

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 May 2011 (12.05.2011)

(10) International Publication Number
WO 2011/056877 A1

PCT

(51) International Patent Classification:
A61K 39/00 (2006.01)

DC 20009 (US). **ZAHN, Bin** [US/US]; 11508 Alcinda Lane, North Potomac, MD 20878 (US).

(21) International Application Number:
PCT/US2010/055305

(74) Agent: **WEISSMAN, Peter, S.**; Blank Rome LLP, 600 New Hampshire Ave., NW, Washington, DC 20037 (US).

(22) International Filing Date:
3 November 2010 (03.11.2010)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/257,573 3 November 2009 (03.11.2009) US

(71) Applicants (for all designated States except US): **THE GEORGE WASHINGTON UNIVERSITY** [US/US]; Rice Hall, Suite 601, 2121 I Street, NW, Washington, DC 20052 (US). **JOHN HOPKINS UNIVERSITY** [US/US]; 100 N. Charles Street, Baltimore, MD 21202 (US). **ALBERT B. SABIN VACCINE INSTITUTE** [US/US]; 2000 Pennsylvania Avenue, NW, Washington, DC 20006 (US).

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DINGLASAN, Rhoel, Ramos** [PH/US]; 14904 McKnew Road, Burtonsville, MD 21205 (US). **HOTEZ, Peter, J.** [US/US]; 5213 Portsmouth Road, Bethesda, MD 20816 (US). **SHAH-BROWN, Ami** [US/US]; 4601 Lincoln Drive, Halethorpe, MD 21227 (US). **BOTTAZZI, Maria, Elena** [US/US]; 1721 21st Street, NW, Apt 201, Washington,

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: MALARIA TRANSMISSION-BLOCKING VACCINE

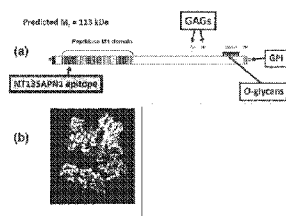
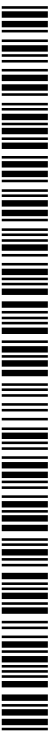


FIG. 1

(57) Abstract: The present invention relates to malaria vaccines. Particularly, the present invention relates to antigens that are effective to prevent the transmission of malaria. The antigen comprises a fragment of AnAPN1.



WO 2011/056877 A1

MALARIA TRANSMISSION-BLOCKING VACCINE

This application claims the priority of U.S. Provisional Patent Application Serial No. 61/257,573, filed November 3, 2009, which is incorporated herein by reference.

5

FIELD OF THE INVENTION

The present invention relates to malaria vaccines. Particularly, the present invention relates to antigens that are effective to prevent the transmission of malaria.

10 BACKGROUND OF THE INVENTION

Malaria continues to be a tremendous public health burden worldwide, with more than 2 to 4 million people dying from the disease each year. One of the most prevalent forms of the disease is caused by the protozoan parasite *Plasmodium vivax*, which is found in tropical and sub-tropical regions. Interestingly the parasite can complete its
15 mosquito cycle at temperatures as low as 15 degrees Celsius, which has allowed the disease to spread in temperate climates. The acute form of malaria is caused by the protozoan parasite, *Plasmodium falciparum* which is responsible for most of the mortality attributable to malaria.

The life cycle of *Plasmodium* is complex, requiring two hosts, man and mosquito
20 for completion. The infection of man is initiated by the inoculation of sporozoites into the blood stream through the bite of an infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes where they differentiate, via the exoerythrocytic intracellular stage, into the merozoite stage which infects red blood cells (RBC) to initiate

cyclical replication in the asexual blood stage. The cycle is completed by the differentiation of a number of merozoites in the RBC into sexual stage gametocytes, which are ingested by the mosquito, where they develop through a series of stages in the midgut to produce sporozoites which migrate to the salivary gland.

5 Vaccines have been at the forefront of global research efforts to curb the disease, yet despite several vaccine candidates, this goal has not been completely realized. To date, only a single preventative vaccine against malaria has been developed (RTS,S). The RTS, S vaccine has been shown to confer between ~38-54% protection in vaccinated individuals in Sub-Saharan Africa.

10 Other vaccines include those disclosed in WO 2006/088597, WO 2006/088597, and WO 2008/009650, which are incorporated herein by reference.

 Although disease severity can be lessened with the afore-mentioned malaria vaccine, the fact remains that individuals who receive the vaccine are still carrying infectious parasites that can be transmitted by mosquitoes. Such deficiencies will
15 unlikely lead to malaria elimination or eradication. In order to reach such a goal, a transmission-blocking component to the vaccine is necessary. The global research effort has since focused on developing a “cocktail” vaccine, wherein different life stages of the parasite can be targeted specifically by separate vaccine components. The key
20 component to such an eradication vaccine would be one that blocks the cascade of secondary infections from immunized but parasitized human hosts. The current paradigm for developing a malaria transmission-blocking vaccine (TBV) involves developing antibodies against gametocyte or ookinete surface molecules. This approach is indeed effective but since there are two main human parasite species (*P. falciparum* and *P.*

vivax), two separate vaccines must be produced. One cannot claim malaria eradication a success if one parasite species remains prevalent in human populations.

Therefore, there remains a need to effectively combat the spread of malaria.

SUMMARY OF THE INVENTION

The present invention relates to a composition to combat the spread of malaria by using transmission-blocking vaccines (TBVs), which prevent the development of malarial parasites within its mosquito vector; thereby abrogating the cascade of secondary

5 infections in humans. The TBV contains a fragment of the Anopheline midgut specific membrane-bound alanyl aminopeptidase (AnAPN1). The present inventors have discovered that AnAPN1 is an excellent TBV due to its highly conserved molecule present across diverse *Anopheles* vector species and is a putative ligand for *P. falciparum* and *P. vivax* ookinetes. Antibodies raised in rabbits against the N-terminal domain of

10 AnAPN1 (NT135APN1) (SEQ ID NO.: 1) are capable of completely (100%) blocking *P. falciparum* invasion of *An. gambiae* in Cameroon. The same antibodies exhibit virtually complete blocking (95-98%) of *P. vivax* invasion of *An. dirus* in Thailand. Anti-AnAPN1 antibodies exhibit no cross-reactivity with normal human lung, kidney, liver, and small intestine tissue sections. These results strongly support the use of AnAPN1

15 fragments as TBVs. Preferably, the AnAPN1 fragment is NT135APN1 (SEQ ID NO.: 1); more preferably, the fragment is amino acids 32-64 of SEQ ID NO.: 1. The fragment of AnAPN1 can be used as a vaccine for preventing the transmission of the parasites that cause malaria. Once an immune response against AnAPN1 is stimulated in a subject, preferably a person, the antibodies produced against the AnAPN1 is effective to disrupt

20 the development of the parasites. Thus, without being held to a particular theory, when a mosquito ingests the blood of the subject, it is believed that the antibodies disrupt the parasites in the mosquito gut, thereby preventing the transmission of the parasites to other subjects.

In an embodiment the TBVs of the present invention can be used with currently available vaccine for malaria, including the RTS,S vaccine, and those disclosed in WO 2006/088597, WO 2006/088597, and WO 2008/009650. The TBV can be administered concurrently with the malaria vaccine. Alternatively, the TBV can be admixed with the
5 malaria vaccine to produce a combination vaccine and administered as a single dose.

The TBV can also include an adjuvant. The term “adjuvant” generally refers to any material that increases the humoral or cellular immune response to an antigen. Adjuvants are used to accomplish two objectives: 1) slow the release of antigens from the injection site; and 2) stimulate the immune system. Synthetic and subunit vaccines are
10 expensive to produce. The addition of an adjuvant may permit the use of a smaller dose of antigen to stimulate a similar immune response, thereby reducing the production cost of the vaccine. Thus, the effectiveness of some injectable medicinal agents may be significantly increased when the agent is combined with an adjuvant.

In another embodiment, the present invention also relates to methods for using the
15 TBV, by itself or in a combination vaccine, to stimulate an immune response against AnAPN1. The method involves administering the AnAPN1 fragment to a subject. The amount of AnAPN1 fragment administered should be sufficient to stimulate an immune response against AnAPN1. The TBV may be administered by any known method, such as by intramuscular injection, intradermal injection, gene gun or electroporation. A
20 skilled person in this art would readily be able to determine the appropriate dosage and schedule for administering the vaccine.

In yet another embodiment, the present invention also provides methods for making the TBVs. The TBV can be isolated from natural sources or, preferably, made

recombinantly. Recombinant methods are well known in the art; however, *Pichia* and *E. coli* are the preferred systems for making the AnAPN1 fragment, particularly NT135APN1.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the details of AnAPN1. (a) Schematic of the AnAPN1 glycoprotein, a highly conserved molecule found on the apical face of midguts of anopheline vectors as determined by Western blot. The N-terminal 135 amino acid fragment target antigen (NT135APN1) is indicated. Predicted motifs for glycosaminoglycans (GAG) and O-linked glycans and GPI-anchors are shown. The GPI anchor was confirmed by releasing AnAPN1 into the supernatant with PI-PLC. (b) Homology model of AnAPN1 based off the A4 hydrolase crystal structure indicating the NT135 epitope (orange) and GAGs (aqua). Image portrays view from the top looking down on AnAPN1. The predicted Mr of AnAPN1 is indicated to the left by the apparent Mr is 125 kDa.

Fig. 2 shows that anti-AnAPN1 IgG blocks *P. falciparum* development in *An. Gambiae* mosquitoes in Cameroon. (a) Antibodies were mixed with gametocytetic blood from volunteers (case codes indicated below each bar panel) and fed to mosquitoes. Asterisks indicates statistical significance ($\alpha=0.05$). Dilution series of IgG are indicated between panels (a) and (b). A 1:2 dilution is equivalent to 10 $\mu\text{g/mL}$ of AnAPN1-specific IgG. (b) An alternative presentation of the inhibition data depicted in (a) is reduction in mosquito infection prevalence. Data is from a second cohort of volunteers during the following year's transmission season. The data clearly show that the antibodies reduce mosquito infection prevalence to zero, i.e., no oocysts form in the mosquito midguts. This suggests that the antibodies are in fact more potent against field isolates of *P. falciparum* than against laboratory stains and represent a true transmission-blocking vaccine approach.

Fig. 3 shows that anti-AnAPN1 IgG blocks *P. vivax* development in *An. Dirus A* mosquitoes in Thailand. Experiments conducted as was described for Fig. 2 above. Asterisks indicates statistical significance ($\alpha=0.05$). Dilution series of IgG are indicated on top of the graph. A 1:2 dilution is equivalent to 10 $\mu\text{g/mL}$ of AnAPN1-specific IgG.

