, alkaline phosphatase, and β -galactosidase. Green Fluorescence Protein (GFP), which has no enzyme activity but can be easily detected, can also be used. GPI-anchored protein C-terminal sequences include the α -agglutinin C-terminal sequence, the CWP2 C-terminal sequence, and so on. Furthermore, it is preferable to insert an appropriate selection marker, such as LEU2 and URA3, into the vector comprising the constructed reporter gene.

The constructed reporter gene is inserted into fungi using an appropriate method, such as the lithium acetate method (Gietz D et al., Nucl. Acids Res. 20: 1425, 1992). The fungi are then cultured, as necessary, using a method that suits the selection marker (e.g. using Leu⁻ medium for LEU2 and Ura⁻ medium for URA3), and then fungi into which the DNA has been introduced are selected.

The effect of a test sample on the transport of GPI-anchored proteins to the cell wall is examined by the following method:

The reporter gene-introduced fungi are cultured under appropriate conditions, for example at 30°C for 48 hours, in the presence of a test sample. After culturing, the culture supernatant is centrifuged, and the reporter enzyme activity of the culture supernatant fraction is measured. The resulting cell fraction is washed, the cell wall components are separated using an appropriate method, such as degrading the cell wall glucan with glucanase, and then the reporter enzyme activity of the cell wall fraction and cytoplasmic fraction is measured. The assay can be simply carried out by using centrifugation to determine the amount of reporter enzyme in the cell fraction, then without washing the cells, using proportional calculations to determine the amount of reporter enzyme derived from the culture supernatant fraction that remains in the cell fraction, and subtracting this from the amount of reporter enzyme

of the cell fraction.

If the test sample exhibits the activity of increasing reporter enzyme activity within the culture supernatant fraction (activity per cell), or the activity of decreasing the reporter enzyme activity in the cell wall fraction (activity per cell), the test sample is judged to have influenced the transport process of GPI-anchored proteins to the cell wall.

(2) A method using an antibody that reacts with the surface glycoprotein of fungal cell walls

A test sample's ability to influence the expression of a GPI-anchored protein at the fungal surface layer can be determined by quantification using an antibody that reacts with that GPI-anchored protein in the fungal cell wall.

Antibodies can be obtained by predicting the antigenic determinant using the amino acid sequence of, for example, a GPI-anchored protein such as α -agglutinin, Cwp2p, or Als1p (Chen MH *et al.*, J. Biol. Chem., 270:26168-26177, 1995; Van Der Vaat JM *et al.*, J. Bacteriol., 177:3104-3110, 1995; Hoyer LL *et al.*, Mol. Microbiol., 15:39-54, 1995), and then synthesizing the peptide of that region, binding it to an antigenic substance such as a carrier protein, and then immunizing a rabbit or such to obtain polyclonal antibodies, or a mouse or such to obtain a monoclonal antibody. A rabbit polyclonal antibody against the Als1p peptide is preferable.

In an alternative method, a monoclonal antibody against a GPI-anchored protein may be obtained by immunizing mice and such with fungi, preferably fungi which overexpress a GPI-anchored protein such as α -agglutinin, Cwp2p, and Als1p, (in some cases by immunizing further with a partially purified GPI-anchored protein), and then using ELISA, Western blot analysis, and so on to select resultant clones based on the antibody that they produce.

The following method can be used to determine the influence of a test sample on the process that transports a GPI-anchored protein to the cell wall, and on the amount of protein derived from that GPI-anchored protein in the cell wall.

Fungi are cultured in the presence of a test sample under appropriate conditions such as 30°C for 48 hours. The cultured fungi

are collected by centrifugation and the cells are disrupted, preferably using glass beads. The washed, disrupted cells are preferably subjected to centrifugal extraction with SDS, and then the precipitate is washed. After extraction, the disrupted cells are treated with an enzyme that degrades glucan, preferably glucanase, and the centrifuged supernatant thereof is the GPI-anchored protein sample.

The anti-Als1p peptide antibody is coated onto a 96-well plate by overnight incubation at 4°C. The plate is washed with a washing solution, preferably PBS comprising 0.05% Tween 20 (PBST), and blocking is carried out using a reagent that blocks the non-specific adsorption sites of the 96-well plate, preferably a protein such as BSA or gelatin, more preferably BlockAce (Dainippon Pharmaceutical Co., Ltd.). The plate is again washed with a washing solution, preferably PBST, and an appropriately diluted GPI-anchored protein sample is added. The reaction is then carried out for an appropriate time such as two hours at room temperature. After washing with a washing solution, preferably with PBST, an antibody against the enzyme-labeled *C. albicans*, preferably HRP-labeled anti-*Candida* antibody, is reacted for an appropriate time such as two hours at room temperature. The labeling method may be enzyme labeling or radioactive isotope labeling. After washing with a washing solution, preferably PBST, the amount of Als1p in the GPI-anchored protein sample is calculated by a method appropriate to the type of label, i.e. for an enzyme label, by adding a substrate solution and then, upon stopping the reaction, measuring absorbance at 490 nm.

