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Tiphaine ROUSSEL-GAILLARD

IDENTIFICATION ET VALIDATION DE MARQUEURS MOLECULAIRES DE LA RESISTANCE DE *PLASMODIUM FALCIPARUM* A LA DOXYCYCLINE

Pour obtenir le grade de DOCTORAT D'AIX-MARSEILLE UNIVERSITE

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Unité de Parasitologie
Institut de Recherche Biomédicale des Armées, Antenne Marseille
Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes
Unité Mixte de Recherche 7278, Marseille

Membres du Jury de la Thèse :

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M. le Docteur Henry VIAL	Rapporteur
M. le Professeur Sandrine HOUZE	Examinateur
M. le Docteur Jean-Marie PAGES	Examinateur
M. le Docteur Bruno PRADINES	Directeur de thèse

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Cette thèse vous est dédiée

« Ceux qui sont férus de pratique sans posséder la Science sont comme le pilote qui s'embarquerait sans timon ni boussole, et ne saurait jamais avec certitude où il va »

Leonard de Vinci

RESUME

Les voyageurs non immuns en zone d'endémie palustre peuvent contracter le paludisme lors du séjour ou à leur retour. Parmi les 30 millions de voyageurs enregistrés chaque année dans le monde, environ 15 000 militaires français sont exposés au paludisme qui reste une grande menace comme l'ont récemment démontré les déploiements au Mali ou en Centrafrique. Des taux d'attaque de 7% étaient estimés lors des premiers mois d'installation et ce malgré la disponibilité de moyens de protection individuels et collectifs. La recherche de molécules utilisables en prophylaxie et dans traitement contre le paludisme reste un axe majeur de recherche, la mise au point de nouvelles molécules répondant à la nécessité de contourner l'émergence d'isolats résistants. Le génome de *Plasmodium falciparum* est complexe et la mise en évidence de nouveaux mécanismes de résistance est classiquement réalisée par l'évaluation du nombre de copies d'un gène, ou par l'identification de polymorphismes dans les gènes codant plus particulièrement des protéines cibles, impliquées dans le mode d'action des antipaludiques ou des protéines de transport. La doxycycline est l'une des molécules recommandées par l'OMS en prophylaxie pour les voyageurs dans les zones d'endémie palustre, en particulier dans les zones de multirésistance. C'est la chimioprophylaxie choisie par de nombreuses armées, dont l'Armée Française. Une étude récente avait suggéré que les isolats de *P. falciparum* présentaient différents niveaux de sensibilité à la doxycycline et que l'augmentation du nombre de copies de deux gènes, *pfmdt* ou *pftetQ,* pouvait être associée à une baisse de sensibilité à la doxycycline d'isolats d'origine africaine.

Le premier objectif de ce travail a consisté à valider ce modèle à partir d'un nouvel échantillonnage d'isolats africains. Le second objectif était d'évaluer le nombre de copies de ces deux gènes sur des isolats originaires d'une zone de multirésistance située en Thaïlande. Le troisième objectif a consisté à rechercher d'autres sources de résistance en investiguant le polymorphisme des gènes codant l'ARN ribosomal plasmodial potentiellement impliqués dans la résistance *in vitro* à la doxycycline.

Les résultats nous ont permis de confirmer que le nombre de copies des gènes *pfmdt* ou *pftetQ* pouvait être impliqué dans la résistance *in vitro* à la doxycycline en Afrique. Les

résultats concernant les isolats Thaï n'ont pas permis de corréler le nombre de copies des gènes *pfmdt* et *pftetQ* au phénotype CI_{50}. Ces éléments montrent que ce mécanisme de résistance seul est insuffisant pour expliquer la résistance à la doxycycline ; les résultats sont en faveur d'une résistance médiée par plusieurs gènes, les marqueurs génétiques pouvant varier en fonction du continent d'origine.

La recherche de points de mutation sur le gène *pfssrRNA* codant pour la petite sous-unité ribosomale de l'ADN plasmodial n'a pas abouti. D'autres cibles moléculaires sont en cours d'étude pour expliquer les mécanismes de résistance de *P. falciparum* à la doxycycline.

ABSTRACT

Non immune travellers visiting malaria-endemic areas are at risk of malaria transmission and may become clinically ill during of after their travel. Among the 30 million travellers registered annually from nontropical regions, about 15,000 French soldiers are exposed each year to malaria that still represents an important threat as have shown the recent deployments in Mali or in Central African Republic. Malaria has occured despite the availability of means of individual protection. Prophylaxis and chemotherapy remains a major area of research in malaria and new molecules are constantly being developed before the emergence of resistant parasite strains. The genome of *Plasmodium falciparum* is complex and the search for mechanisms of resistance of *P. falciparum* for antimalarial drugs comes from the evaluation of genes copy number or the identification of polymorphism in genes encoding target proteins specifically involved in the mode of action of the antimalarial molecules or transport proteins. Doxycycline is currently one of the recommended chemoprophylactic regimens for travellers visiting malaria-endemic, particularly in countries with a high prevalence of resistance to chloroquine and multiresistance. This drug is the chemoprophylaxis used by many armies, including the French Army. A previous study suggested that increased *pfmdt* or *pftetQ* copy number could be associated with a lower susceptibility to doxycycline in African *P. falciparum* isolates.

The first aim of this study was to validate the pre-established model involving these two molecular markers with other African isolates. The second was to evaluate these markers in *P. falciparum* isolates coming from a multiresistance area in Thaïland. The third was to investigate the eventual association between the polymorphism in genes encoding ribosomal rRNA and *in vitro* resistance to doxycycline.

The results confirm that *pfmdt* or *pftetQ* copy numbers should be involved in *in vitro* susceptibility to doxycycline in African *P. falciparum* isolates. The results concerning the Thai isolates indicate that there is no correlation between the *pfmdt* and *pftetQ* genes copy numbers and the belonging to the high doxycycline IC_{50} phenotype; this implies that this mechanism of resistance is not enough by itself to explain resistance to doxycycline; it

augurs that the resistance to doxycycline should be controlled by multiple genes, and that these genetic markers could be continent-dependent. The search for points of mutation in isolates from the different doxycycline IC_{50} phenotypic groups has not resulted with *pfssrRNA*. Other therapeutic targets are being considered to explain *P. falciparum* resistance to doxycycline.

TABLE DES MATIERES

LISTE DES ABBREVIATIONS

ACT (CTA)	Artemisinin-based combination therapy (Combinaisons thérapeutiques à base d'artémisinine)
ADN (DNA)	Acide désoxyribonucléique (Deoxyribonucleic acid)
ARN (RNA)	Acide ribonucléique (Ribonucleic acid)
ART	Dérivés de l'artémisinine
CDC	Center of Disease Control
CI_{50} (IC_{50})	Concentration Inhibitrice 50 (Inhibitory Concentration 50)
CSF	Cerebrospinal fluid
DHFR	Dihydrofolate déshydrogénase
DHPS	Dihydroptéroate synthétase
DOX	Doxycycline
EF-G	Elongation factor-G
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
HRP2	Histidin Rich Protein 2
K13	Kelch 13
OMS (WHO)	Organisation Mondiale de la Santé (World Health Organisation)
SP	Sulfadoxine-pyriméthamine
STI	Sex Transmitted Infection

INTRODUCTION

Le paludisme, pathologie à transmission vectorielle, est l'une des grandes menaces sanitaires des régions tropicales, malgré l'existence de moyens de protection individuelle efficaces par utilisation de répulsifs et de moustiquaires imprégnées d'insecticide, venant compléter une chimioprophylaxie spécifique. L'identification et le développement de nouvelles molécules à visée prophylactique ou thérapeutique contre le paludisme sont des axes majeurs de recherche pour faire face à l'émergence de parasites résistants. L'utilisation de molécules antipaludiques est conditionnée non seulement par le niveau de résistance de *Plasmodium falciparum* dans le pays de destination, mais également par leurs contre-indications, leur tolérance clinique et le coût financier. Parmi les composés potentiellement utilisables, des antibiotiques tels que les cyclines, ont été étudiés *in vitro* ou *in vivo*.