5 Fig. 4 shows mouse immunizations with recombinant AnAPN1 elicit a potent transmission-blocking response against *P. berghei*. (a) Antigen specific antibody titers elicited in BALB/c (5 mice/cohort) from three bleeds (pooled) at two week intervals following the first (Bleed 1, ●), second (Bleed 2, ■) and third (Bleed 3, Δ) booster immunizations as determined by ELISA. This data is representative of replicated
10 experiments. Optical density (O.D. 630 nm) and serum dilutions are plotted. Error bars indicate the standard error of the mean from quadruplicate wells. End point titers were defined as the highest serum dilution giving an O.D. reading greater than that of pre-immune serum +2 standard deviations. (b) mice (M1-M3) immunized with AnAPN1 elicited *P. berghei* transmission-blocking antibody titers in two biological experiments
15 indicated by the stippled (cohort 1) and solid vertical bars (cohort 2). Asterisks denote statistical significance (*) at $P<0.05$ (Mann-Whitney U Test). Error bars indicate 1 standard deviation.

Fig. 5 shows data generated by Epitope Identification Suite (Merck Research labs) suggest that the NT135APN1 sequence is “clean” of potential cross-reactive human
20 epitopes. A search for potential T-cell epitopes with significant sequence identity with human 9-mer peptides suggests (1) that the NT135APN1 has an immunogenic hot spot between amino acids 32-64 and (2) there is little to no homology with human peptides currently annotated in the human proteome. Three MHC II alleles common in Tanzanian

populations are indicated in the gray box on the right. The eight MHC II alleles used in the query (shown on the left) are common across Caucasian populations in the US. The complete NT135APN1 sequence is shown in the red box on the bottom with the peptide sequence corresponding to a potential cross-reactive epitope in humans underlined.

5 Fig. 6 shows immunofluorescence staining of normal human kidney, lung, liver and small intestine sections with anti-AnAPN1 IgG. Sections were stained with polyclonal rabbit anti-AnAPN1 IgG and detected with anti-rabbit Texas red-conjugated antibody. Secondary antibody alone was not used as a control in follow up assays since there was no detectable signal to primary antibody. Note that the red signal in the
10 overlay image for the liver is completely due to leakage of autofluorescence into the red channel. Images were acquired at 200X. Bright field images are provided for orientation. DAPI was used to stain nuclei and appear blue.

 Fig. 7 shows H&E staining and immunofluorescence microscopy of a normal lung section with anti-AnAPN1 IgG. Lung sections were stained with polyclonal rabbit anti-
15 ANAPN1 IgG and anti-actin (human) monoclonal antibodies and detected with anti-rabbit Texas Red-conjugated and anti-mouse Alexa 488-conjugated secondary antibodies, respectively. Two different compartments of the lung were examined, the alveolus and smooth muscle/connective tissues, which are also shown by H&E staining below. Actin appears green and AnAPN1 cross reactivity appears red. DAPI was used to stain nuclei
20 and appear blue. Note that the actin signal and AnAPN1 signal overlap (orange) but that the fluorescence signal is predominantly contributed by bleed-through of the green signal into the red channel from detection of actin, not unlike what was observed in Fig. 6. H&E staining of compartments of human lung from a serial section are shown in panels

(a) and (b). In panel (a), the arrow indicates staining of resident alveolar lumen macrophages. AS indicates alveolar sac and AD indicates alveolar duct. In panel (b), the arrow indicates junction between sub-endothelial connective and smooth muscle tissues within the lung. Bright field images are provided for orientation. All images were
5 acquired at 400X.

Fig. 8 shows expression of recombinant AnAPN1 in different yeast constructs. 1. AnAPN1/pPink α -HC/*Pichia*Pink#4. 2. AnAPN1-M/pPICZaA/*P.pastoris* X-33. 3. AnAPN1-D/pPICZaA/*P.pastoris* X-33. Expression gels suggest that using strains that are deficient of proteases is a better strategy than changing the makeup of the protein.

10 Fig. 9 shows SDS PAGE of product purification efforts from *Pichia*. (a) In process samples from Butyl HP column (SDS PAGE/CBB). See Blue MW marker (M), Starting material (SM), Flow-through (FT), peak fraction A4 (elution with buffer containing no salt), and peak fraction A5 (elution with buffer containing no salt) were run on a 14% Tris-Glycine gel (Invitrogen) and stained with Coomassie Brilliant Blue
15 (CBB). Starting material was diluted and very faint on the gel. No AnAPN1 was seen in the collected fractions. (b) In process samples from QXL column (SDS PAGE/CBB). See Blue MW marker (M), Starting material (SM), peak fraction A8 (elution with buffer containing 200 mM NaCl), peak fraction A11 (elution with buffer containing 400 mM NaCl) and peak fraction B2 (elution with buffer containing 600 mM NaCl) were run on a
20 14% Tris-Glycine gel (Invitrogen) and stained with CBB. No AnAPN1 was seen in the collected fractions. (c) In process samples from QXL column (SDS PAGE/SS). See Blue MW marker (M), Starting material (SM), peak fraction A8 (elution with buffer containing 200 mM NaCl), peak fraction A11 (elution with buffer containing 400 mM

NaCl) and peak fraction B2 (elution with buffer containing 600 mM NaCl) were run on a 14% Tris-Glycine gel (Invitrogen) and silver stained. There is very little AnAPN1 seen in the collected fractions and is relatively unpure.

Fig. 10 shows *E. coli* in process samples during the homogenization step (SDS PAGE /CBB) of the recovery of NT135APN1 from *E. coli*. To determine if the *NT135APN1* was solubilized, 10 ul of each in-process sample were run on a reduced 14% Tris-Glycine gel (Invitrogen). For each cycle with the homogenizer samples were centrifuged at 14,000 RPM for 5 minutes at room temperature to separate the soluble portion in the supernatant and the insoluble pellet. A whole cell sample (total cell suspension after Cycle 1) was also included to show the total protein contained in both supernatant and pellet.

Fig. 11 shows *E. coli* in process samples from Ni Column (SDS PAGE / CBB). Starting material (SM), flow-through (FT), wash of unbound sample (W), wash with corresponding buffer containing 20 mM Imidazole (Labeled as 20 mM), and elution (Labeled as 500 mM).

Fig. 12 shows *E. coli* purification yield based on densitometry (SDS PAGE / CBB). For *NT135APN1*, 2.5 ul of sample was determined to be within the linear range of the machine, and for BSA 4 ug was determined to be within the linear range of the machine (1. See Blue; 2. NT135APN1 – 2.5µl; 3. BSA, 4µg).

Fig. 13 shows an SDS-PAGE of pre-dialyzed material 2 µg (non-reduced: Lane 1; reduced: Lane 2) and 2 µg Lot 032410JLP (non-reduced: Lane 3; Reduced: Lane 4) followed by Coomassie Staining (A), Silver Staining (B), Western blot analysis with anti-APN1 (C) and anti-His (D).

Fig. 14 shows binding of APN-1 to Alhydrogel® in 10% glucose/10 mM imidazole/10 mM SB 3-12 or 10% glucose/10 mM imidazole/1 mM SB 3-12. Vaccine supernatants were analyzed by SDS-PAGE alongside *NT135APN1* standards (unformulated). The standards show the amounts of *AnAPN1* that would be present in the vaccine supernatants if 50%, 25%, 10% and 1% of the formulated *NT135APN1* was unbound and free in solution. No protein at the expected size of approximately 15 kDa migrates in the formulated vaccine supernatant lanes, indicating that *NT135APN1* was 100% adsorbed to Alhydrogel® in these formulations. MW markers (in kDa) are shown in the far left lane.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have discovered that fragments of AnAPN1, preferably NT135APN1 (SEQ ID NO.: 1), is an attractive malaria TBV because it is highly immunogenic, can block *P. falciparum* and *P. vivax* transmission through mosquitoes in the field, and can be easily microencapsulated in slow-release polymers for delivery. The features that allow AnAPN1 fragments to be an effective TBV includes:

- Mosquito-based antigen as opposed to a parasite antigen which is difficult to express correctly;
- Mosquito-based antigen is a highly conserved (i.e., present in all species of anopheline mosquitoes) mosquito midgut specific membrane bound alanyl aminopeptidase (APN)
- The selected protein fragment to be used as antigen contains immunological hotspots corresponding to common HLA epitopes that would be recognized across different human populations (targeted for vaccination);
- Rabbit IgG completely blocks (100% inhibition) field isolates of *P. falciparum*;
- Rabbit IgG is a potent inhibitor (95-98% inhibition) of field isolates of *P. vivax*;
- Rabbit IgG works against parasites in different mosquito species from different parts of the world;
- At present, polymorphisms in APN in these mosquitoes do not appear to effect potency;
- IgG shows no cross-reactivity with human APN in several different tissues;
- At present, mouse-immunization experiments do not result in death;

- Mouse IgG exhibits potent inhibitory activity against *Plasmodium berghei* (murine model of malaria);
- Antigen can be expressed as a completely soluble product ~ 100 mg/L in *Pichia pastoris* or *E. coli*;
- 5 • *E. coli* codon-harmonized gene can be used to express protein in bacteria;
- Antigen can be microencapsulated in biodegradable micro- and/or nanoparticles for delivery. Additional proof of principle data shows that a single dose of the microparticle:antigen:alum formulation in mice results in comparable inhibition profiles to that of controls which were immunized following the standard prime
10 and 3 boost schedule;
- Antigen can be co-administered with other vaccine candidates targeting parasite proteins from the sporozoite, gametocyte, gamete and ookinete stages;
- Antigen can be easily complexed with other adjuvants, e.g., CpG oligonucleotides, cholera toxin, synthetic bacterial lipopolysaccharides, TLR
15 agonists as well as other adjuvants currently available (e.g., from Infectious Disease Research Institute); and
- Antigen:adjuvant delivery systems can take the form of needle-free, microparticle sheet delivery, liposomes, as naked DNA, via adenoviral vectors, as well as other novel vehicles and standard emulsions.

20 Because malarial parasites are transmitted among human hosts by the bite of its obligate insect vector, the *Anopheles* mosquito, targeting mosquito midgut antigens that serve as ligands for different *Plasmodium* parasite species and strains is one viable approach to breaking the transmission cycle. The present inventors have recently

identified in AnAPN1 as a predominant ligand for different malaria parasite species and strains. Rabbit antibodies directed against a 135 amino acid N-terminal fragment (NT135APN1) prevented both the murine malaria parasite *Plasmodium berghei* and the human malaria parasite, *P. falciparum* from developing in the mosquito vector. The antibodies are quite potent, blocking field isolates of *P. falciparum* (100% inhibition) and *P. vivax* (95-98% inhibition) in completely divergent anopheline vectors (*An. gambiae* s.s. and *An. dirus* A, respectively). Moreover, immunization of mice following a standard regimen, which are then subsequently infected with *P. berghei*, showed that the mice elicit a high titer of IgG that completely block parasite transmission to mosquitoes. It is important to note that the critical goal for TBVs should be the complete prevention of mosquito infection and not simply the reduction in oocyst intensity in the mosquito. After all, a single oocyst is all that is needed to continue the transmission cycle of *Plasmodium*.