(3) A method using the ability to adhere to animal cells

The test sample's influence on the expression of a GPI-anchored protein on the fungal surface can be determined by measuring the activity of that GPI-anchored protein in the fungal cell wall, and preferably by measuring the ability of fungi to adhere to animal cells and the like. In addition to the activity of Als1p, Hwp1p and such in adhesion to animal cells, GPI-anchored protein activity includes that of α -agglutinin in mating, of Flo1p in yeast aggregation, and so on. Hereinafter, a method using the ability of fungi to adhere to animal cells will be described in detail, but the

present invention is not to be construed as being limited thereto.

A fungus with the ability to adhere to cells is used, and this fungus is preferably *C. albicans*. For mammalian cells, cells that adhere to the fungus, preferably intestinal epithelial cells, are used. The mammalian cells are cultured and fixed using an appropriate method, such as ethanol fixation. The test sample and the fungi are incubated for an appropriate time such as 48 hours at 30°C, then inoculated and cultured for a set time, for example, one hour at 30°C. The culture supernatant is then removed, and the cells are washed with a buffer and overlaid with agar media such as Sabouraud Dextrose Agar Medium (Becton Dickinson Company, Ltd.). After culturing at 30°C overnight, the number of colonies is counted, and the adhesion rate is calculated.

If, when compared to fungi not treated with the compound, a test sample is observed to have the activity of decreasing the number of colonies formed by cell adhesion, that test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall.

(4) A method for observing fungi using an electron microscope or an optical microscope

The influence of a test sample on the expression of the GPI-anchored protein in the fungal surface can be determined by observing the structure of the fungal cell wall using an electron microscope.

In the presence of a test sample, a fungus such as *C. albicans* is cultured for a certain period of time, for example, 48 hours at 30°C, and its ultrafine morphological structure is observed using a transmission electron microscope. Herein, observation using a transmission electron microscope can be carried out, for example by the method according to the Electron Microscope Chart Manual (Medical Publishing Center). The flocculent fibrous structure of the outermost layer of a fungal cell has a high electron density and is observable by transmission electron microscope. This structure is not influenced by other existing antifungal agents and is considered to be a surface glycoprotein layer, including GPI-anchored proteins as its constituents. When this structure disappears, leaving only

a slight layer with a high electron density, the test sample is judged to have influenced the process that transports GPI-anchored proteins to the cell wall, compared to untreated cells.

When observation under both a transmission electron microscope and an optical microscope reveals greatly swollen fungal cells and inhibited budding (division), the test sample is judged to have an influence on the cell wall.

The present invention also provides a method for treating malaria, which comprises the step of administering a compound that inhibits the activity of a GWT1 protein a malaria parasite. Such a compound includes the compounds described in WO 02/04626 (for example, the compounds described herein in (1)-(5)).

The nucleotide sequence for the natural PfGWT1 protein is characterized by an exceedingly high AT content (80.41%), and thus codon usage is biased. In addition, the gene contains sequence stretches comprising six or more consecutive A residues at 23 separate positions, and these sequence stretches may serve as pseudo-poly(A) sites, thus producing truncated proteins. Because of the features described above, the gene was only expressed poorly in yeast, and very difficult to amplify using PCR or to replicate in *E. coli*. It was also difficult to determine the nucleotide sequence. However, the present inventors succeeded in expressing the PfGWT1 protein with a high efficiency by using a degenerate mutant of the DNA (SEQ ID NO: 5), with a lower AT content than the DNA encoding the PfGWT1 protein. The inventors also revealed that the introduction of the degenerate mutant DNA can rescue the phenotype of GWT1-deficient yeast. This finding suggests that the GPI synthase of a malaria parasite is interchangeable with that of a fungus such as yeast.

The AT content of the gene encoding the malaria parasite GPI synthase is, for example, 79.35% for GPI8 and 77.89% for the GPI13 of *P. falciparum*. These AT contents are as high as that of PfGWT1. It is predicted that most *P. falciparum* genes are hardly expressed in other species, because the average AT content over the translated regions of the *P. falciparum* genome is 76.3%. The present inventors succeeded in expressing a degenerate mutant of the DNA with a lower AT content than that of the DNA encoding the PfGWT1 protein, in yeast.

Hence, the malaria parasite GPI synthase can be expressed in a host other than malaria parasites by using such a degenerate DNA mutant. Furthermore, GPI-deficient yeast and GWT1-deficient yeast are known to exhibit similar phenotypes, including the characteristic of lethality and such. Thus, the phenotype of the GPI synthase gene-deficient fungus can be rescued by using the degenerate mutant DNA described above.

The phenotype of the GPI synthase gene-deficient fungus into which the degenerate mutant DNA described above has been introduced depends on the activity of the malaria parasite GPI synthase. Accordingly, compounds that inhibit the activity of the malaria parasite GPI synthase can be selected by screening using the phenotype of the GPI synthase gene-deficient fungus as an index. Thus, antimalarial drugs targeting the GPI biosynthesis pathway can be selected without actually using the malaria parasites themselves.

The present invention provides a degenerate mutant DNA encoding a protein that has the activity of rescuing the phenotype of a GPI synthase gene-deficient fungus, and which has an AT content lower than that of the original DNA encoding the protein involved in the biosynthesis of GPI. Such a DNA can be used in the screening method of the present invention.