Les tétracyclines, famille d'antibiotiques à large spectre découverte au début des années 1940, sont actives sur les protozoaires parmi lesquels *Plasmodium*. Elles ont été utilisées dans cette indication dès 1950 pour des accès palustres simples à *P. falciparum* et *P. vivax*, sur des petites séries de patients. L'émergence de résistances à la chloroquine dans les années 1960 a conduit à des études menées par le Center of Disease Control (CDC) ; en 1985, l'Organisation Mondiale de la Santé (OMS) émettait des recommandations en faveur de l'utilisation de la doxycycline dans la chimioprophylaxie du paludisme à *P. falciparum*. De nos jours, cette molécule est utilisée en thérapeutique en association avec de la quinine, et pour la chimioprophylaxie dans les zones de multirésistance, notamment en Asie du Sud-est. Elle est également utilisée comme chimioprophylaxie de première ligne par de nombreuses armées, et plus particulièrement par l'Armée Française lors des déploiements en zone d'endémie. Cette dernière doit cependant déplorer plus de 3 000 cas de paludisme depuis 2002. Les déploiements récents au Mali ou en République Centrafricaine ont été responsables d'une recrudescence des cas. Ces échecs de prophylaxie avec la doxycycline sont principalement associés à une posologie inadéquate ou une mauvaise observance. Les propriétés pharmacocinétiques de la doxycycline, et en particulier sa demi-vie réduite, peuvent expliquer en partie ces défaillances. Cependant des phénomènes de résistance peuvent être envisagés.

La deuxième famille d'antibiotiques candidats potentiels dans la lutte contre *Plasmodium* est la grande famille des macrolides et de ses dérivés, les lincosamides et azalides. Si les molécules les plus anciennes de cette famille sont peu attrayantes en raison de leurs propriétés pharmacocinétiques, les composés les plus récents : azithromycine et clindamycine ont été étudiés sur des isolats de différents continents. Ces molécules, dont le spectre d'action est semblable à celui des tétracyclines, ont l'avantage de pouvoir être administrées chez l'enfant et la femme enceinte.

La première partie de notre travail a consisté à réaliser une revue de la littérature sur l'utilisation des antibiotiques comme substance antipaludique. Deux familles sont particulièrement décrites, les tétracyclines et les macrolides et dérivés, avec déclinaison de leurs indications, de leurs propriétés pharmacocinétiques, de leur tolérance, de leur mécanisme d'action, des mécanismes de la résistance décrits à ce jour. D'autres molécules antibiotiques sont également évoquées ; elles pourraient dans le futur se retrouver en première ligne dans la lutte contre le paludisme.

L'objectif de la seconde partie de notre travail, expérimentale, a consisté à valider les marqueurs moléculaires de la résistance de *Plasmodium falciparum* aux antibiotiques précédemment décrits. Différentes voies ont été explorées pour mettre en évidence des cibles moléculaires à l'origine de la résistance de *P. falciparum* à la doxycycline. L'étude des mécanismes d'action de cette molécule dans le monde bactérien a conduit à la découverte de cibles parasitaires potentielles; une étude de 2010 avait montré que l'augmentation du nombre de copies des gènes *pftetQ* et *pfmdt* pouvait être associée de façon indépendante à une diminution de la sensibilité à la doxycycline. Nous avons cherché à évaluer et valider ces marqueurs moléculaires de diminution de la sensibilité de *P. falciparum* à la doxycycline en évaluant leur prévalence en Afrique de l'Ouest et sur d'autres isolats africains à l'origine de paludisme d'importation. Nous avons ensuite cherché à valider ce modèle à partir d'isolats thaïlandais pour lesquels les concentrations inibitrices CI_{50} pour la doxycycline avaient été préalablement déterminées.

Nous avons ensuite recherché d'autres mécanismes moléculaires pouvant être à l'origine de la résistance aux molécules antimalariques, toujours par extrapolation à partir du monde bactérien. Plusieurs études ont montré un effet de la doxycycline, de l'azithromycine et de

la clindamycine sur l’apicoplaste de *P. falciparum*; dans certaines bactéries, ces antibiotiques inhibent la synthèse protéique en se fixant sur le site accepteur de la petite sous-unité ribosomique ARNt. L’apicoplaste est capable de synthétiser des protéines grâce à des ribosomes de type procaryote. Ces théories nous ont amené à entreprendre l'identification de cibles moléculaires apicoplastiques à la doxycycline.

Ces projets ont été entrepris en tenant compte de la complexité du génome de *P. falciparum*: l'échange fréquent de séquences chromosomiques (environ 25%) et la composition riche en bases A et T (80,6%) rendent le génome du parasite instable et son étude complexe.

PREAMBULE :
EPIDEMIOLOGIE DU PALUDISME EN 2014

Le paludisme est la première endémie parasitaire mondiale avec 3,2 milliards d'individus exposés, 198 millions de cas estimés [1] et 584 000 décès en 2013. L'Afrique est le continent le plus touché : 90 % des décès liés au paludisme surviennent sur ce continent, essentiellement chez des enfants âgés de moins de 5 ans. La lutte antipaludique repose avant tout sur la lutte anti-vectorielle. Cette dernière s'est intensifiée ces 10 dernières années, notamment en Afrique sub-saharienne, et l'on considérait en 2013 que 49 % de la population exposée avait accès à des moustiquaires imprégnées versus 2 % en 2004. De plus, 38 pays sur les 97 exposés pratiquaient le contrôle larvaire en complément des épandages insecticides anti-vectoriels.

Concernant l'accès aux traitements, fin 2013, les combinaisons thérapeutiques à base d'artémisinine (ACT) étaient adoptées en traitement de première ligne dans 79 pays dans lesquels *P. falciparum* est endémique. Le nombre d'enfants traités restait cependant insuffisant, ce qui explique une mortalité dans cette catégorie de population toujours élevée. L'année 2014 a été marquée par la mise en évidence de résistances à l'artémisinine dans 5 pays : le Cambodge, le Laos, la Birmanie, la Thaïlande et le Viet Nam. Un certain nombre d'isolats de *P. falciparum* du Cambodge et la Thaïlande sont dorénavant résistants à la plupart des molécules antiplasmodiales. Ces nouvelles données ont conduit l'OMS à dissuader l'administration orale de dérivés de l'artémisinine en monothérapie, et en novembre 2014, seuls 8 pays continuaient à les utiliser en monothérapie. Limiter l'émergence et la diffusion des résistances constitue actuellement l'enjeu majeur des systèmes de Santé Publique des pays exposés, l'objectif de l'OMS pour 2015 étant une diminution de la mortalité de 55 % par rapport à l'année précédente.