APN1 antigen appears to be an abundant midgut glycoprotein that is conserved across diverse anopheline vectors and is referred to herein as AnAPN1 (Fig. 1). Anti-AnAPN1 IgG recognition of orthologous aminopeptidase antigens in the midguts of several anopheline vectors and its efficacy in blocking both human malaria parasite species implies a significant utility of AnAPN1 or its fragments as an effective transmission-blocking target that is effective for all malaria causing parasites, especially *P. falciparum* and *P. vivax*.

The AnAPN1 fragments can be obtained from natural sources, or preferably through recombinant techniques known in the art. Appropriate host cells for the expression of the fragments include, but are not limited to, yeast cells, insect cells,

mammalian cells, or bacteria. In exemplary embodiments of the invention, the fragments are produced in yeast cells such as *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces fragilis*, *Kluveromyces lactis*, and *Schizosaccharomyces pombe*. Alternatively, the preferred bacterial system for producing the fragments is *E. coli*. The preferred expression systems offer the advantages of being cost-effective and easily adapted to large-scale growth in fermenters.

Transmission blocking efficiency

Anti-AnAPN1 polyclonal IgG efficiently and effectively (10 µg) confers complete transmission-blocking immunity against field isolates of *P. falciparum* (Fig. 2) and between 95-98% inhibition of *P. vivax* (Fig. 3). In contrast, the matching concentrations of IgG in pre-immune sera from rabbits, mice or malaria-naïve human AB serum exhibit no inhibition of oocyst development in mosquito. It remains unclear why *P. vivax* is not blocked to the same extent at the 10 µg concentration of IgG.

Immunization of mice with AnAPN1 recombinant protein in incomplete Freund's Adjuvant or alum elicits a potent transmission-blocking antibody titer against *P. berghei* (Fig. 4). These results suggest that eliciting transmission –blocking antibody titer in human is highly likely.

In silico analysis

Work leading to the selection of NT135APN1 as an antigen focuses on identifying highly hydrophilic, relatively low structural complexity (e.g. the absence of cysteines), and potentially immunogenic domains using a variety of web-based tools.

Further *in silico* analyses using simple BLAST analysis as well as proprietary software from Merck Research Laboratories (Epitope Identification Suite) has confirmed the initial findings and verified the suitability of NT135APN1 as a highly antigenic fragment.

Briefly, Epitope Identification Suite is a program used by Merck Research labs to
5 identify potential microbial pathogen vaccine targets (e.g. HIV vaccines). We used this software to 1) identify areas of the protein fragment which show high sequence identity with human proteins through a search against the entire human proteome; and 2) identify peptides along NT135APN1 that are predicted to be high-binders to eight of the most frequently found MHC II HLA-DR alleles (DR-1, 3, 4, 7, 8, 11, 13, and 15) in the world
10 population (Fig. 5).

A search for potential T-cell epitopes with significant homology to human proteins (80% or higher amino acid identity to any human peptide, i.e., eight or more identical amino acids in a span of 9), suggests that the NT135APN1 is “clean” (WO2006/124406). We found only two possible peptides (MSSAKVSSL and
15 SSAKVSSLP) that match, albeit poorly. An independent BLASTP analysis using the NT135APN1 as ‘query’ resulted in poor matches to a puromycin-sensitive human aminopeptidase (with a known cytosolic localization) with e-values with low statistical significance (e^{-09}). Alignments with other human APNs show lower e-values ($1e^{-05}$ to 2^{-05}). Furthermore, sequence alignments confirm the absence of a string of amino acids in
20 one linear location that is above 7 aa/10 aa sequence. NT135APN1 shows the highest sequence identity with mosquito APNs only (both *Anopheles* and *Aedes*).

Loosening the stringency (2 mismatches in a 9 amino acid linear location), the total number of possible peptide matches increased to four. It should be noted that highly

safe and acceptable vaccines that are already available, e.g., against tetanus (TeTx) exhibits more sequence identity with human sequences than NT135APN1. Moreover, recent immunizations of mice, with NT135APN1 in alum, did not result in any obvious adverse side-effects in the mice 6 months post-immunization (i.e., no unexpected mortality due to antigen vs. controls). Taken together, the data suggests that NT135APN1 is relatively “clean” and potentially safe. This data suggests NT135APN1 would be a safe vaccine no little human cross-reactivity.

It is important to note that Epitope Identification Suite also found a potential immunogenic “hot spot” in sub-fragment amino acids 39-62 of the NT135APN1 sequence (Fig. 5). Not only is this sub-fragment “clean” (as described above) but contains multiple predicted binders for all eight MHC II alleles. In addition, two other immunogenic sub-fragments are clearly identifiable. At present it is not clear which are the protective epitopes or if polyclonal targeting of these epitopes is the major contributing factor to the potency of the antibodies.

15

Immunohistology

Alanyl aminopeptidases, in mammalian general, are membrane-bound metalloproteases that are expressed on the surface of normal human cells in the lung, liver, small intestine and kidney. We conducted immunohistochemical experiments to assess the potential cross-reactivity of anti-AnAPN1 antibodies against commercially available human tissue sections (corresponding to those listed above). Sections were stained with polyclonal rabbit anti-AnAPN1 IgG followed by detection with Texas Red-conjugated anti-rabbit secondary Abs. Images were acquired in the green/red channel to

20

acquire the background autofluorescence signal and then in the red channel alone to assess anti-AnAPN1 cross-reactivity. Replicate immunofluorescence, staining experiments suggest that any detection of signal in the red channel is largely due to a strong background autofluorescence signal extending into the red channel as determined by overlays (Fig. 6). In spite of this initial data, closer examination (400X) of compartments of the human lung suggest that AnAPN1 IgG staining of alveolar macrophages appeared distinct from background fluorescence. In follow-up experiments, lung sections were stained with both anti-AnAPN1 antibodies (rabbit polyclonal IgG) and anti-Human Actin (mouse MAb) and detected with Texas Red (red channel) conjugated anti-rabbit and Alexa 488 (~FITC/green channel) conjugated anti-mouse secondary antibodies, respectively. These experiments show that the AnAPN1 cross-reactivity signal colocalizes with cytosolic actin staining suggesting that the detection may be either artefactual spill-over from the anti-actin signal in the green channel (as was seen with the autofluorescence data) or that macrophages in the alveolar lumen can be stained with anti-AnAPN1 IgG (Fig. 7).

Expression of NT135APN1 in yeast

The native DNA sequence (SEQ ID NO: 2) encoding NT125APN1 has been cloned into different yeast expression vectors and transformed into different strains of yeast. Preliminary expression feasibility is assessed at the shaker flask level and optimized prior to conducting larger 10L scale fermentations. The expression results are summarized in the Table 1.

Table 1. Status of *NT125APN1* expression in yeast. Summary of a variety of *AnAPN1* constructs that were engineered and evaluated. Stability and yield indicators (+’s) are to compare between strains. +++ for yield is approximately 100mg/L of culture broth.

DNA	His-tag	Vector	Host	Solubility	Yield	Stability	
						pH5.0	pH3.0
AnAPN1	+	pPICZaA	<i>P.pastoris</i> /X-33	soluble	++	+	++
AnAPN1	-	pPICZaA	<i>P.pastoris</i> /X-33	soluble	+++	+	++
AnAPN1	+	pPICZaA	<i>P.pastoris</i> /SMD1168	soluble	+	++	+++
AnAPN1	-	pPICZaA	<i>P.pastoris</i> /SMD1168	soluble	++	++	+++
AnAPN1	+/-	pGAPZaA	<i>P.pastoris</i> /X-33	soluble	++	+	++
AnAPN1	+	pGAPZaA	<i>P.pastoris</i> /SMD1168	soluble	+	+	++
AnAPN1	-	pPinka-HC	<i>PichiaPink</i> Strain#1	soluble	++	+	++

5

As shown in Table 1, *NT125APN1* with or without His-tag at C-terminus was expressed in most of the yeast strains with high yield. The best yield (> 100 mg/liter) was observed when *NT125APN1* without a His-tag was expressed in *Pichia pastoris* X-33 under induction of 0.5% methanol. However, the expressed recombinant *AnAPN1* was not stable in yeast culture, with product derived (confirmed by western blot and amino terminal sequencing) break-down fragments readily forming. Amino acid sequencing results of different fragments showed that the cleavage happened at multiple sites on the C-terminus of the recombinant protein, possibly resulting from the hydrolytic activities of *Pichia* derived protease(s).

15

In an attempt to evaluate how to inhibit or prevent the proteolytic breakdown of recombinant *NT135APN1*, experiments were initiated by transforming *NT135APN1* into protease deficient *Pichia* strains like *P. pastoris* SMD1168 or *PichiaPink* Strain #4 and

by lowering pH of culture to 3-5. The best condition for expressing *NT135APN1* with high yield and high stability has been optimized as being induced in minimal methanol Adenine media (MMA) with pH 4 for clone of *AnAPN1* /pPink-HC transformed PichiaPink strain#4 (shown as pink in the table 1, Fig. 8). The construct without the His-
5 tag was evaluated in PichiaPink, strain#4.

In addition and to reduce the degradation of recombinant full-length *NT135APN1* caused by yeast derived protease(s) during induction, the actual and predicted cleavage sites at C-terminus of *NT135APN1* based on the amino acid sequencing results were mutated to create a mutated clone (*AnAPN1* -M), or the C-terminus containing all
10 cleavage sites was deleted to create a deletion clone (*AnAPN1*-D). These two clones were transformed into *P. pastoris* X-33 to create high yield transformants with improved stability, but still minor degradation remains (Fig. 8).

Finally and to increase stability of recombinant *NT135APN1*, the dimer form of *NT135APN1* (Di- *NT135APN1*) was expressed in *P. pastoris* X-33 by linking two
15 *AnAPN1* coding sequences with a EcoRI site (GAATTC encoding for EF). However, the expressed recombinant dimer form of *NT135APN1* remained problematic in terms of stability with the dimer becoming separated and the proteolytic cascade continuing.

Yeast fermentation

20 *Seed Culture.* For a 10 L-scale fermentation process, a 2.5 L Tunair shake flask (Shelton Scientific, CT), containing 1 L of buffered minimal glycerol was inoculated with 2 mL of the *P. pastoris* PichiaPink™ Strain 4 seed stock. The flask was rotated on a shaker at 180 RPMs and incubated at 30 ± 2 °C for 24-28 hours to a final OD₆₀₀ of 5-15.

Approximately 0.5 L of this culture was used to inoculate 5 L of heat-sterilized basal salt media (BSM) containing 3.5 mL/L (17.5 mL total) of a filter-sterilized trace element (PTM4) solution.