As used herein, the term "AT content" refers to the content of adenine and thymine in the entire nucleotide sequence of the coding region of the GPI synthase gene. The AT content in the degenerate mutant DNA of the present invention preferably ranges from 50% to 70%, more preferably from 53% to 65%, and still more preferably from 55% to 62%.

The phenotype of the GPI synthase gene-deficient fungus includes temperature sensitivity (preferably, sensitivity to high temperatures) and lethality.

The proteins of the present invention involved in the biosynthesis of GPI in malaria parasites include GWT1, GPI1, GPI8, GPI3/PIG-A, GPI10/PIG-B, YJR013W/PIG-M, GPI13/PIG-O, GAA1/GAA-1, DPM1, GPI2, GPI15, YDR437W, GPI12, MCD4, GPI11, GPI7, GPI17, GPI16, CDC91, DPM2, DPM3, and SL15. Of the proteins indicated above, GPI1 and GPI8 have been found to be present in malaria parasites, and

GPI3/PIG-A, GPI10/PIG-B, YJR013W/PIG-M, GPI13/PIG-O, GAA1/GAA-1, and DPM1 have been suggested to be present in malaria parasites (Delorenzi et al., Infect. Immun. 70: 4510-4522, 2002). The nucleotide sequences of GWT1, GPI1, GPI8, GPI3/PIG-A, GPI10/PIG-B, YJR013W/PIG-M, GPI13/PIG-O, GAA1/GAA-1, and DPM1 of *P. falciparum* are shown in SEQ ID NO: 1 and the even sequence identification numbers in SEQ ID NOs: 6-21, respectively. Each corresponding amino acid sequence is shown in SEQ ID NO: 2 and the odd sequence identification numbers in SEQ ID NOs: 6-21. In addition, the nucleotide sequence of *P. vivax* GWT1 is shown in SEQ ID NO: 3, and the corresponding amino acid sequence is shown in SEQ ID NO: 4. Using a method known to those skilled in the art, for example, a method using hybridization or PCR, GWT1, GPI1, GPI8, GPI3/PIG-A, GPI10/PIG-B, YJR013W/PIG-M, GPI13/PIG-O, GAA1/GAA-1, or DPM1 of other malaria parasites can be cloned using DNA comprising any one of the nucleotide sequences shown in SEQ ID NO: 1 and 3, and the even-numbered SEQ ID NOs: 6-21.

Furthermore, GPI synthase genes other than GWT1, GPI1, GPI8, GPI3/PIG-A, GPI10/PIG-B, YJR013W/PIG-M, GPI13/PIG-O, GAA1/GAA-1, and DPM1 of malaria parasites can be cloned by using yeast or human GPI synthase genes. The nucleotide sequences of GPI2, GPI15, YDR437W, GPI12, MCD4, GPI11, GPI7, GPI17, GPI16, and CDC91 of yeast (*S. cerevisiae*) are shown in the even sequence identification numbers in SEQ ID NOs: 22-41 respectively; and each corresponding amino acid sequence is shown in the odd sequence identification numbers in SEQ ID NOs: 22-41. In addition, the nucleotide sequences of human DPM2, DPM3, and SL15 are shown in the even sequence identification numbers in SEQ ID NOs: 42-47 respectively; and each corresponding amino acid sequence is shown in the odd sequence identification numbers in SEQ ID NOs: 42-47.

The production of a degenerate mutant DNA encoding a protein involved in the biosynthesis of the GPI of malaria parasites, and with a lower AT content than that of the original DNA, consists of two steps: design, and synthesis. In the design step, the amino acid sequence of a protein of interest is first reverse-translated and then possible codons for each amino acid residue are listed. Reverse translation can be achieved by using commercially available gene

analysis software (for example, DNASIS-Pro; Hitachi Software Engineering Co., Ltd). Of the codons listed, those meeting the purpose (for example, codons whose AT content is lower and codons frequently used in the host to be used for gene expression) are selected for each amino acid. The degenerate mutant DNA can be designed by rearranging the amino acid sequence of the protein of interest using these selected codons.

The DNA thus designed can be synthesized by a method known to those skilled in the art. The degenerate mutant DNA of the present invention can be synthesized based on the designed nucleotide sequence by, for example, using a commercially available DNA synthesizer.

The present invention also provides vectors in which the above-described degenerate mutant DNA has been inserted, and transformants (preferably GPI synthase gene-deficient fungi) that retain the DNA or the vector in an expressible state. The vector and the host can be those described above.

As used herein, the expression "deficient in the GPI synthase gene" means that the functional product of the gene is not expressed, or that the expression level is decreased. The GPI synthase gene-deficient fungus of the present invention can be prepared by disrupting the GPI gene. The disruption can be achieved by inserting DNA unrelated to the gene, for example a selection marker, based on homologous recombination technology, and the like. More specifically, such a mutant fungus can be prepared by introducing into yeast a selection marker cassette which comprises the *his5* gene or the kanamycin resistance gene of *S. pombe* (Longtine *et al.*, Yeast, 14: 953-961, 1998) amplified with primers, each of which comprises a nucleotide sequence homologous to a portion of the gene (ranging from 50 to 70 nucleotides).