Les forces armées françaises déploient chaque année environ 15000 hommes en zone d'endémie palustre, la majorité d'entre eux exerçant en zone de chloroquinorésistance voire même de multirésistance. Les déploiements au Mali ou en République Centrafricaine ont été responsables d'une recrudescence des cas de paludisme, avec respectivement 284 cas en 2013 et 518 cas en 2014, soit des taux d'attaque respectifs de 7.5 % et 12.5 %. En 2014, le paludisme a été à l'origine de 5 évacuations sanitaires sur la métropole, de 72 évacuations médicales tactiques et a généré 1500 jours d'indisponibilité.

En 2014, un total de 2 299 cas de paludisme a été déclaré au Centre National de Référence (CNR) du Paludisme par les correspondants du réseau métropolitain (rapport CNR paludisme 2014). Le nombre de cas de paludisme d'importation a été estimé à environ 4370 cas pour l'ensemble de la France métropolitaine. Les pays de contamination sont majoritairement situés en Afrique subsaharienne (96 %), les cas surviennent principalement chez des sujets d'origine africaine (76,5 %), résidant en France ou arrivant d'Afrique, et ils sont dus en majorité à l'espèce *Plasmodium falciparum* (86 %). L'année 2014 confirme la tendance à l'augmentation des cas de paludisme importés en France métropolitaine observée en 2013. La doxycycline est le premier traitement chimioprophylactique (62,6 %) déclaré par les patients qui ont fait un accès palustre sous chimioprophylaxie adaptée à la zone visitée.

LES MOLECULES UTILISABLES DANS LA LUTTE ANTIPALUDIQUE

Les molécules antipaludiques sont utilisées en prophylaxie ou lors d'un traitement, qu'il s'agisse du traitement d'un accès palustre simple ou d'un paludisme grave (**Tableau 1**). Jusqu'aux années 1960, les molécules antipaludiques étaient peu nombreuses : en Afrique et en Amérique étaient utilisées la chloroquine et l'association sulfadoxine-pyriméthamine et dans le sud-est asiatique : la méfloquine. Progressivement, la chloroquine et la sulfadoxine-pyriméthamine ont perdu leur efficacité, et des associations de molécules démontrées synergiques se sont vite imposées.

L'arsenal antipaludique actuel se compose de cinq grandes familles (**Tableau 2**) aux propriétés pharmacologiques variées (**Tableau 3**) : les quinoléines, grande famille de molécules, également la plus ancienne ; les dérivés de l'artémisinine, les hydroxynaphtoquinones, les antifoliniques et antifoliques et enfin, les antibiotiques parmi lesquels les tétracyclines et les macrolides.

Parmi les quinoléines, la quinine reste le principal antipaludique recommandé en deuxième ligne dans le traitement du paludisme grave et chez la femme enceinte en Afrique. Elle est efficace cliniquement contre les souches résistantes à la chloroquine ou à la méfloquine. Les premiers cas cliniques de résistance à la quinine ont été rapportés dans les années 60 en Amérique du sud et en Asie du sud-est [2]. Ils sont actuellement décrits dans l'ensemble des continents où sévit le paludisme. En Asie du sud-est et en Guyane, la quinine est utilisée dans le traitement du paludisme en association avec les tétracyclines ou la clindamycine dès les années 90 [3][4][5]. La quinine exerce son action antimalarique en se liant à l'hème. Le complexe hème–quinine est capable d'endommager les membranes parasitaires par peroxydation lipidique et libération d'hème en présence de glutathion. De nombreuses études ont montré l'indépendance de la sensibilité des isolats à la quinine par rapport à celles de la chloroquine ou de la méfloquine, ce qui suggère que les mécanismes de résistance à la quinine sont différents de ceux de la résistance à la chloroquine ou à la méfloquine. Le phénotype de réponse à la quinine semble être affecté par les gènes *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) [6], impliqué dans la résistance à la chloroquine et *pfmdr1* (*Plasmodium falciparum* multi-drug resistance 1) impliqué ans la résistance à la méfloquine [7]. L'introduction expérimentale de mutations sur le gène *pfmdr1* a été associée *in vitro* à une résistance à la quinine [8] et la surexpression *in vitro* de *pfmdr1* entraîne une résistance à la fois à la quinine et à la

méfloquine [9][10]. La quinine restant efficace contre les souches résistantes à la chloroquine, il est probable que les phénotypes de réponse à la quinine soient liés à d'autres gènes. Cette hypothèse est étayée par l'identification de cinq gènes supplémentaires associés significativement à la réponse à la quinine d'isolats de *P. falciparum* originaires de trois continents différents. Le polymorphisme du gène *pfnhe-1* semble associé à la résistance à la quinine [11][12]. Ce gène code un échangeur Na+/H+ situé dans la membrane plasmique du parasite (contrairement aux autres transporteurs impliqués dans la résistance aux quinoléines qui sont localisés dans la membrane vacuolaire) qui pourrait réguler le pH cytoplasmique ou de la vacuole digestive du parasite. Des perturbations de ce pH liées à ce transporteur pourraient altérer l'activité de la quinine [13].

La chloroquine est une amino-4-quinoléine de synthèse qui a fait son apparition après la seconde guerre mondiale. Efficace, rapide et peu onéreuse, elle s'est imposée comme un remarquable antipaludique. Cependant, les premiers cas de résistance à la chloroquine sont apparus en Asie et en Amérique du sud dès la fin des années 50 [2]. Cette résistance s'est ensuite répandue en Afrique et elle concerne aujourd'hui la totalité des zones d'endémie palustre. Elle est néanmoins restée pendant trois décennies le médicament de première ligne pour prévenir et traiter le paludisme. La résistance à la chloroquine s'est accompagnée d'une augmentation importante de la mortalité due au paludisme [14]. C'est la molécule antipaludique dont le mécanisme d'action est le plus documenté. La molécule est active exclusivement sur les formes érythrocytaires du parasite. La chloroquine, base soluble, traverse les différentes membranes de l'érythrocyte puis du parasite et s'accumule dans la vacuole digestive acide dans laquelle elle exerce son action létale. C'est également la molécule la plus étudiée concernant les marqueurs moléculaires de résistance, grâce notamment aux techniques de biologie moléculaire [15][16][17][18][19][20]. Des mutations dans le gène *pfmdr1* (chromosome 5) codant pour une protéine de la superfamille des ABC transporteurs, sont associées à une multiplication du risque de résistance *in vivo* à la chloroquine [21][22][23]; certaines conclusions sont néanmoins contreversées [7][24]. Des résultats de transfection [25][26][27] ont conduit à mettre en évidence l'association entre le génotype d'isolats et leur phénotype : le gène *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter), situé sur le chromosome 7,

est ainsi directement impliqué dans la résistance à la chloroquine. Enfin, deux mutations dans le gène *pfmrp* (chromosome 1) semblent être associées à une diminution de la sensibilité *in vitro* à la chloroquine [8][28][29][30].