Fermentation Parameters. Fermentation is conducted in a BioFlo 3000 fermentor (New Brunswick Scientific Co. Inc.). The pH of the 5 L of BSM is adjusted and maintained with a 14% ammonium hydroxide feed. The following fermentation parameters are set and verified.

Table 2. Initial fermentation parameters

Parameter	Value
Agitation	500 $\pm$ 10 RPM
Temperature	30 $\pm$ 1 °C
Aeration	Air Flow 7.5 $\pm$ 0.5 LPM, (100% Air)
Dissolved oxygen	30%
pH	5.0 $\pm$ 0.5

10

Batch Phase. After inoculation, a dissolved oxygen spike to a value of 50% for 5 minutes marks the depletion of glycerol (17-20 hours after inoculation) and the end of the batch phase.

Glycerol Fed-Batch Phase. After the dissolved oxygen spike, 50% (v/v) glycerol is introduced into the cell culture media at a set flow rate of 15mL/L of BSM/hr for 8 hours. The pH of the cell culture media is decreased linearly from 5.0 to 4.5 and the temperature decreased linearly from 30 °C to 26 °C over the last two hours of this 8 hour period. Excessive foaming is controlled with 10% (v/v) KFO673 antifoam (Emerald Performance Materials) in DI water.

Methanol Induction Ramp-Up Phase. The methanol induction phase, inducing the expression and secretion of *AnAPN-1*, is initiated when the wet cell weight (WCW) reached approximately 200 g/L. 100% Methanol is added at an initial flow rate of 1.5 mL/L of BSM/h increasing to 8.0 mL/L of BSM/h (ramp) over an 8-hour period.

5 *10X YPYNB Feed.* During the methanol induction phase, a feed of 10X Yeast Extract, Phytone Peptone, Yeast Nitrogen Base (10X YPYNB) is initiated to try to prevent proteolytic cleavage of *AnAPN-1*. The 10X YPYNB is added at a flow rate of 3 mL/L/h over a period of 65 hours to the end of the fermentation.

10 *Methanol Soaking Phase.* Methanol induction is continued for another 57 hours by adding 100% Methanol at a flow rate of 8 mL/L of BSM/Hour. The dissolved oxygen concentration is maintained at >20% using a 30% set point and monitored to provide information on methanol accumulation.

Harvest. The fermentation was harvested with a final wet cell weight of approximately 258 g/L.

15

Recovery and purification from yeast fermentation

Approximately 7.5 L of fermentation cell culture is centrifuged for 30 minutes at 7000 RPM and 4 °C. The ~6.5 L of supernatant is filtered with a 0.22 µm Millipak-60 filter (Millipore) and stored at -80°C, until further purification.

20 Purification is performed on AKTA Explorer (GE Healthcare) Air 100 purification system. The chromatography columns evaluated for the first purification step were a hydrophobic interaction Butyl Sepharose HP column, a cation exchange SP Sepharose XL column, and an anion exchange Q Sepharose XL column. Filtered

fermentation supernatant was the starting material for the following chromatographic experiments

Butyl Sepharose HP. Butyl Sepharose HP is a hydrophobic affinity column that binds proteins based upon their hydrophobicity; material is then eluted from the column with a chaotropic agent. All steps of the Butyl Sepharose HP chromatography are performed at room temperature at a flow rate of ~155 cm/h (1 mL/min) while monitoring the absorbance of the column eluate at 280 nm.

A pre-packed HiTrap Butyl HP 1 mL column (GE Healthcare) was equilibrated with 50 mM Succinic acid, pH 6.0. A solution of 50mM Succinic acid/3 M ammonium sulfate was added to the fermentation supernatant to achieve a concentration of 1.5 M ammonium sulfate, and the pH was adjusted to 6.0 with 5N NaOH. The adjusted supernatant was filtered with a 0.22 um filter (Millipore).

The *AnAPN1* starting material was loaded onto the HiTrap Butyl HP 1 mL column, and unbound sample was washed with buffer (50 mM Succinic acid, 1.5 M ammonium sulfate, pH 6.0). The column was then washed with 50 mM Succinic acid, pH 6.0 applying step wise gradient of six different concentrations of ammonium sulfate (1.35M, 1.2M, 1.05M, 0.9M, 0.75M, and 0M). Two fractions were collected during a wash with buffer containing no ammonium sulfate while monitoring the absorbance at 280 nm. They were also analyzed by SDS-PAGE (Fig. 9a).

SP Sepharose XL. SP Sepharose XL binds proteins in a low ionic strength buffer; material is then eluted from the column by increasing the ionic strength of the buffer. All steps of the SP Sepharose XL chromatography are performed at room temperature at a

flow rate of ~155 cm/h (1 mL/min) while monitoring the absorbance of the column eluate at 280 nm.

A pre-packed HiTrap SPXL 1 mL column (GE Healthcare) was equilibrated with 50 mM Succinic acid, 1M NaCl, pH 4.0. A solution of 50mM Succinic acid, pH 4.0 was added to the fermentation supernatant to reduce the sample conductivity to <5 mS/cm. The pH was approximately 4.0 and did not need adjustment. The diluted supernatant was filtered with a 0.22 um filter (Millipore).

After filtration of the sample, the material was loaded onto the HiTrap SPXL 1 mL column, and unbound sample was washed with buffer (50 mM Succinic acid, pH 4.0). The column was then washed with 50 mM Succinic acid, pH 4.0 containing six different concentrations of NaCl (20 mM, 50 mM, 150 mM, 300 mM, 400 mM, and 800 mM). No peak fractions were seen while monitoring the absorbance at 280 nm.

Q Sepharose XL. *Q Sepharose XL* binds proteins in a low ionic strength buffer; material is then eluted from the column by increasing the ionic strength of the buffer. All steps of the *Q Sepharose XL* chromatography are performed at room temperature at a flow rate of ~155 cm/h (1 mL/min) while monitoring the absorbance of the column eluate at 280 nm.

A pre-packed HiTrap QXL 1 mL column (GE Healthcare) was equilibrated with 20 mM Tris-HCl, 1M NaCl, pH 8.5. A solution of 20mM Tris-HCL, pH 8.5 was added to the fermentation supernatant to reduce the sample conductivity to <5 mS/cm and the pH was adjusted to 8.5 with 5N NaOH. The adjusted supernatant was filtered with a 0.22 um filter (Millipore).

After filtration, the material was loaded onto the HiTrap QXL 1 mL column, and unbound sample was washed with 20 mM Tris-HCL, pH 8.5. The column was then washed with 20 mM Tris-HCl, pH 8.5 containing seven different concentrations of NaCl (25 mM, 50 mM, 100 mM, 200 mM, 400 mM, 600 mM, and 1M). Peak fractions were
5 collected during the washes containing 200 mM, 400 mM, and 600 mM of NaCL while monitoring the absorbance at 280 nm. They were also analyzed by SDS-PAGE (Fig. 9b and 9c).

Expression of NT135APN1 in *E. Coli*

10 In order to produce soluble *NT135APN1* in *E. coli*, the coding sequence was harmonized based on the *E. coli* codon preference and protein structure analysis. The harmonized DNA sequence (SEQ ID NO.: 3) was synthesized and cloned into *E. coli* expression vector pET41a with the deletion of the vector GST tag. The recombinant *NT135APN1* with 6His-tag at C-terminus was expressed in *E. coli* BL21(DE3) under
15 induction of 1 mM IPTG. However, the majority of recombinant *NT135APN1* that was expressed in *E. coli* formed insoluble inclusion bodies. Initial experiments have been performed to refold the denatured recombinant *NT135APN1* by rapid dilution into various refolding buffers. The best recovery of about 40% soluble protein was achieved by diluting guanidine denatured inclusion bodies into 1.1 M guanidine, 0.44 Arginine and
20 5mMDTT, however, the recovered soluble *NT135APN1* precipitated out after being further dialyzed against PBS, pH7.4.

To increase the solubility of recombinant *NT135APN1* expressed in *E. coli*, the induction condition was optimized by decreasing the concentration of IPTG, and

lowering induction temperature. The induced *E. coli* cells were lysed in the presence of ionic detergent sarkosyl. The results showed that more than 10% of recombinant *NT135APN1* expressed in *E. coli* became soluble when *NT135APN1/E. coli* was induced with 0.5 mM IPTG at 25°C for 6 hours and recovered in the presence of sarkosyl. The total yield of soluble *NT135APN1* was about 80 mg/L under this induction condition. However, the efficiency and specificity of His-tag *NT135APN1* binding on nickel column was low during initial experiments (possibly due to aggregation and subsequent lack of His-tag accessibility).

Due to the initial promising results, the strain AnAPN/pET41aΔGST/BL21 was identified to undergo evaluation for process development and scale-up. A Research seed stock was made by growing AnAPN/pET41aΔGST/BL21 in LB till the OD₆₀₀ reached 2.1, adding glycerol to 20% and storing at -80°C. Process development and scale-up of soluble *NT135APN1* were accelerated and evaluated by performing fermentation and purification at the 10 liter scale.

15

E. coli fermentation

Fermentation is conducted in a BioFlo 3000 fermentor (New Brunswick Scientific Co. Inc.). The pH of the 9 L of heat-sterilized LB media is adjusted and maintained with a 20% phosphoric acid feed. Prior to fermentor inoculation the LB media in the fermentor is supplemented with 30μg/ml of Kanamycin by adding 27ml of 10mg/ml Kanamycin stock solution. Approximately 130 mL of seed culture was used to inoculate 9 L of LB media. Target OD in fermentor after inoculation is 0.05-0.1 (Table 3).

Table 3. 10-L Production Fermentor Operating Parameters

PROCESS PARAMETERS	PROCESS DATA	EFT	DURATION	
Shake Flask				
Volume of autoclaved Luria-Bertani (LB) Media	1L		14.0 h	
Concentration of Kanamycin in LB media	30 ug/ml			
Volume of Inoculum	100 ul			
Incubator Rotation Speed	180 RPM			
Temperature of Incubator	37 ± 0.5 °C			
Target OD in Shake Flask	3.0 ± 0.5			
Time of Incubator	14 ± 3 h	14:00		
Fermentor				
Cell Grow Stage				
Volume of LB Media	9 L		2.3 h	
Concentration of Kanamycin in LB media	30 ug/ml			
Target OD in 10L Fermentor after inoculation	0.05-0.1			
Volume of Inoculum	~130 ml			
Volume of 10% antifoam added after inoculation	5 mL			
pH adjusted and maintained with Phosphoric acid	7±0.1			
Temperature	37± 0.5 °C			
Agitation	400 RPM			
DO	>30%			
Air Flow	7.5 LPM			
Target OD Before Temperature Ramp	0.4 ± 0.1	15:40	6 h	
Temperature Ramp / hour	37° to 25°C	16:20		
Induction Stage				
Target OD before Induction	0.6±0.1	16:20		
Conc. of IPTG in Fermentor	0.5 mM			
Volume of IPTG added	9 mL			
Hours of Induction	6±0.1 h		22:20	
Harvesting (volume of cell culture)	~ 9 L			

5

Cell Growth Phase. After fermentor inoculation the level of dissolved oxygen

(DO) is maintained above 30%. Cells culture was growing at 37 ± 0.5 °C, at 400 RPM for

1.3 hours to target OD₆₀₀ of 0.4. The temperature of the cell culture media is decreased

linearly from 37 to 25 °C in one hour period. Excessive foaming is controlled with 10% (v/v) KFO673 antifoam in DI water.