The GPI synthase gene-deficient fungus of the present invention includes, for example, the GWT1 temperature-sensitive mutant strain gwt1-20, GPI7 disruptant strain, GPI8 mutant strain gpi8-1, and GPI10 temperature-sensitive mutant strain per13-1.

A GPI synthase gene-deficient fungus which has been transformed with the degenerate mutant DNA of the present invention can be prepared by introducing into a fungus a vector into which the degenerate mutant

DNA has been inserted. pRS316, YEp351, or such can be used as the vector for *S. cerevisiae*, and pcL, pALSK, or such can be used as the vector for *S. pombe*.

The present invention also provides a method of screening for antimalarial drugs, which comprises using GPI synthase gene-deficient fungi described above.

In such a method, the first step comprises contacting a test sample with a GPI synthase gene-deficient fungus that has been transformed with degenerate mutant DNA with a lower AT content than the DNA encoding a protein involved in the biosynthesis of GPI of malaria parasites. The "contact" can be achieved by adding a test sample to the culture of the above-mentioned fungus. When the test sample is a protein, a vector comprising DNA encoding the protein can be introduced into the above-mentioned fungus.

In the method of the present invention, the next step comprises measuring the degree of growth of the above-mentioned fungus. More specifically, the fungus is inoculated under typical culture conditions, specifically, the fungus is inoculated onto a liquid culture medium such as Yeast extract-polypeptone-dextrose medium (YPD medium) or onto an agar plate, and then incubated at 25 to 37°C for 4 to 72 hours. Thus GPI synthase gene-deficient fungus transformed with the degenerate mutant DNA of the present invention can be assessed for growth. The degree of growth can also be determined using the turbidity of the culture liquid, the number of colonies, or the size or color of the spots formed on the agar plate as an index. In the method of the present invention, the next step comprises selecting compounds that inhibit the growth of the above-mentioned fungus.

In an alternative method, the first step comprises contacting a test sample with a GPI synthase gene-deficient fungus in which the above-described degenerate mutant DNA has been introduced. The next step comprises determining the amount of GPI-anchored protein transported onto the yeast cell wall. The detection method includes: (1) methods using a reporter enzyme; (2) methods using an antibody that reacts with a surface glycoprotein on the fungal cell wall; (3) methods using the ability to adhere to animal cells; and (4) methods using a light microscope or an electron microscope to observe the

fungi. In the method of the present invention, the next step comprises selecting a sample that decreases the amount of GPI-anchored protein transported to the cell wall.

The present invention provides a method of screening for antimalarial drugs using a protein involved in the biosynthesis of GPI, which is prepared using a degenerate mutant DNA of the present invention. Such methods include, for example, a binding assay system where screening is carried out to select compounds that bind to a protein involved in GPI biosynthesis in competition with a labeled compound bound to the protein. Specifically, a degenerate mutant DNA of the present invention is introduced into the GPI synthase gene-deficient fungus, the protein encoded by the DNA is expressed in the fungus, and the expressed protein is prepared. The prepared protein is then contacted with a test sample and with a labeled compound that can bind to the protein. In the next step, the labeled compound bound to the protein is detected, and test samples that decrease the amount of labeled compound bound to the protein are selected.

The present invention also provides an assay system for GlcN-PI acyltransferase. Such a system comprises using a GWT1 protein which is prepared using a DNA encoding a protein that has the activity of complementing the phenotype of GWT1-deficient yeast, which the DNA is a degenerate mutant of a DNA encoding a malaria parasite GWT1 protein that has a lower AT content than the original DNA. Specifically, the degenerate mutant DNA is introduced into GWT1-deficient fungus, the protein encoded by the degenerate mutant DNA is expressed in the fungus, and the expressed protein is prepared. This protein is then contacted with a test sample, GlcN-(acyl)PI is detected, and a test sample that decreases the amount of GlcN-(acyl)PI is selected.

Any patents, patent applications, and publications cited herein are incorporated by reference in their entireties.

Brief Description of the Drawings

Fig. 1 depicts photographs showing the results of tetrad analysis. The gwt1-disrupted strain became viable after the

introduction of the opfGWT1-overexpressing plasmid. The four spores derived from a single diploid cell were spotted vertically.

If one copy of the GWT1 gene was disrupted, only half of the spores grew. Thus, the ratio of [colony-forming spots]: [spots exhibiting no growth] is 2:2 in such cases. In the columns marked with an arrow, the lethal phenotype of the gwt1 disruptant was complemented by the introduced opfGWT1, and hence all four spots grew, each forming a colony.

Fig. 2 depicts a diagram showing the inhibitory activity of a compound with respect to the growth of yeast expressing the opfGWT1 gene. Either the yeast GWT1 gene or opfGWT1 gene was expressed in GWT1 gene-disrupted yeast.

A compound having the activity of inhibiting the GWT1-dependent growth of yeast also showed inhibitory activity with respect to the opfGWT1-dependent growth of yeast in which opfGWT1 was expressed.