L'amodiaquine a récemment connu un regain d'intérêt dans le traitement de l'accès simple, en association avec les dérivés de l'artémisinine et plus particulièrement en association avec l'artésunate, leur combinaison ayant un pouvoir synergique puissant. Le mode d'action de l'amodiaquine semble être le même que celui de la chloroquine. Des résistances croisées à la chloroquine et amodiaquine ont été observées *in vivo* et *in vitro*, mais ne concernent pas la totalité des isolats. L'amodiaquine semble plus efficace que la chloroquine, notamment dans les zones où la résistance à la chloroquine est élevée [31]. L'utilisation de l'amodiaquine dans les associations avec les dérivés de l'artémisinine sélectionnerait des parasites de sensibilité diminuée à la monodéséthylamodiaquine, le métabolite actif de l'amodiaquine, suggérant une perte d'efficacité rapide de cette association en Afrique [32]. Du fait de sa toxicité pour le foie et la moelle osseuse dans les traitements de longue durée, elle n'est pas recommandée en prophylaxie. Peu d'études ont exploré les bases moléculaires de la résistance à l'amodiaquine, il existerait des mécanismes communs à l'amodiaquine et à la chloroquine [33].

La méfloquine est un arylaminoalcool synthétisé à la fin des années 70. Elle demeure une des molécules recommandées pour la prophylaxie en zone de multirésistance. La méfloquine a été utilisée avec succès sur des souches multirésistantes de *P. falciparum*, ainsi que comme traitement de première ligne d'accès simples de paludisme en Thaïlande. Il a été observé depuis une diminution de son efficacité dans certaines régions et l'apparition et propagation de souches résistantes en Asie, où elle reste très largement utilisée, associée à l'artésunate ; des résistances à l'association artésunate-méfloquine se sont d'ailleurs développées sur ce continent. Des études ont montré une association entre la résistance à la méfloquine et l'amplification du nombre de copies de *pfmdr1* dans le génome, une mutation sur *pfmdr1* ou les deux. L'augmentation du nombre de copies du gène *pfmdr1* (de 1 à 2 copies ou plus) a été associée à la résistance à la méfloquine avec un risque de résistance *in vivo* multiplié par 8.6 (OR ; IC95% : 3.3-22.9) et à un risque significativement élevé de résistance *in vivo* à l'association artésunate-méfloquine [21].

Cependant, l'association entre le nombre de copies de *pfmdr1* dans le génome ou des mutations sur *pfmdr1* n'est pas absolue, suggérant l'existence d'autres mécanismes de résistance pour la méfloquine, impliquant des gènes encore non connus [34]. De plus, l'association entre diminution de la sensibilité à la méfloquine et l'augmentation du nombre de copies du gène *pfmdr1* n'a pas été démontrée sur l'ensemble des continents.

De nouvelles quinoléines ont fait leur apparition sur le marché des antipaludiques ces dernières années, essentiellement utilisées en association avec les dérivés de l'artémisinine ; il s'agit de la luméfantrine, de la pyronaridine, de la pipéraquine. L'augmentation du nombre de copies du gène *pfmdr1* (de 1 à 2 copies ou plus) a été associée à la résistance à la luméfantrine [35].

L'artémisinine, également dénomée qinghaosu, est un alcaloïde naturel extrait de l'armoise *Artemisia annua*. Bien que les vertus de cette plante soient connues en Chine depuis plus de 2000 ans, elle n'a été étudiée en Occident qu'à partir des années 1970. Il a pourtant fallu attendre le début des années 1990 et les graves problèmes de chloroquinorésistance pour qu'elle soit utilisée hors de Chine et de Birmanie. En 2001, l'OMS considérait que l'artémisinine était « le plus grand espoir mondial contre le paludisme ». Bien que son action soit rapide (pic plasmatique par voie orale obtenu en moins de 2 heures), sa demi-vie faible et son action létale partielle obligent à l'associer à d'autres antipaludiques. Depuis 2001, plus de 60 pays ont adopté officiellement les ACT (artemisinin-based combination therapy) en traitement de première ligne. Il s'agit des dérivés trioxane, dihydroartémisinine et arthemeter, ou de la forme soluble artésunate [36]. Différentes associations sont commercialisées ou en cours d'évaluation (artésunate-sulfadoxine-pyriméthamine, artésunate-amodiaquine, arthéméter-luméfantrine, artésunate-méfloquine, artésunate-chloroproguanil-dapsone, dihydroartémisinine-pipéraquine et artésunate-pyronaridine). L'utilisation des ACT s'est accompagnée d'une diminution drastique de la transmission, de la morbidité et de la mortalité par paludisme dans de nombreuses zones d'endémie [37]. Mais les premiers cas d'échecs cliniques aux ACT ont été identifiés en Asie du sud-est très rapidement [38][39]. Le mécanisme d'action de l'artémisinine et de ses dérivés repose sur leur propriété pro-oxydante par existence d'un pont endopéroxyde (O-O) qui se rompt au contact du fer de l'hème. Le radical libre de type alkyl ainsi produit

est à l'origine de l'action anti-plasmodiale. Un deuxième mécanisme d'action des ACT repose sur leur capacité à alkyler des protéines spécifiques : l'alkylation de la PfATPase6 (PfSERCA), enzyme indispensable à la survie du parasite, conduirait à la mort de ce dernier. La mise en évidence de mutations sur des gènes codant pour des protéines essentielles est compliquée par la grande diversité génétique des parasites en fonction de la zone géographique. Les mutations portant sur le locus K13 pourraient être impliquées dans la résistance à l'artémisinine [40][41]. Le rôle de K13 a été montré en Asie mais pas encore en Afrique ou en Amérique du Sud.

La troisième grande famille de molécules antipaludiques est la famille des hydroxynaphtoquinones, dont le représentant est l'atovaquone. Les premières évaluations de l'atovaquone dans les accès simples de *P. falciparum* ont montré une bonne réponse, mais associée à un taux de recrudescence élevé pouvant atteindre 30 % [42]. Afin d'éviter l'apparition de résistances à l'atovaquone, sa combinaison avec le proguanil, antifolinique, a été développée. Cette association est synergique *in vitro* et l'efficacité clinique de cette association a largement été démontrée [43]. Cette association est recommandée en prophylaxie dans les zones de résistance à la chloroquine et de multi-résistance et dans le traitement de l'accès simple à *P. falciparum* en France. La fixation de l'atovaquone sur le cytochrome b mitochondrial plasmodial conduit à la mort du parasite [33]. Le proguanil, qui seul n'a aucune action sur le potentiel membranaire de la mitochondrie, augmente la capacité de l'atovaquone à interagir. Ce mécanisme d'action explique l'efficacité de l'association dans des régions où de nombreux échecs au proguanil sont observés [42]. Deux mutations (Tyr268Asn et Tyr268Ser) portées par le gène *Pfcytb* codant le cytochrome b, sont associées à la résistance de *P. falciparum* à l'atovaquone [44][45]. Ces mutations sont très rares dans les populations générales et elles ne sont généralement détectées qu'à l'occasion des échecs thérapeutiques ou prophylactiques de l'association atovaquone-proguanil [46].