IPTG Induction Phase. The IPTG induction phase, inducing the expression of *NT135APN1*, is initiated when the OD₆₀₀ of cell culture reached approximately 0.6.

- 5 Concentration of 0.5 mM IPTG in fermentor was achieved by adding 9 mL of 5M IPTG stock solution. After 6 hours of induction at 25 °C final OD₆₀₀ of cell culture in fermentor was 2.8.

Recovery and purification from *E. coli* fermentation

- 10 *Centrifugation.* The cell culture is subjected to a centrifugation step in order to separate cells from fermentation media. After centrifugation of ~9 L cell culture at 7000 RPM, 30 minutes at 4°C, approximately 20 g of cell pellet was collected. Cell pellet was resuspended in 0.8L of ice-cold buffer 20 mM Tris-HCl, pH 8.5 (40 mL buffer/gram of cell pellet) and centrifuged at 7000 RPM, 30 minutes at 4°C.
- 15 *Homogenization.* Approximately 5g of the cell pellet (1/4 of the full scale) was resuspended in 250 mL buffer 20 mM Tris-HCl, 2% Sarkosyl, pH 8.5. Cells were opened and homogenized by passing the resuspended cell culture three times through Emulsiflex C-55B machine. Homogenization was performed at 20,000 psi, maintaining the temperature of cell suspension below 13 °C. Disrupted cell pellet was centrifuged at 7000
- 20 RPM, for 30 minutes at 4°C in order to separate supernatant from inclusion bodies. The supernatant, containing soluble portion of *AnAPN1* is filtrated (0.8 - 0.2 um) and it serves as the starting material for downstream purification. In process samples were taken and analyzed (Fig. 10).

Purification. The current purification process for *NT135APN1* consists of one column chromatography step. Purification was performed on AKTA Explorer (GE Healthcare) Air 100 purification system.

Sample was adjusted with the Imidazole concentration to 5 mM, by adding 62mL
5 of 5X 5mM Imidazole, 20mM Tris-HCl, 500mM NaCl, pH 8.5 solution, 300 mL of supernatant (1/4 of the full scale) was loaded onto the Ni column at flow rate of 10 mL/min. Unbound sample was washed from column with 50 mL (10 CV) of 20 mM Tris-HCL, 5 mM Imidazole and 500 mM NaCl, pH 8.5 to remove all unbound material. Weak bounded impurities are washed from the column with 50 mL (10 CV) of 20 mM
10 Tris-HCL, 20 mM Imidazole and 500 mM NaCl, pH 8.5.

The *NT135APN1* is then eluted from the column with 20 mM Tris-HCl, 500 mM Imidazole, 500 mM NaCl, pH 8.5. The eluate (19 mL) is collected as a single peak. The eluted protein was stored at 2-8°C for the subsequent purification or formulation step.

This purification steps are performed at room temperature at flow rate of 300cm/h
15 (10 mL/min). The column eluate is monitored for protein content by absorbance at 280 nm.

To assess the purity of the protein eluted with 20 mM Tris-HCl, 500 mM Imidazole, 500 mM NaCl, pH 8.5 (eluate), 3.5 ul of sample from each step of the purification were loaded on a non-reduced 14% Tris-glycine gel (Invitrogen) and stained
20 with Coomassie Brilliant Blue (CBB) (Fig. 11). The majority of impurities seen in the starting material (SM) did not bind to the Ni column and were found in the flow-through (FT) and wash of unbound sample (W), leaving the eluate with an approximate relative

purity more than 90%. The wash with 20 mM Tris-HCL, 20 mM Imidazole and 500 mM NaCl, pH 8.5 did not show significant removal of protein from the column on this gel.

Yield was determined by analyzing protein bands from SDS-PAGE with a GS-800 self-calibrating densitometer. The *NT135APN1* eluate from the Ni column and BSA, a known standard, were run on a reduced 14% Tris-Glycine gel (Invitrogen) and stained with Coomassie Brilliant Blue (Fig. 12). Based on calculations, the concentration of *NT135APN1* in the Ni column eluate was 1.65 mg/ml. The volume of the eluate was 19 mL, therefore the yield was 31.3 mg.

10 mg of protein was dialyzed in 15% Sucrose, 10 mM Tris, 0.2% Tween-80 pH 7.8 buffer (GWU Lot# 032410JLP). The protein was characterized by SDS-PAGE (non-reducing, reducing) followed by coomassie blue staining (with densitometry purity analysis), silver staining, western blotting with anti-His and anti-APN1 (Supplied by Rhoel) antibodies, and LAL endotoxin testing. The lot had a relative purity (by densitometry after coomassie blue staining) of 95.2% and an endotoxin content of < 25 EU/mL. The characterization is presented in the following Fig. 13 and Table 4.

Table 4. Research COA for Lot 032410JLP of AnAPN1 in 15% Sucrose, 10 mM Tris, 0.2% Tween-80, pH 7.8

Assay	Method	Results
LAL Endotoxin	0.25 EU/mL Gel Lot	< 25 EU/mL
Protein Concentration	A280 (GWU SOP 196.2)	1.7 mg/mL
Relative Purity	SDS-PAGE followed by Coomassie staining and Densitometry analysis (GWU SOPs 174.6, 175.6 and 179.2)	95.2% (main product doublet band)
Identity	SDS-PAGE followed by Western Blot analysis with anti-APN1	Main bands react to polyclonal anti-APN1 antibody

Formulation

Initial formulation studies based on SE-HPLC and visual color and appearance analysis showed that *NT135APN1* was highly insoluble in 1x PBS, but that a buffer composed of 10% glucose/10 mM imidazole/10 mM sulfobetaine 3-12 (SB 3-12, a non-ionic detergent) was beneficial in terms of reducing precipitation and aggregation of APN-1.

Once this initial buffer was identified, a preliminary Alhydrogel® (aluminum hydroxide gel adjuvant) binding study was performed. Based upon the theoretical pI of APN-1 (approx. 6.4), it was predicted that *NT135APN1* would readily adsorb onto Alhydrogel®. This prediction was based on the fact that at physiological pH, *NT135APN1* is relatively negatively charged and Alhydrogel® is relatively positively charged. Electrostatic interactions are the most important attractive forces between protein and aluminum hydroxide when the protein and adjuvant have opposite charges (as is the case here).

With this in mind, 0.1 mg/mL *NT135APN1* was formulated with 0.8 mg/mL Alhydrogel® in two different buffers: 10% glucose/10 mM imidazole/10 mM SB 3-12, pH 7.4; and 10% glucose/10 mM imidazole/1 mM SB 3-12, pH 7.4. Binding of *NT135APN1* was allowed to proceed with gentle rotation at room temperature for 30 minutes. Samples of each formulation were then centrifuged at 16,100 x g for 2 minutes to pellet the Alhydrogel® (along with adsorbed *AnAPN1*). The supernatants were aspirated and run in duplicate on a 4-20% Tris-glycine SDS-PAGE gel in the presence of *NT135APN1* standards (unformulated) representing the amount of *NT135APN1* that would be present in the vaccine supernatants if 50%, 25%, 10% and 1% were unbound

(i.e., free in solution) (Fig. 14). *NT135APN1* was 100% adsorbed to Alhydrogel® in both formulation buffers since no protein is visible at approximately 15 kDa on the gel. The level of detection of this assay is approximately 600 pg, which allows for detection of 0.05% unbound *NT135APN1*. This study clearly shows that *NT135APN1* readily binds to
5 Alhydrogel® at physiological pH and in the presence of the stabilizing excipient glucose and the non-ionic detergent SB 3-12.

Although certain presently preferred embodiments of the invention have been specifically described herein, it will be apparent to those skilled in the art to which the invention pertains that variations and modifications of the various embodiments shown
10 and described herein may be made without departing from the spirit and scope of the invention. Accordingly, it is intended that the invention be limited only to the extent required by the appended claims and the applicable rules of law.

What is claimed is

1. An immunogenic composition for use in prevention of malaria comprising a fragment of AnAPN1.
- 5 2. The method of claim 1, further comprising a malaria antigen.
3. The immunogenic composition of claim 2, wherein the malaria antigen is an antigen from *Plasmodium falciparum* or *Plasmodium vivax*.
- 10 4. The immunogenic composition of claim 2, wherein the malaria antigen is an immunogenic particle RTS,S.
5. The immunogenic composition of claim 1, wherein the malaria antigen is an antigen from the sporozoite, gametocyte, gamete, or ookinete stages of
- 15 *Plasmodium falciparum* or *Plasmodium vivax*.
6. The immunogenic composition of claim 1, wherein the fragment of AnAPN1 is NT135APN1.
- 20 7. The immunogenic composition of claim 1, further comprising an adjuvant.
8. The immunogenic composition of claim 1, wherein the fragment of AnAPN1 comprises amino acids 32-64 of SEQ ID NO.: 1.

9. The immunogenic composition of claim 1, wherein the fragment of AnAPN1 is a recombinant polypeptide.
10. A method for preventing malaria comprising the step of administering an
5 effective amount of the composition of claim 1 to an individual.
11. A method for preventing the transmission of malaria comprising the step of stimulating an immune response against AnAPN1.
- 10 12. The method of claim 11, wherein the stimulating step comprises administering to an individual a fragment of AnAPN1.
13. The method of claim 12, wherein the fragment of AnAPN1 is NT135APN1.
- 15 14. The method of claim 12, wherein the fragment of AnAPN1 comprises amino acids 32-64 of SEQ ID NO.: 1.
15. The method of claim 12, wherein the fragment of AnAPN1 is a recombinant polypeptide.
- 20 16. A method for making an immunogenic composition for use in prevention or treatment of malaria comprising the step of admixing a fragment of AnAPN1 and a malaria antigen.

17. The method of claim 16, further comprising the step of adding an adjuvant to the admixture.

5 18. The method of claim 16, wherein the malaria antigen is an antigen from *Plasmodium falciparum* or *Plasmiodium vivax*.