Fig 3 depicts a diagram showing antimalarial activity. Human red blood cells were infected with *P. falciparum*. A GWT1-inhibiting compound was added to these red blood cells, and inhibition of malaria parasite infection was determined.

All five compounds exhibiting antifungal activity also inhibited the malaria parasite infection of red blood cells.

Best Mode for Carrying out the Invention

Herein below, the present invention will be specifically described using Examples, but it is not to be construed as being limited thereto.

[Example 1] *P. falciparum* GWT1 (PfGWT1)

(1) The nucleotide sequence of *P. falciparum* GWT1 (PfGWT1) (SEQ ID NO: 1) has been disclosed in the database of the *P. falciparum* genome (PlasmoDB database, <http://plasmodb.org/>). The PfGWT1 gene was cloned by PCR using genomic DNA purified from *P. falciparum* (the 3D7 strain) as a template. The 5' half and 3' half of the PfGWT1 gene were prepared separately, and the two halves were assembled at an XbaI (TCTAGA) restriction enzyme site. Thus, the full-length PfGWT1 gene was prepared. In addition, restriction enzymes sites outside the coding region were included, thus allowing insertion into an

expression vector.

(2) The 5' half of the PfGWT1 gene was amplified by PCR using *P. falciparum* genomic DNA as a template and the primers pf152F (SEQ ID NO: 48) and pf136R (SEQ ID NO: 49). The 3' half was amplified by the same procedure described above, using the primers pf137F (SEQ ID NO: 50) and pf151R (SEQ ID NO: 51). The DNA fragments amplified were subcloned into the pT7-Blue vector (Novagen), and the nucleotide sequences of the inserts were sequenced to confirm homology to SEQ ID NO: 1. Clones containing the 5' half of the PfGWT1 gene were named PF15-5 clones. Clones containing the 3' half were named PF20-9 clones.

(3) Using PCR, cleavage sites for restriction enzymes were added outside the coding region to enable the PfGWT1 gene to be inserted into an expression vector. An EcoRI cleavage site was added to the 5' half by PCR using PF15-5 as a template and the primers pf154FE (SEQ ID NO: 52) and pf157R (SEQ ID NO: 53). The amplified DNA fragment was subcloned into the pT7-Blue vector (Novagen) to prepare the clone pT7-plasmN2. Likewise, the 3' half was amplified by PCR using PF20-9 as a template and the primers pf168BK (SEQ ID NO: 54) and pf155RK (SEQ ID NO: 55). The amplified DNA fragments were subcloned to prepare pT7-plasmBK5 clones.

(4) The full-length PfGWT1 gene was prepared by the procedure described below. The yeast expression vector YEp352GAPII was digested with the restriction enzymes EcoRI and KpnI. The EcoRI-XbaI fragment (about 1500 bp) derived from pT7-plasmN2, and the XbaI-KpnI fragment (about 1100 bp) derived from pT7-plasmBK5, were inserted into the vector at a cleaved site. The expression vector YEp352GAPII-PfGWT1 containing the full-length PfGWT1 was then constructed.

[pf152F] ATGACAATGTGGGGAAGTCAACGGg (SEQ ID NO: 48)

[pf136R] TGTGTGGTTACCGTTCTTTGAATACATAGA (SEQ ID NO: 49)

[pf137F] ATAGAAAATGATTTATGGTACAGCTCAAA (SEQ ID NO: 50)

[pf151R] AGACCAAATTAATTATGCCTTTACATGTAC (SEQ ID NO: 51)

[pf154FE] agaattcaccATGAGCAACATGAATATACTTGCGTATCTT (SEQ ID NO: 52)

[pf157R] GAAATTC AATGTATTCCATATTC ACTTAT (SEQ ID NO: 53)

[pf168BK] AAGATCTAATACATTAAACATTTTAGATTAATGAATATGTG (SEQ ID NO: 54)

[pf155RK] aggtaccGTACACTCCACTCTATGATGATCATTC (SEQ ID NO: 55)

[Example 2] A fully synthetic PfGWT1 gene

The adenine and thymine (AT) proportion is exceedingly high (80% or higher) in *P. falciparum* DNA, and thus routine biological techniques (PCR, *E. coli*-based gene engineering, expression systems for recombinant proteins, and so on) are often unavailable (Sato and Horii; Protein, Nucleic acid, and Enzyme Vol. 48, 149-155, 2003). Likewise, the AT content of PfGWT1 DNA was 80.41% including many consecutive A or T stretches. Thus, the gene was predicted to be difficult to replicate and express as a protein in yeast. Indeed, when native PfGWT1 ligated with a yeast overexpression vector was introduced into GWT1 disrupted yeast, the PfGWT1 did not rescue the lethal phenotype of the GWT1 disruptant at all. To reduce AT content, codons were replaced with synonymous codons without changing the original amino acid sequence.

The codon substitution was carried out based on the nucleotide sequence of *P. falciparum* GWT1 (SEQ ID NO: 1) disclosed in the *P. falciparum* genome database (PlasmoDB database, <http://plasmodb.org/>). The resulting nucleotide sequence was named "optimized PfGWT1 (opfGWT1)" (SEQ ID NO: 5).