La quatrième famille est celle des antifoliniques. L'invasion de l'Indonésie par les Japonais pendant la seconde guerre mondiale a privé les armées alliées de leur unique source d'antipaludique, la quinine. Ceci a conduit à une recherche intensive et au développement du proguanil. Le succès de cette molécule a conduit à stimuler la recherche sur les dérivés

des pyrimidines et la synthèse de la pyriméthamine. Cependant, leur efficacité a rapidement diminué en raison du développement rapide de la résistance du parasite en Asie contre cette famille de molécules dans les années soixante, résistance qui s'est finalement propagée au continent africain [47]. Dans les années cinquante, dans une étude menée en Tanzanie [48], la pyriméthamine était administrée tous les mois en prophylaxie à des habitants d'un village rural. Les échecs de prophylaxie sont apparus au troisième mois, et n'ont cessé de progresser les mois suivants. 20 à 40 % d'échecs cliniques sont apparus dès l'introduction de la molécule en prophylaxie dans un village situé à 20 km du premier huit années plus tard. Un seul échec était constaté dans un village situé à plus de 225 km du lieu original de pression médicamenteuse. Cette émergence très rapide de la résistance à la pyriméthamine en réponse à une pression locale a été suivie rapidement par une propagation de la résistance. Cette dernière a alors été associée à un sulfonamide, la sulfadoxine. La résistance à l'association sulfadoxine/pyriméthamine s'est également étendue rapidement ; l'hypothèse d'un clone ancien asiatique ayant migré en Afrique et répondant très rapidement à une pression de sélection reste privilégiée [49]. Cette association reste utilisée en prophylaxie chez les enfants et les femmes enceintes en zone d'endémie [50][51]. Concernant le proguanil, il a été associé à la chloroquine en prophylaxie. Progressivement, de plus en plus de souches sont devenues résistantes à la chloroquine et au métabolite actif du proguanil, le cycloguanil. Ces molécules agissent sur la voie des folates et inhibent la dihydrofolate déshydrogénase (DHFR) chez *P. falciparum*. Il est vraisemblable que des mutations sur le gène *dhfr* altèrent la structure de la protéine et diminuent son affinité pour la pyriméthamine et le cycloguanil conduisant à une résistance aux inhibiteurs de la DHFR [52][53]. L'enzyme de souches modérément et très résistantes à la pyriméthamine fixent de 15 à 500 fois moins la pyriméthamine que celle de souches sensibles. La mutation Ser108Asn (sérine à la place de l'asparagine sur le codon 108) sur *Pfdhfr* codant pour la DHFR est associée à la résistance de *P. falciparum* aux antifoliniques. Les mutations additionnelles Asn51 IIe, Cys59Arg ou IIe164Leu augmentent cette résistance, l'association des quatre mutations étant responsable du niveau le plus élevé de résistance aux antifoliniques et à l'association sulfadoxine/pyriméthamine. La triple mutation des codons 108, 51 et 59 est souvent observée en Afrique et en Asie chez les souches résistantes à la sulfadoxine/pyriméthamine. Cette triple mutation multiplie le risque de résistance *in vivo* à la sulfadoxine/pyriméthamine par 4,3 (OR ; IC95% : 3,0-6,3, méta-analyse de 16 études) [21]. Cette triple mutation est considérée comme le

meilleur facteur prédictif de la résistance *in vivo* à la sulfadoxine/pyriméthamine. La détection de la mutation Ser108Asn est quant à elle prédictive de la présence des deux autres mutations. En Amérique du sud, la mutation du codon 59 est moins fréquente et la mutation Cys50Arg la remplacerait. La mutation IIe164Leu est plus rare et peut être associée à des niveaux élevés de résistance à la sulfadoxine/pyriméthamine. Une autre mutation a été décrite sur le gène *pfmrp* qui pourrait être impliquée dans la sensibilité à l'association sulfadoxine/pyriméthamine [54]. La protéine du gène *pfmrp* serait un transporteur de folates et la forme mutée permettrait un efflux plus important du folate intraérythrocytaire, diminuant ainsi la compétition entre le folate et la pyriméthamine et une meilleure efficacité clinique de la sulfadoxine/pyriméthamine.

Parmi les antifoliques, les sulfones, représentés par la dapsone, et les sulfonamides ont été très utilisés pendant la seconde guerre mondiale puis nettement moins avec l'utilisation de la chloroquine et de la pyriméthamine. Le plus utilisé des sulfonamides en Afrique est la sulfadoxine en association avec la pyriméthamine. Les sulfones et les sulfonamides inhibent la dihydroptéroate synthétase (DHPS) de *P. falciparum* [55]. La DHPS est une enzyme de la voie des folates inhibée par la sulfadoxine et la dapsone dont elle est la cible moléculaire [52][53]. Ces composés, analogues de l'acide para-aminobenzoïque (PABA), agissent comme des inhibiteurs compétitifs de la DHPS. Les molécules agissant à deux niveaux de la même voie métabolique, elles génèrent un effet synergique lorsqu'elles sont associées [20][25]. L'analyse des séquences du gène de la DHPS montre des différences d'acides aminés entre les souches sensibles et résistantes à la sulfadoxine [56][57]. Les mutations Ser436Ala, Ser436Phe, Ala437Gly et Lys540Glu du gène *dhps* confèrent une résistance à la sulfadoxine [58][59]. En particulier, la mutation isolée Ala437Gly et la double mutation Ala437Gly + Lys540Glu sont respectivement associées à une multiplication du risque de résistance *in vivo* à l'association sulfadoxine / pyriméthamine. Les mutations Ala581Gly et Ala613Thr/Ser sont rares mais pourraient être associées à des degrés élevés de résistance. La combinaison de la triple mutation *dhfr* Ser108Asn + Asn51 IIe + Cys59Arg et de la double mutation *dhps* Ala437Gly + Lys540Glu (« quintuple mutation ») multiplie le risque de résistance *in vivo* à l'association sulfadoxine / pyriméthamine par 5,2 (OR ; IC95% :3,2-8,8, méta-analyse de 3 études) [21].

Enfin, les antibiotiques, parmi lesquels les tétracyclines, jouent un rôle non négligeable dans la prévention et le traitement du paludisme. La doxycycline est actuellement recommandée par l'OMS en prophylaxie dans les zones de multirésistance ; elle est également recommandée en deuxième ligne dans le traitement de l'accès simple à *P. falciparum* ou dans le traitement de l'accès grave en association à l'artésunate, la méfloquine ou la quinine. Les macrolides et certains dérivés tels que l'azithromycine présentent également une action antiplasmodiale en inhibant la synthèse protéique par altération de certaines fonctions de l'apicoplaste chez *P. falciparum*. Le rôle des antibiotiques dans la lutte contre le paludisme sera développé dans le chapitre suivant.

Malgré les efforts déployés pour la découverte de nouveaux médicaments antiplasmodiaux et la mise en place effective par les systèmes de santé de combinaisons thérapeutiques pour le traitement antipaludique, *P. falciparum* s'adapte en permanence et développe des résistances, y compris contre les combinaisons thérapeutiques à base d'artémisinine. Ceci s'explique par la grande diversité génétique de *P. falciparum* due à un taux élevé de mutations dans son génome et par les masses importantes de parasites portés par les individus infectés. La pression de sélection d'une molécule antipaludique conduit à la résistance, à l'image de ce qui se passe dans le monde bactérien.