19. The method of claim 16, wherein the malaria antigen is an immunogenic particle RTS,S.

10

20. The method of claim 16, wherein the malaria antigen is an antigen from the sporozoite, gametocyte, gamete, or ookinete stages of *Plasmodium falciparum* or *Plasmiodium vivax*.

15

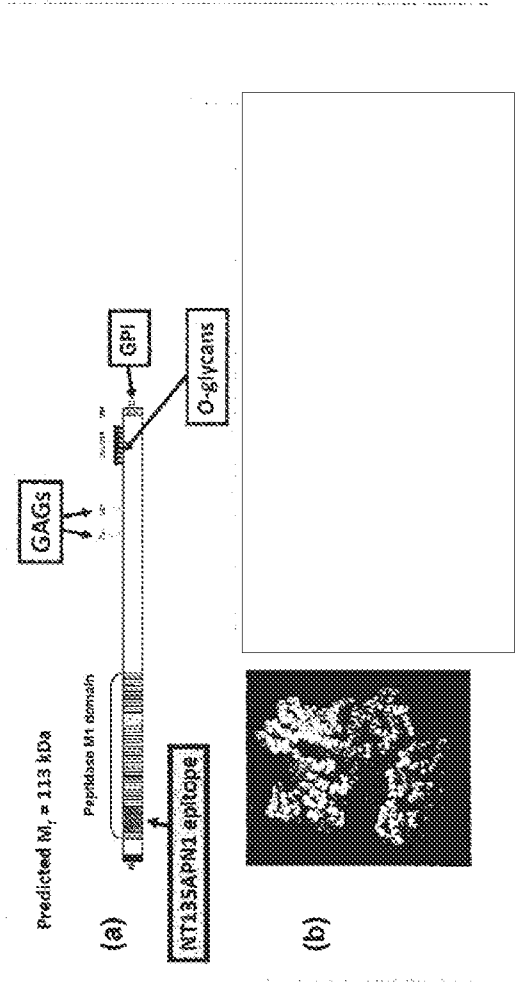


FIG. 1

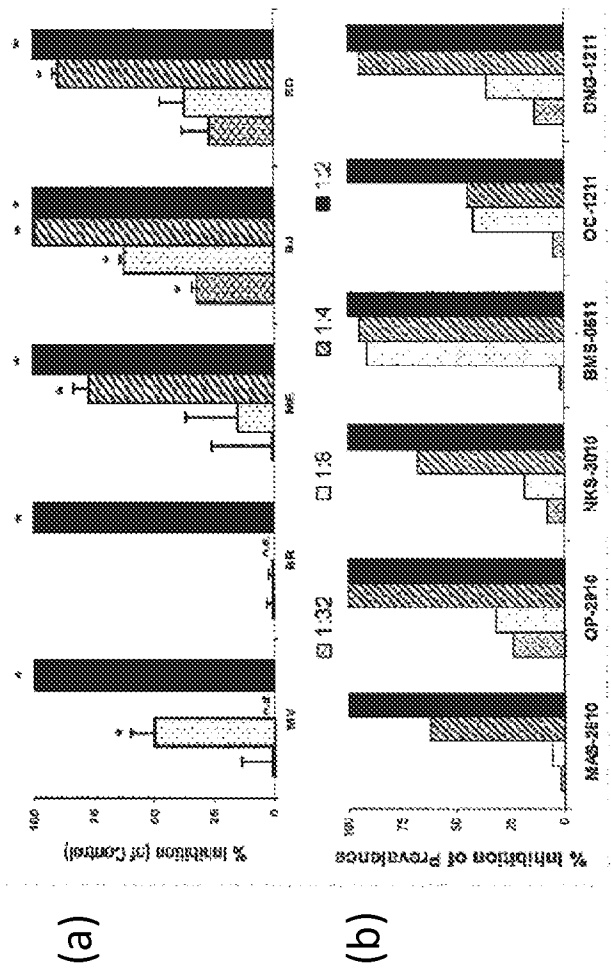


FIG. 2

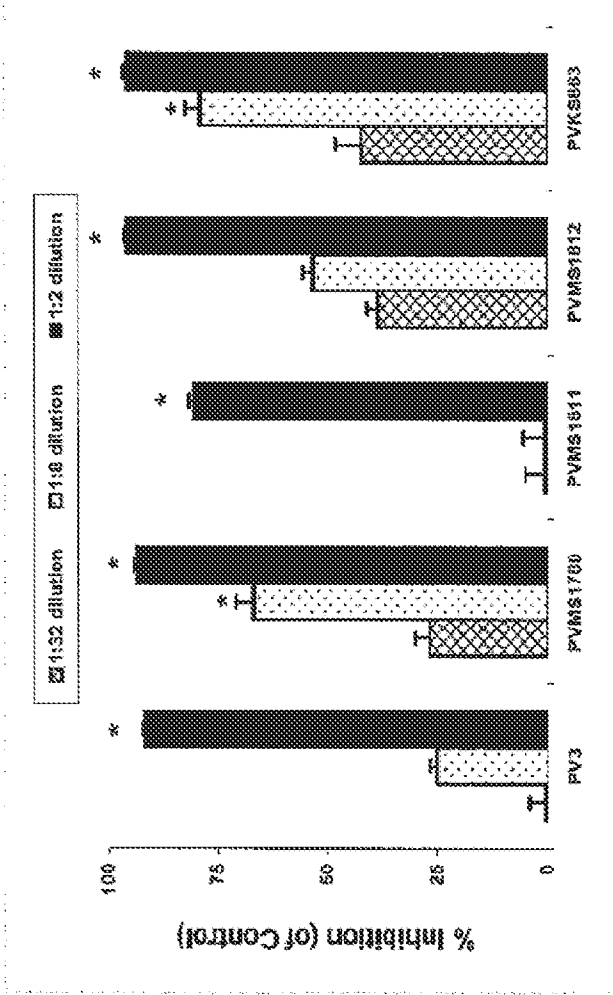


FIG. 3

FIG. 4

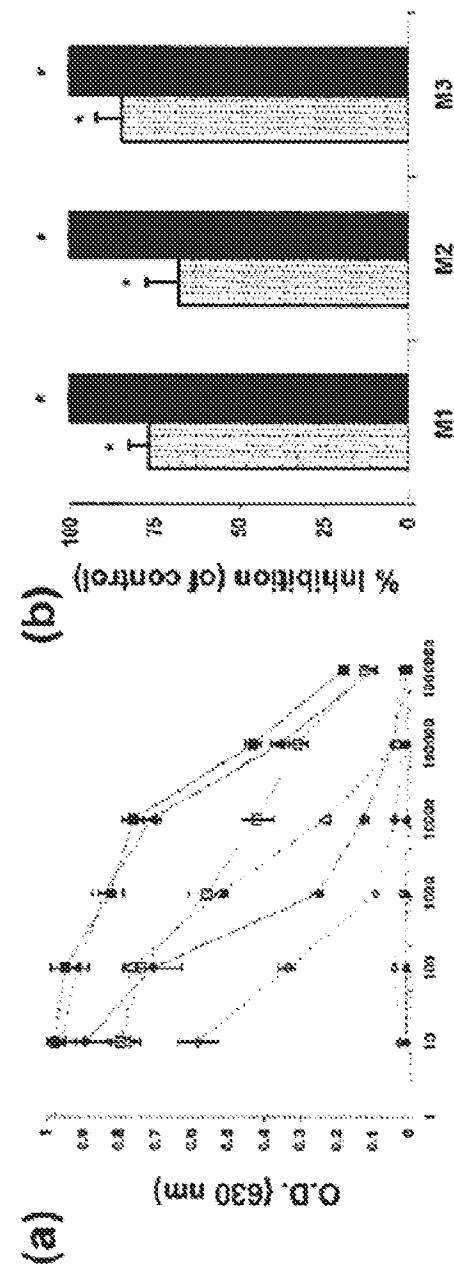


FIG. 4

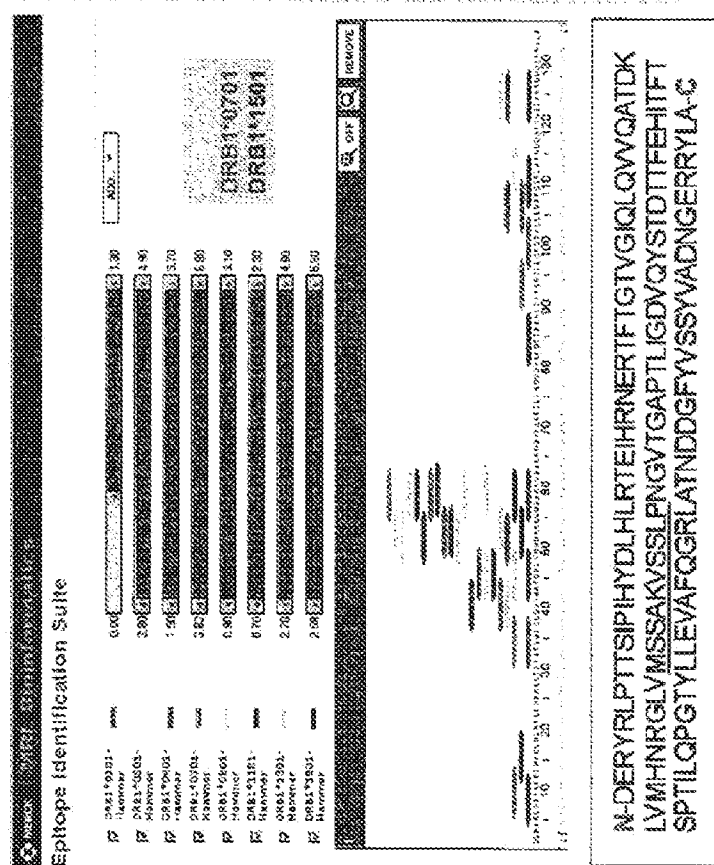


FIG. 5

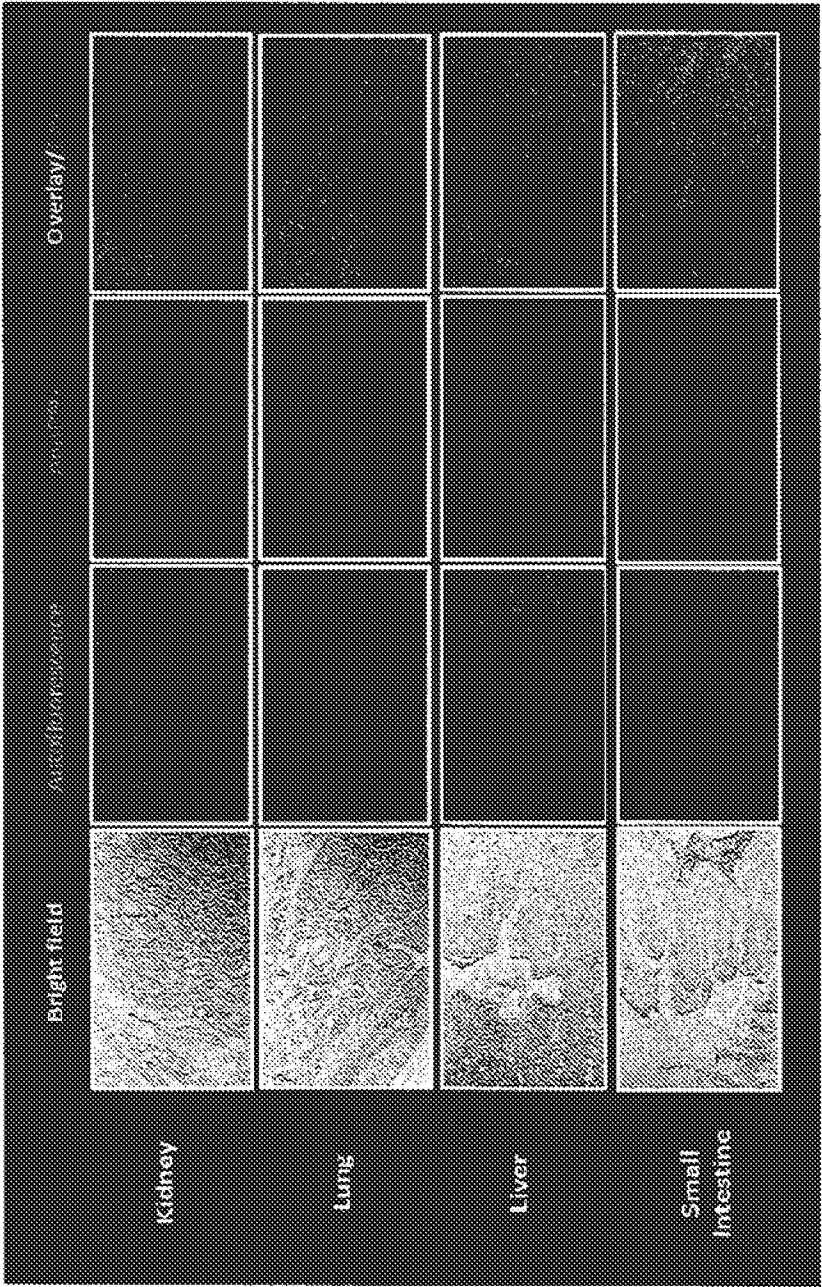


FIG. 6

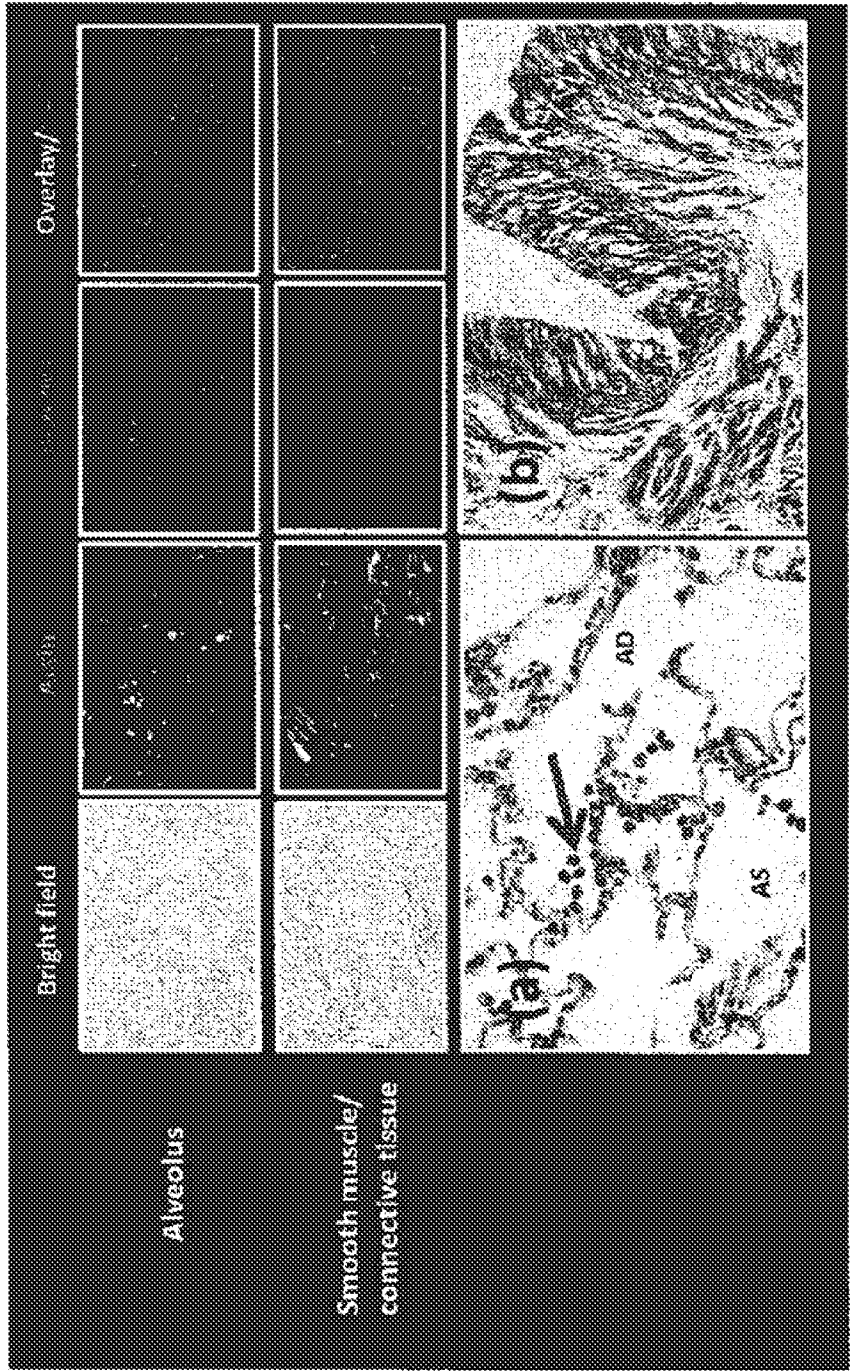


FIG. 7

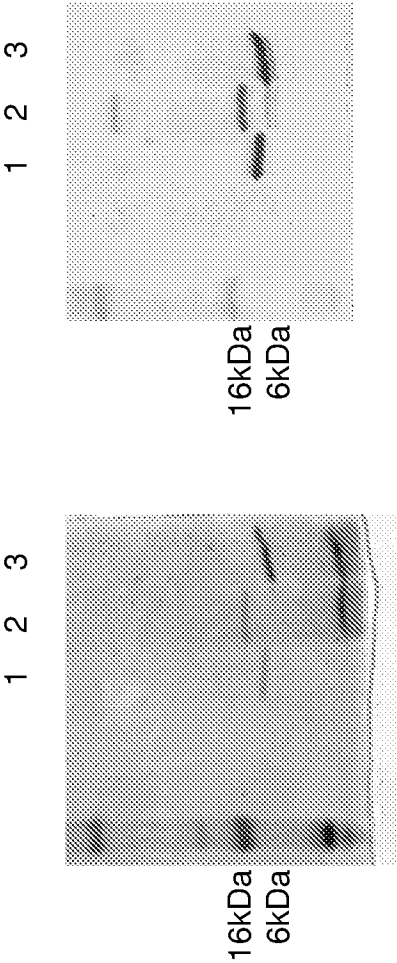


FIG. 8

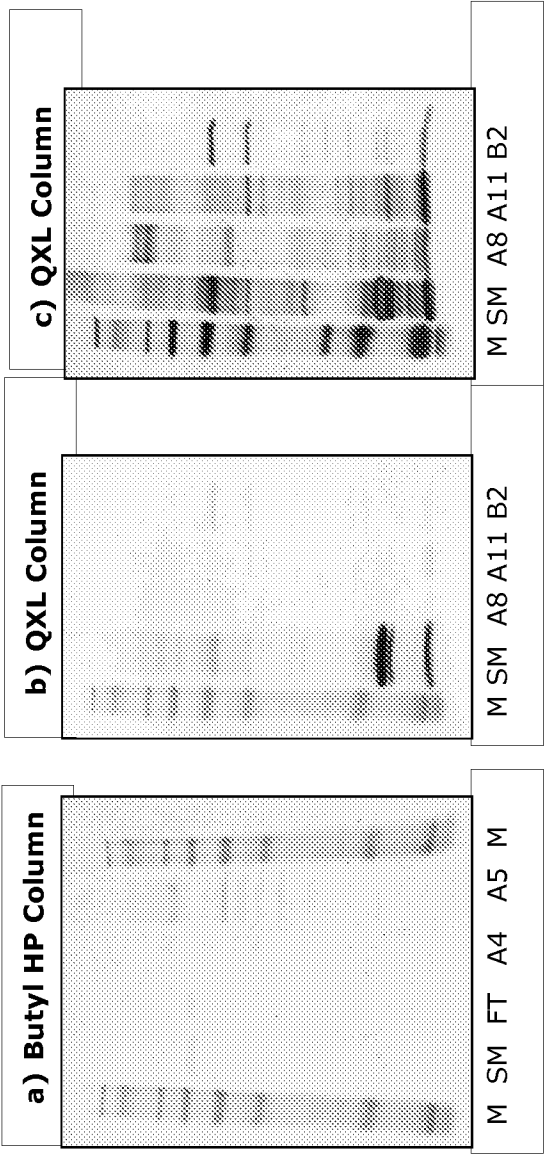


FIG. 9

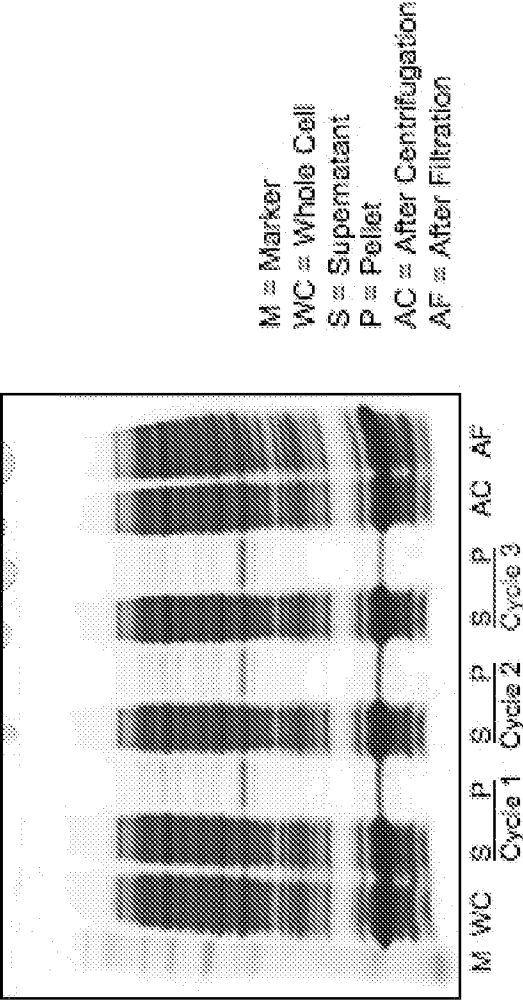


FIG. 10

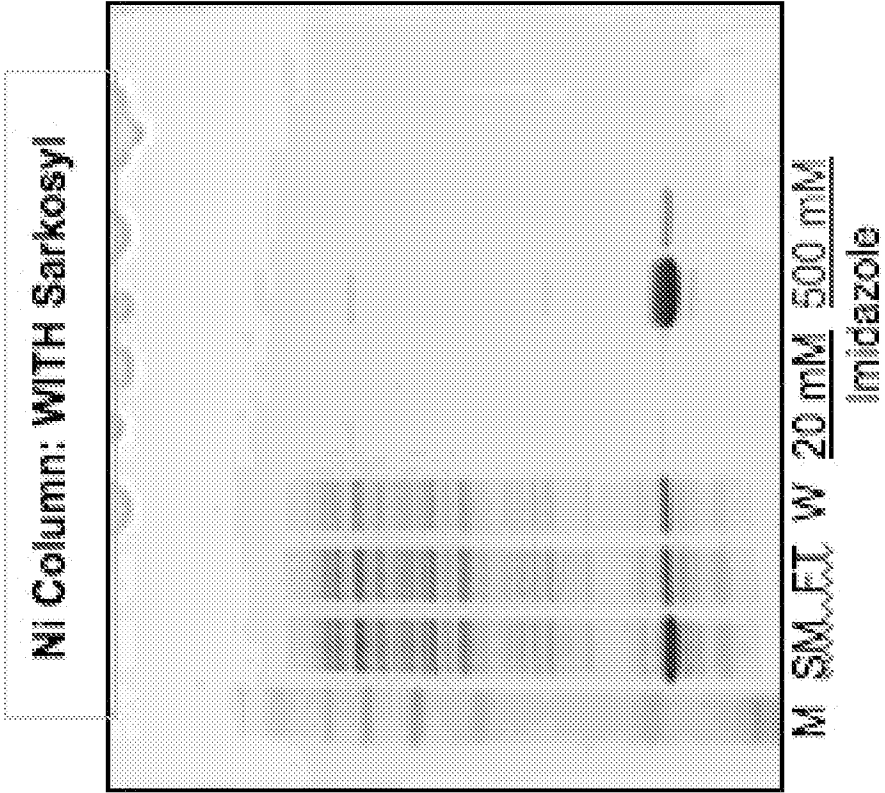