The sequence described above was designed to include additional sequences outside the coding region; namely an EcoRI cleavage site sequence (GAATTC, at the 5' end), Kozak's sequence (ACC, at the 5' end), and a KpnI cleavage site sequence (GGTACC, at the 3' end). The synthesis of the resulting sequence was consigned to Blue Heron Inc. in the U.S.A. These additional restriction enzyme sites were used to ligate the fully synthetic opfGWT1 into the YEpl352GAPII vector to construct an overexpression plasmid for opfGWT1. The construct was introduced into diploid cells (WDG2) in which only a single copy of the GWT1 gene had been disrupted. The resulting transformants were cultured on plates containing a sporulation medium to form spores for tetrad analysis.

The AT content of the newly designed codon-modified opfGWT1 was

reduced to 61.55%. The results of tetrad analysis are shown in Fig. 1. The gwt1-disrupted strain became viable after introduction of the opfGWT1 overexpression plasmid. The findings described above indicate that the PfGWT1 gene can be expressed in yeast cells when its AT content is reduced by codon modification.

[Example 3] An assay for antimalarial activity using opfGWT1-expressing yeast

A screening system for compounds having antimalarial activity was constructed using opfGWT1-expressing yeast.

An expression cassette was constructed by inserting the *S. cerevisiae* GWT1 terminator, and the *S. cerevisiae* GAPDH promoter and multi-cloning site into the SacI-KpnI site of the single-copy vector pRS316. *S. cerevisiae* GWT1 and opfGWT1 were inserted into the multi-cloning site to prepare pGAP-ScGWT1 and pGAP-opfGWT1 plasmids, respectively. These plasmids were introduced into the GWT1 disruptant. Serial two-fold dilutions of compound (1) were prepared using YPAD to make the highest final concentration 50 µg/ml. A 50 µl aliquot of the diluted compound was added to each well of a 96-well plate. Overnight cultures of yeast cells comprising each plasmid were diluted 1000-fold and then a 50 µl aliquot of the dilution was added to each well. The plates were incubated at 30°C for two days, and then culture turbidity was determined at 660 nm (Fig. 2 and Table 1).

[Table 1]

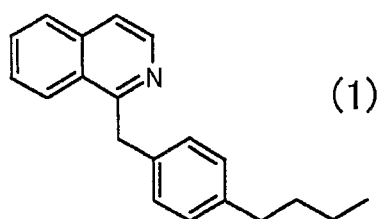
	0	6.25	12.5	25	50
pGAP-ScGWT1	0.7560	0.7370	0.6670	0.1140	0.0420
pGAP-opfGWT1	0.7150	0.6990	0.6910	0.3630	0.0530

Although the GWT1 disruptant was nonviable, the strain became viable after introduction of each plasmid (as shown at 0 µg/ml of compound concentration). The growth of ScGWT1-expressing yeast was inhibited by compound (1), a GWT1-specific inhibitor. The use of the

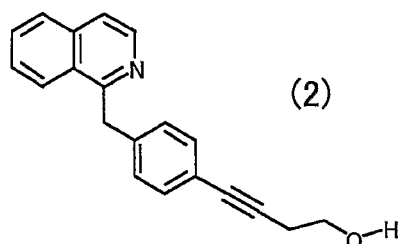
compound at 25 µg/ml resulted in about 85% inhibition of growth. When the compound was used at 50 µg/ml, the yeast was completely nonviable. The growth of opfGWT1-expressing yeast was also inhibited by compound (1). The use of the compound at 25 µg/ml resulted in about 50% inhibition of growth. When the compound was used at 50 µg/ml, the yeast was completely nonviable. Since growth of opfGWT1-expressing yeast depends on the activity of the introduced opfGWT1, growth inhibition can be attributed to the inhibition of the opfGWT1 function by compound (1). These findings suggest that compounds with *P. falciparum* GWT1-specific inhibitory activity GWT1 can be identified by screening compounds using this assay system.

[Example 4] Antimalarial activity

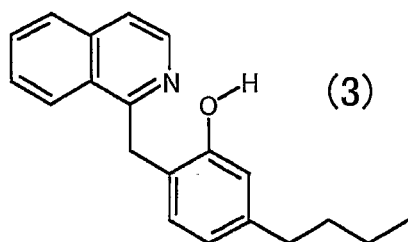
Representative compounds (1) to (5), that inhibit yeast GWT1, were assayed for antimalarial activity using a red blood cell culture system.