Tableau 1. Synthèse des antipaludiques utilisés chez l'adulte

ANTIPALUDIQUE	PROPHYLAXIE	TRAITEMENT PALUDISME A *P. FALCIPARUM*	
		Accès simple	Accès grave
Amodiaquine / Flavoquine® * (> 6 ans)		X	
Artémether IM / Paluther® *			X
Artémether-luméfantrine / Riamet® Coartem® (pédiatrique) *		X	
Artésunate IV / Malacef® *			X
Artésunate-amodiaquine / AS AQ® Coarsucam® Arsucam® *		X	
Artésunate-méfloquine AS MQ® Artequin® *		X	
Artésunate-pyronaridine / Pyramax® (Corée) *		X	
Artésunate-sulfadoxine-pyriméthamine / Arsudar® *		X	
Atovaquone-proguanil / Malarone® *	X	X	
Chloroquine / Nivaquine® *	X	X	
Chloroquine-proguanil *	X		
Chlorproguanil - dapsone / Lapdap® *		X	
Dihydroartémisinine-pipéraquine / Eurartesim® *		X	
Doxycycline / Doxypalu® Vibraveineuse®	X		X
Halofantrine / Halfan® *		X	
Méfloquine / Lariam® *	X	X	
Méfloquine-sulfadoxine-pyriméthamine / Fansimef® (ASE) *		X	
Quinine / Quinimax® *	X	X	X (Référence)
Quinine + clindamycine (ASE et Amazonie) *		X	X
Quinine + doxycycline (ASE)		X	X
Sulfadoxine-pyriméthamine / Fansidar® *		X	

- * utilisable chez l'enfant à posologie adaptée
- ASE : Asie du Sud-est

Tableau 2. Synthèse des antipaludiques :
Classification en fonction de leur structure chimique

FAMILLE THERAPEUTIQUE		MOLECULE (DCI)
Quinoléines	Amino-4-quinoléines	Chloroquine, amodiaquine, pipéraquine
	Amino-8-quinoléines	Primaquine, tafénoquine
	Amino-alcools	Quinine, méfloquine, halofantrine, luméfantrine
Dérivés de l'artémisinine		Artémisinine, dihydroartémisinine, artéméther, artésunate
Hydroxynaphtoquinones		Atovaquone
Antimétaboliques	Antifoliniques	Proguanil, pyriméthamine
	Antifoliques	Sulfadoxine, dapsone
Antibiotiques		Tétracyclines, macrolides

Tableau 3. Synthèse des antipaludiques :
Classification en fonction de leurs propriétés pharmacologiques

PROPRIETE PHARMACOLOGIQUE	STRUCTURE CHIMIQUE	MOLECULE
Schizonticide érythrocytaire	**Amino-4-quinoléines**	Chloroquine, amodiaquine, pipéraquine
	Amino-alcools	Quinine, méfloquine, halofantrine, luméfantrine
	Sesquiterpènes	Artémisinine, dihydroartémisinine, artéméther, artésunate
	Antimétabolites	
	Antifoliques Antifoliniques Antibiotiques	Sulfadoxine, dapsone Proguanil, pyriméthamine Doxycycline, clindamycine
	Analogues de l'ubiquinone	Atovaquone
Schizonticide intra-hépatique	**Amino-8-quinoléines**	Primaquine, tafénoquine
	Antimétabolites	Proguanil, doxycycline
Gamétocytocides	**Amino-8-quinoléines**	Primaquine, tafénoquine

L'action synergique schizonticide de plusieurs molécules permet d'augmenter l'efficacité des médicaments antimalariques et de réduire l'acquisition de résistances, essentiellement de *P. falciparum*.

PREMIERE PARTIE :
ANTIBIOTIQUES ET PALUDISME

Les molécules utilisées dans la prophylaxie ou le traitement restent un axe majeur de recherche dans la lutte contre le paludisme et de nouveaux composés sont constamment développés. Le choix d'une molécule antipaludique est conditionné par le niveau de résistance de *Plasmodium falciparum* dans le pays de destination, ses contre-indications, sa tolérance et son coût financier. Parmi les composés potentiellement utilisables, certains antibiotiques ont été particulièrement étudiés, *in vivo* ou *in vitro*.

Les tétracyclines, famille d'antibiotiques à large spectre, sont actives sur les protozoaires y compris *Plasmodium*. L'émergence de la chloroquino-résistance a conduit à des études initiées par le Center of Disease Control (CDC) puis à la diffusion de recommandations par le World Health Organization (WHO) reposant sur l'utilisation de la doxycycline en prophylaxie du paludisme à *P. falciparum* en 1985. Cette molécule est actuellement préconisée en chimioprophylaxie dans les zones de multirésistance, particulièrement dans le sud-est asiatique, mais également en thérapeutique associée à la quinine. Elle est la molécule de choix de nombreuses armées et plus particulièrement celle utilisée par l'Armée Française lors des déploiements en zone de chloroquino-résistance. Des échecs de prophylaxie peuvent néanmoins survenir, essentiellement associés à des posologies insuffisantes et/ou une mauvaise observance. Les propriétés pharmacocinétiques de la doxycycline, et notamment sa demi-vie réduite, pourraient expliquer ces échecs. Mais de possibles résistances doivent être évoquées. Le premier cas clinique d'échec prophylactique a été publié.

La seconde famille d'antibiotiques candidate potentielle dans la lutte contre *Plasmodium* est la grande famille des macrolides et de ses dérivés, les lincosamides et les azalides. Les composés les plus récents : l'azithromycine et la clindamycine, ont été étudiés sur des isolats de différents continents. Ces molécules, dont le spectre d'action est similaire à celui des tétracyclines, ont l'avantage de pouvoir être administrés chez l'enfant et la femme enceinte.

D'autres antibiotiques, plus confidentiels, sont utilisés ou sont testés contre le paludisme.

Une revue de la littérature a été menée pour déterminer la place des antibiotiques dans la prophylaxie et le traitement du paludisme. Pour chaque famille, ont été développés les propriétés pharmacocinétiques, la tolérance, le mécanisme d'action de ces molécules et les mécanismes de résistance connus et publiés à ce jour.

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Tetracyclines in malaria

Tiphaine Gaillard[1,2,3], Marylin Madamet[2,4,5] and Bruno Pradines[1,2,5,6*]

Abstract

Malaria, a parasite vector-borne disease, is one of the greatest health threats in tropical regions, despite the availability of malaria chemoprophylaxis. The emergence and rapid extension of *Plasmodium falciparum* resistance to various anti-malarial drugs has gradually limited the number of potential malaria therapeutics available to clinicians. In this context, doxycycline, a synthetically derived tetracycline, constitutes an interesting alternative for malaria treatment and prophylaxis. Doxycycline is a slow-acting blood schizontocidal agent that is highly effective at preventing malaria. In areas with chloroquine and multidrug-resistant *P. falciparum* parasites, doxycycline has already been successfully used in combination with quinine to treat malaria, and it has been proven to be effective and well-tolerated. Although not recommended for pregnant women and children younger than 8 years of age, severe adverse effects are rarely reported. In addition, resistance to doxycycline is rarely described. Prophylactic and clinical failures of doxycycline have been associated with both inadequate doses and poor patient compliance. The effects of tetracyclines on parasites are not completely understood. A better comprehension of the mechanisms underlying drug resistance would facilitate the identification of molecular markers of resistance to predict and survey the emergence of resistance.