FIG. 11



FIG. 12

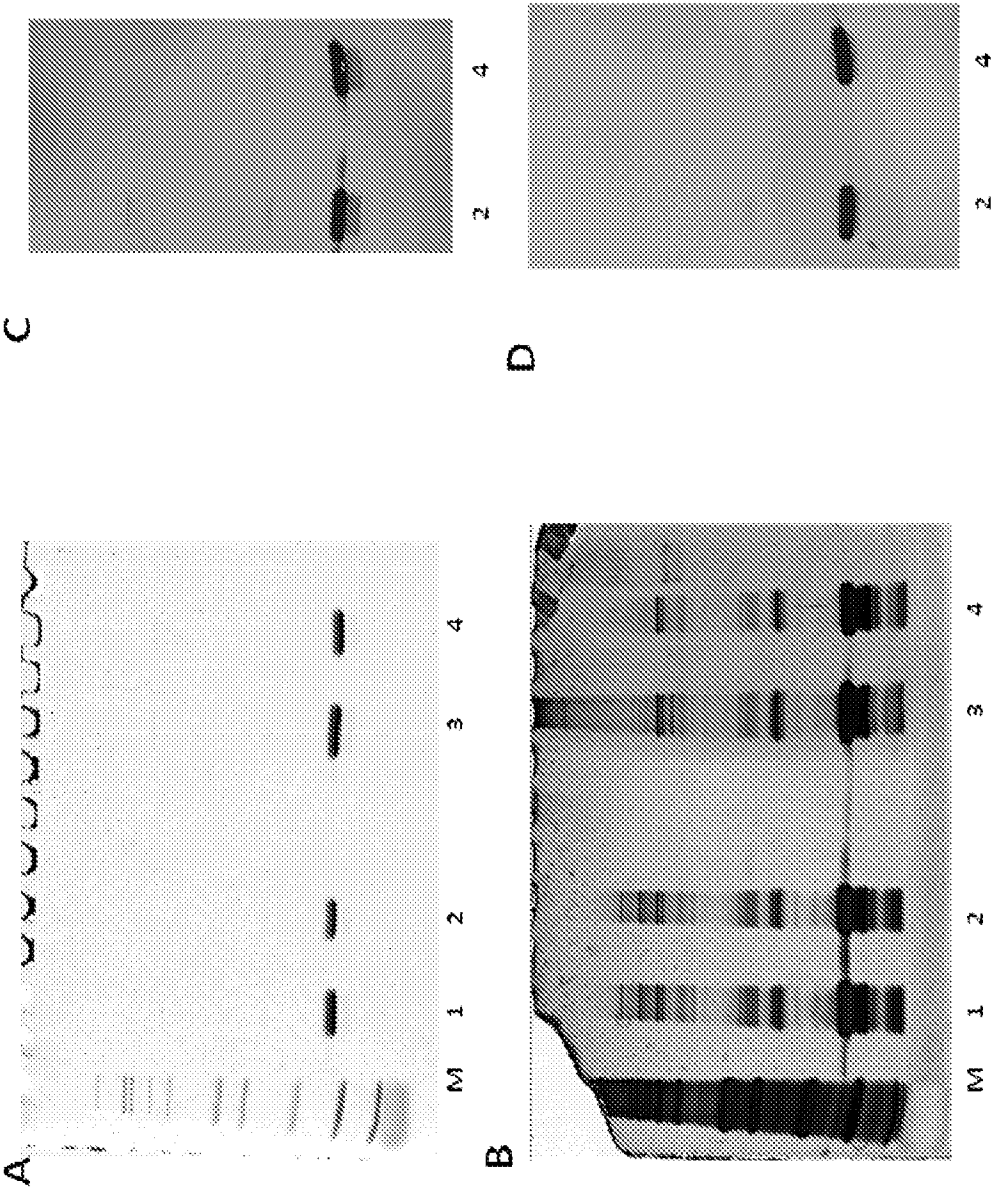


FIG. 13

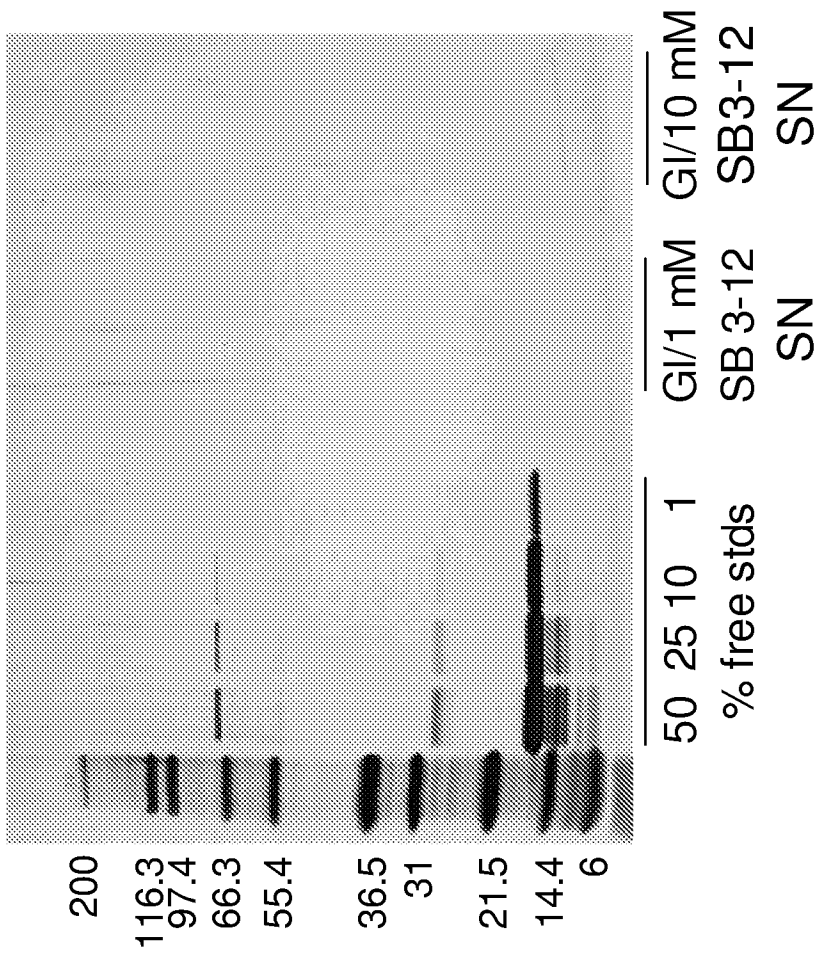


FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/55305

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00 (2010.01)

USPC - 424/265.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8)-A61K 39/00 (2010.01)

USPC-424/265.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar

malaria, vaccine, antigen, falciparum, vivax, plasmodium, transmission blocking, aminopeptidase, anapn1, anapn-1, rts,s, TBV, liver, sporozoite or gametocyte or gamete or ookinete, Dinglasan

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DINGLASAN et al., Disruption of Plasmodium falciparum development by antibodies against a conserved mosquito midgut antigen. PNAS, 14 August 2007, Vol. 104, No. 33, pp 13461-13466.	1, 6-15
Y	abstract, pg 13461 para 1-4, pg 13462 Table 1; pg 13464 col1 para 1; pg 13465 col 2 para 4	2-5, 16-20
Y	DINGLASAN et al., Flipping the paradigm on malaria transmission-blocking vaccines. Trends in Parasitology, August 2008, Vol.24 No.8. pg 364 col 1 para 1; pg 364 col 2 para 1,2; pg 365 Fig.1 Box 1; pg 367 col 1 para 1)	2-5, 16-20
Y	HOLT et al., The Genome Sequence of the Malaria Mosquito Anopheles gambiae. Science 4 October 2002, Vol 298, No 5591, pp129-149 (2002). (uniprot acc# Q7Q2T8.1) 8, 14	8, 14
Y	US 2005/0260219 A1 (LAVAZEC et al.) 24 November 2005 (24.11.2005) (para [0003], [0010]-[0012], [0097])	1-20

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 January 2011 (04.01.2011)

Date of mailing of the international search report

21 JAN 2011

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774