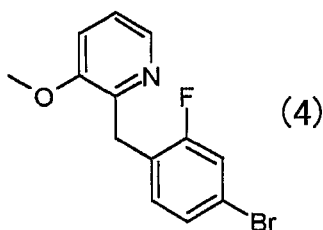
compound (1): 1-(4-butyl benzyl) isoquinoline



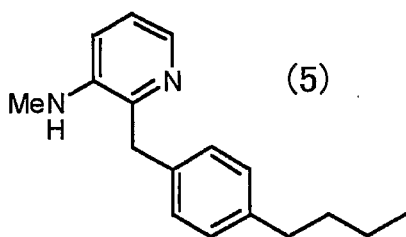
compound (2): 4-[4-(1-isoquinolyl methyl) phenyl]-3-butyne-1-ol



compound (3): 5-butyl-2-(1-isoquinolyl methyl) phenol



compound (4): 2-(4-bromo-2-fluorobenzyl)-3-methoxypyridine



compound (5): N-[2-(4-butyl benzyl)-3-pyridyl]-N-methylamine

Specifically, a test compound was dissolved in 100% DMSO, diluted with a medium, and an 80 μ l aliquot of the dilution was added to each well of a 96-well culture plate. *P. falciparum* FCR3 strain was pre-cultured in RPMI1640 medium containing 10% human serum at 37°C, and then 20 μ l of the cultured cells (containing 10% red blood cells) was added to each well. At this time, 0.47% of red blood cells were infected. After culturing under 5% O₂, 5% CO₂, and 90% N₂ at 37°C for 48 hours, the malaria parasites were stained using Giemsa staining. The number of protozoan-infected red blood cells was determined in order to estimate infection rate (Fig 3). As a result, compound (3) was revealed to have strong antimalarial activity. The other four compounds also showed antimalarial activity. Compound (4) exhibited the lowest activity. Therefore, compounds inhibiting yeast GWT1 include compounds which have the activity of inhibiting *P. falciparum*.

GWT1, suggesting that antimalarial drugs can be synthesized based on such compounds.

Industrial Applicability

The present invention succeeded in producing fungi that express malaria parasite GWT1. Using such fungi, antimalarial drugs targeting the pathway of GPI biosynthesis can be screened without using malaria parasites.

To date, no attempt has been made to express a malaria parasite gene in fungal cells and screen substances which inhibit the function of that gene. The methods of the present invention remove the need to actually using malaria parasites themselves, and thus this method proves the possibility of entirely new screening methods for drug discovery using comparative genomics in the post-genome era.

CLAIMS

1. A DNA according to any one of (a) to (d), which encodes a protein of a malaria parasite having GlcN-PI acyltransferase activity:

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 or 4,

(b) a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3,

(c) a DNA hybridizing to DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3 under stringent conditions, and

(d) a DNA encoding a protein which comprises the amino acid sequence of SEQ ID NO: 2 or 4, in which one or more amino acids have been added, deleted, substituted, and/or inserted.

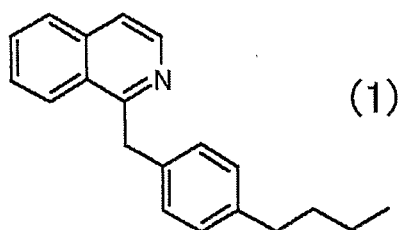
2. A protein encoded by the DNA according to claim 1.

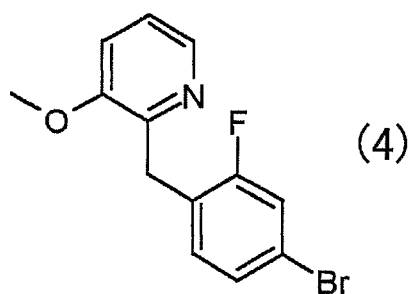
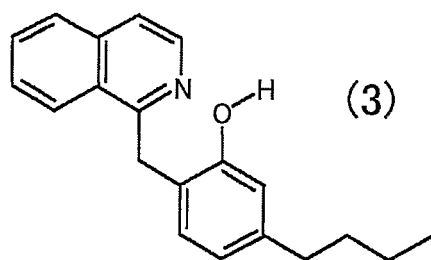
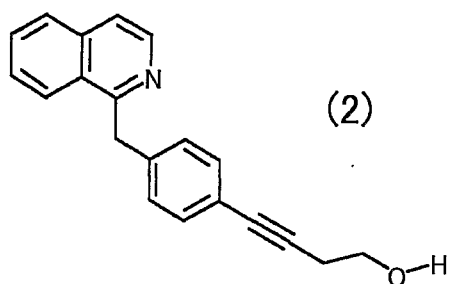
3. A vector into which the DNA according to claim 1 is inserted.

4. A transformant which retains, in an expressible state, the DNA according to claim 1 or the vector according to claim 3.

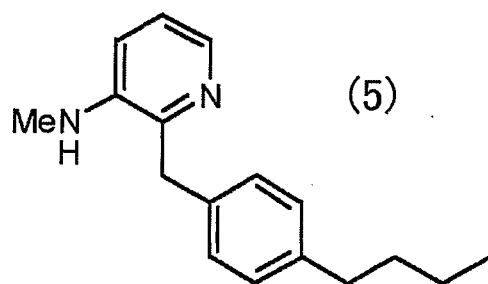
5. An antimalarial drug which comprises as an active ingredient a compound that inhibits the activity of the protein according to claim 2.

6. The antimalarial drug according to claim 5, wherein the compound that inhibits the activity of the protein according to claim 2 is at least one selected from the group consisting of the following compounds (1) to (5):





and,



7. A method of screening for a compound having antimalarial activity, which comprises the steps of:

(1) contacting the protein according to claim 2 with a test sample and a labeled compound that has the activity of binding to the protein,

(2) detecting the labeled compound that binds to the protein,
and,

(3) selecting a test sample that decreases the amount of labeled compound that binds to the protein.