Keywords: Malaria, *Plasmodium falciparum*, Anti-malarial drug, Resistance, Tetracycline, Doxycycline, Prophylaxis, Treatment

Background

Malaria, a parasite vector-borne disease, is one of the greatest health threats in tropical regions, despite the availability of malaria chemoprophylaxis and the use of repellents and insecticide-treated nets. Malaria prophylaxis and chemotherapy remain a major focus of research, and new molecules are constantly being developed prior to the emergence of drug-resistant strains of the malaria parasite. The use of anti-malarial drugs is conditioned on the resistance level of *Plasmodium falciparum* in endemic areas, as well as the contraindications, clinical tolerance and financial costs of these drugs. Among the compounds potentially used against *Plasmodium*, antibiotics have been examined in vitro or in vivo.

Tetracyclines, a family of broad-spectrum antibiotics discovered in the early 1940s, are active in protozoa, including *Plasmodium*. In a small series of patients in 1950, tetracyclines were used to treat *P. falciparum* and *Plasmodium vivax* uncomplicated malaria. The emergence of chloroquine resistance in the 1960s led to studies conducted by the Centers for Disease Control and Prevention (CDC) and the development of the World Health Organization (WHO) recommendations that were based on the use of doxycycline for chemoprophylaxis of falciparum malaria in 1985. Currently, doxycycline is used in combination with quinine in treatment therapies and for chemoprophylaxis in multidrug resistance areas, particularly Southeast Asia. Finally, many armies use it as first-line chemoprophylaxis in areas with chloroquine resistance, including French military forces deployed in malaria-endemic areas. Since 2002, the French Army has, regrettably, had 3000 malaria cases. Recent deployments in Mali and Central African Republic showed high incidence rates, with a significant risk of contracting malaria for the 2000 soldiers. The attack rates were estimated at 7.5 % in 2013 and 12.5 % in 2014. These failures of prophylaxis with doxycycline are mainly associated with inadequate dosing or poor compliance. The pharmacokinetics of doxycycline, including a reduced half-life, may partly explain these failures; however, resistance phenomena may also be a factor.

*Correspondence: bruno.pradines@free.fr
[1] Unité de Parasitologie, Département d'Infectiologie de Terrain, Institut de Recherche Biomédicale des Armées, Marseille, France
Full list of author information is available at the end of the article

Classification

Tetracyclines are synthetic antibiotics derived from a cycline that is naturally produced by bacteria from the genus *Streptomyces* [1]. Tetracycline consists of three groups, based on pharmacological differences: the long-acting group, which includes doxycycline and minocycline, are the most active against *Plasmodium* in vitro. The antibiotic action, common to all tetracycline, is bacteriostatic and inhibits bacterial protein synthesis; their spectrum of activity is large [2].

Pharmacological properties

The pharmacokinetics properties of doxycycline have been investigated in numerous studies with healthy volunteers. One important property of doxycycline is its ability to be rapidly absorbed orally; it is detectable in the blood 15–30 min after its administration [3, 4]. After an oral dose of 200 mg, peak plasma levels are obtained in approximately 2 h; its half-life ranges from 15 to 25 h [5]. There are great individual variations, depending on the age of the patient and any coadministered substances [6]. Only one study of the pharmacokinetics of doxycycline was conducted during infections. It involved a case of uncomplicated malaria in combination with quinine or artesunate [7]. The authors concluded that there was a need for an initial dose of 400 mg twice daily to maintain plasma concentrations at therapeutic levels during the treatment for malaria infection.

Side effects and warnings against doxycycline

Tetracyclines are well known for their use in treating bacterial infections, and their adverse effects have been well documented [8, 9]. At the usual doses prescribed for malaria chemoprophylaxis, the published data are limited, and the reported adverse events vary widely. Comparative studies of the tolerance of doxycycline have been contradictory. Several retrospective studies of military teams have reported increased digestive and skin disorders and headaches with chemoprophylaxis [10–14]. A detailed analysis of studies reporting high numbers of side effects makes it possible to objectify pitfalls in the data interpretation: the dosage form is rarely specified and doxycycline is often co-administered with other substances, such as quinine. Thus, it is difficult to attribute an adverse event to cyclines only. In 1996 in sub-Saharan Africa, the French Army Health Service conducted an efficacy study of doxycycline hyclate salt versus chloroquine-proguanil [15].

Doxycycline hyclate was more efficacious than chloroquine-proguanil. However, with a 6 % withdrawal rate due to gastrointestinal side effects, it was considered to be unacceptable as chemoprophylaxis. The gastrointestinal side effects (e.g., diarrhoea and epigastralgia) were attributed to the hyclate salt acidity (pH 3) and the galenic form (capsule). According to the French Drug Agency recommendations, doxycycline hyclate has been replaced by doxycycline monohydrate, a less acidic salt (pH 6) with the same bioavailability [16]. Gastrointestinal side effects, mouth ulcers, and sun sensitization occurred less frequently in the doxycycline monohydrate group than in the chloroquine-proguanil group [17]. Fifty-seven per cent of deployed Australian soldiers using mefloquine prophylaxis in East Timor reported at least one adverse effect, compared to 56 % using doxycycline [18]. In Turkish troops deployed in Afghanistan, the total number of side effects in the doxycycline group was significantly higher than that in the mefloquine group [19]. However, among non-immune travellers to Sub-Saharan Africa, the total number of side effects in the doxycycline group was significantly lower compared with the chloroquine-proguanil or mefloquine groups [20].

The use of an antibiotic for several months for prophylaxis always triggers opposition from a number of bacteriologists, who note the risk of selecting resistant bacteria cyclines [21]. In 1988, a publication reported tetracycline-resistant cases of *Campylobacter jejuni* gastroenteritis among American soldiers based in Thailand [22]. A subsequent study by the same team showed that taking doxycycline for malaria prophylaxis resulted in less exposure to resistant bacteria than the acquisition of already resistant bacteria cyclines, which has long been widespread in this country [23]. The increase in multidrug-resistant gram-negative bacteria colonization among US military personnel in Afghanistan is likely due to environmental exposures rather than doxycycline exposure [24]. Methicillin-susceptible *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* colonization of military personnel under deployment was not associated with doxycycline exposure [25]. However, outbreaks of Panton-Valentine leukocidin-positive, doxycycline resistant, methicillin-susceptible *Staphylococcus aureus* infections associated with doxycycline prophylaxis have been reported in the French Army at the Ivory Coast [26]. Except for these military clinical cases, no study has been published about the risk of bacterial resistance to tetracyclines associated with their prophylaxis use. Doxycycline is contraindicated in patients with allergies to tetracyclines, pregnant women (from the second trimester of pregnancy due to the risk of abnormal tooth bud) and children under 8 years of age because of the risk of discolouration and enamel hypoplasia.