8. The method according to claim 7, wherein the labeled compound that has the activity of binding to the protein is produced by labeling at least one compound selected from the group consisting of the compounds (1) to (5) according to claim 6.

9. A method of screening for a compound having antimalarial activity, which comprises the steps of:

(1) contacting a test sample with the protein according to claim 2,

(2) detecting GlcN-(acyl)PI, and,

(3) selecting a test compound that decreases the level of GlcN-(acyl)PI.

10. A method of screening for a compound having antimalarial activity, which comprises the steps of:

(1) contacting a test sample with a cell overexpressing the protein according to claim 2,

(2) determining the amount of GPI-anchored protein transported to the cell wall in the cell, and,

(3) selecting a test sample that decreases the amount of the GPI-anchored protein transported to the cell wall, as determined in step (2).

11. A method for treating malaria, which comprises administering a compound that inhibits the activity of the protein according to claim 2.

12. The method according to claim 11, wherein the compound that inhibits the activity of the protein according to claim 2 is the compound according to claim 5.

13. A DNA encoding a protein that has the activity of complementing the phenotype of a GPI synthase gene-deficient yeast, which is a degenerate mutant of a DNA encoding a protein involved in GPI biosynthesis in malaria parasites, and that has a lower AT content than the original DNA.

14. A DNA encoding a protein that has the activity of complementing the phenotype of a GPI synthase gene-deficient yeast, which is a degenerate mutant of a DNA encoding a protein involved

in the biosynthesis of GPI in malaria parasites, and that has an AT content which is reduced by 70%.

15. The DNA according to claim 13 or 14, which is selected from the group consisting of:

(a) a DNA encoding a protein that comprises any one of the amino acid sequences of SEQ ID NOs: 2 and 4, and odd sequence identification numbers in SEQ ID NOs: 6-47,

(b) a DNA comprising any one of the nucleotide sequences of SEQ ID NOs: 1 and 3, and even sequence identification numbers in SEQ ID NOs: 6-47,

(c) a DNA hybridizing under stringent conditions to the DNA that comprises any one of the nucleotide sequences of SEQ ID NOs: 1 and 3, and even sequence identification numbers in SEQ ID NOs: 6-47, and

(d) a DNA encoding a protein which comprises any one of the amino acid sequences of SEQ ID NOs: 2 and 4, and odd sequence identification numbers in SEQ ID NOs: 6-47, in which one or more amino acids have been added, deleted, substituted, and/or inserted.

16. A DNA comprising the nucleotide sequence of SEQ ID NO: 5.

17. A vector in which the DNA according to any one of claims 13 to 16 is inserted.

18. A transformant which retains, in an expressible state, the DNA according to any one of claims 13 to 16, or the vector according to claim 17.

19. The transformant according to claim 18, which is a GPI synthase gene-deficient fungus.

20. The transformant according to claim 18, which is a GPI synthase gene-deficient yeast.

21. A method for producing a protein encoded by the DNA according to any one of claims 13 to 16, which comprises the steps of culturing the transformant according to any one of claims 18 to 20, and recovering the expressed protein from the transformant or the culture supernatant.

22. A method of screening for a compound having antimalarial activity, which comprises the steps of:

(1) contacting a test sample with a GPI synthase gene-deficient fungus that expresses the DNA according to any one of claims 13 to

16,

- (2) assaying the growth of that fungus, and,
- (3) selecting a test compound that inhibits the growth of that fungus.

23. A method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) contacting a test sample with a GPI synthase gene-deficient fungus expressing the DNA according to any one of claims 13 to 16,
- (2) determining the amount of a GPI-anchored protein transported to the fungal cell wall, and,
- (3) selecting a test sample that decreases the amount of the GPI-anchored protein transported to the cell wall, as determined in step (2).

24. A method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) introducing the DNA according to any one of claims 13 to 16 into a GPI synthase gene-deficient fungus and expressing the protein encoded by the DNA,
- (2) preparing the protein expressed in step (1),
- (3) contacting the prepared protein with a test sample and a labeled compound that has the activity of binding to the protein,
- (4) detecting the labeled compound that binds to the protein, and,
- (5) selecting a test sample that decreases the amount of labeled compound that binds to the protein.

25. A method of screening for a compound having antimalarial activity, which comprises the steps of:

- (1) introducing into a GWT1-deficient fungus, (i) a DNA encoding a protein that has the activity of complementing the phenotype of a GWT1-deficient yeast, wherein the DNA is a degenerate mutant of a DNA encoding a malaria parasite GWT1 protein that has a lower AT content than the original DNA, or (ii) a vector into which the degenerate mutant of DNA has been inserted, and expressing the protein encoded by the degenerate mutant DNA,
- (2) preparing the protein expressed in step (1),
- (3) contacting the prepared protein with a test sample,

- (4) detecting GlcN-(acyl)PI, and
- (5) selecting a test compound that decreases the level of GlcN-(acyl)PI.