Mechanism of action

Cyclines are a family of antibiotics that act by inhibiting bacterial protein synthesis. Their mechanisms of action have been described at the molecular level [27]. Cyclines

act by binding to several proteins in the 30S ribosomal small subunit and to different ribonucleic acids in the 16S ribosomal RNA. Their mechanisms of action on *Plasmodium* have not been as well described, although a number of studies have addressed this issue. There are three categories of ribosomes in *Plasmodium*: mitochondrial, plastid and nuclear [28]. As suggested by three studies [29–31], tetracycline may directly inhibit mitochondrial protein synthesis and also decrease the activity of a mitochondrial enzyme (i.e., dihydroorotate dehydrogenase) involved in de novo pyrimidine synthesis [32]. Doxycycline inhibits the synthesis of nucleotides and deoxynucleotides in *P. falciparum* [33], but the concentration used (200 μM) is much higher than that used clinically. *In vitro* exposure of *P. falciparum* to minocycline also decreases the transcription of mitochondrial genes (subunit I of cytochrome c oxidase and apocytochrome b) and apicoplast genes (subunit rpoB/C of RNA polymerase), suggesting some activity with these two organelles [34]. A more recent study [35] has shown that doxycycline would specifically act on the apicoplast of *P. falciparum* and, to a lesser extent, on the mitochondrial whose division is inhibited at the end of the cycle; according to the authors, this finding could be attributed to the apicoplastic target (the two organelles present common metabolic pathways). The most recently published study confirms the action of doxycycline on the apicoplast in two stages, with an immediate toxic effect and a toxic effect (measurable after cell division): the first effect is considered to be non-specific, acting on collateral targets that are not located in the apicoplast; the second effect is characteristic of cell death, as observed after an offset effect on the apicoplast [36]. A proteomic approach confirmed the specific deregulation of the proteins involved in apicoplast metabolism after doxycycline treatment [37].

Antiplasmodial activities

Activity on sporogony

All studies of the antiplasmodial activity of doxycycline have shown that this molecule, at a dose of 100 mg daily, was a schizonticide agent, with a slow-acting duration [1]. The lack of an in vivo effect of tetracyclines on the development of gametocytes, suggested by Ruiz Sanchez [38, 39], was confirmed by a study performed in 1971 with healthy volunteers infected with *P. falciparum* or *P. vivax* and treated using tetracycline or doxycycline [40]. Tetracyclines have no effect on the sporogony in *Anopheles*: they do not reduce the infectivity of mosquitoes infected with gametocyte carriers under treatment [41].

Activity on hepatic forms

Several in vivo studies performed with simian models (rhesus monkeys and chimpanzees) infected by *Plasmodium cynomolgi bastianellii*, *P. vivax* or *P. cynomolgi ceylonensis* have shown that terramycin, minocycline or demeclocycline also affected their hepatic forms [42–44]. In a murine model, doxycycline also proved to be effective in the hepatic stages of *Plasmodium berghei* and *Plasmodium yoelii yoelii* [45], as the administration of 1.4 mg of doxycycline simultaneously or 3 h after the injection of sporozoites prevented the appearance of a parasitaemia in 100 % of the rodents (n = 10), while the untreated controls became infected.

However, the activity of doxycycline on the liver forms of *P. falciparum* was demonstrated to be partially effective in several studies of the hepatic forms of *P. falciparum* [46, 47]. Of the twelve subjects who received 100 mg of doxycycline per day for 3 days prior to exposure to infected mosquitoes and for the six following days, four developed malaria [46]. Moreover, the regular uptake of doxycycline did not alter the level of antibodies against the pre-erythrocytic stages of *P. falciparum* [48]. The findings of these studies have justified the recommendation of the currently approved doxycycline regimen (i.e., once daily for 4 weeks after returning from an endemic area).

Activity on erythrocytic forms

According to Geary et al. [49], cyclines are active during the three developmental asexual erythrocytic stages of *P. falciparum*, equivalently. According to Dahl et al. [35], the aged trophozoites and young schizonts were more susceptible to doxycycline than the young trophozoites and older schizonts, with a dose and time-dependent relationship observed for the effectiveness of the doxycycline on erythrocytic stages. The effectiveness of doxycycline on the erythrocytic stages is evaluated by identifying the concentration necessary to inhibit the growth of 50 % of the parasites, or the IC_{50} [50, 51]. When comparing the IC_{50} value of doxycycline to the values of other antimalarial drugs, which are sub-micromolar, doxycycline appears to be much less active. Considering its delayed onset of action [52, 53], this finding justifies its therapeutic use in combination with a fast schizonticide.

Clinical effectiveness

Among tetracyclines, doxycycline is the only one recommended as an anti-malarial prophylaxis [41]. In 1994, 34 years after its development, doxycycline was approved as prophylaxis against malaria by the Food and Drug Administration. In multidrug resistance zones, doxycycline is used as malaria chemoprophylaxis against *P. falciparum* at a dose of 100 mg/day starting at the day of arrival in endemic areas and continuing for up to 4 weeks after returning. This scheme was originally recommended by the WHO in 1985, based on the previously mentioned

studies [40, 41]. The primary studies (Table 1) of the efficacy and safety of doxycycline prophylaxis were performed with different populations living in endemic areas [54–58] and non-immune travellers, primarily soldiers from different armies [15, 47, 59]. Most of the failures observed in the prophylaxis of falciparum malaria were related either to inadequate dosages (confirmed by low plasma concentrations of doxycycline) [60], the use of half-doses [55] or poor adherence [59, 61–63]. True prophylactic failures (verified by plasma dosage of doxycycline) are rarely reported. Two Australian soldiers presented with falciparum malaria 2 weeks after returning from Papua New Guinea, despite good adherence [59]. *In vitro* chemosensitivity tests to doxycycline were not performed in these cases. However, the prophylaxis was stopped 3 days after returning from the endemic area; the recommendation is that prophylaxis should be continued 4 weeks after returning. There has been one recent report of the death of a French soldier due to a prophylaxis failure caused by doxycycline resistance [64]. Cyclines are inactive on hypnozoites. Indeed, the occurrence of malaria caused by *P. vivax* or *P. ovale* returning from an endemic area requires a radical cure with primaquine [65].

Doxycycline at a dose of 100 mg/day starting at the day of arrival in endemic areas and continuing for up to 4 weeks after returning, still remains highly effective as *P. falciparum* prophylaxis. Concerning the treatment of malaria, studies conducted in the 1950s [28, 39] and in 1970 [40, 41, 66, 67] have shown the effectiveness of cycline monotherapy in treating simple access to *P. falciparum*. Later, the need for a minimum 7-day treatment was demonstrated; the disappearance of parasites was effective only after 5 days at a dose of 200 mg daily [68].

With the risk of rapid progression from uncomplicated *Plasmodium falciparum* malaria to severe disease and the slow schizonticide action of the cyclines, they should not be used as monotherapy (Table 2). Their combination with other anti-malarial drugs has been studied many times, particularly in areas of multidrug resistance, such as Southeast Asia (Table 3) [69–73]. The most described associations are doxycycline (200 mg) with quinine (10 mg/kg/day) for 7 days, which operates with a therapeutic efficacy of 91–100 % in multi-resistant areas, even if the in vitro susceptibility of isolates to quinine is decreasing [74]. All other tested associations are lower or equal in terms of their efficacy, parasite clearance or resolution of fever, and they are often more expensive.

Due to its slow schizonticide action and short half-life, doxycycline should not be use in monotherapy in the treatment of uncomplicated malaria. Doxycycline remains still effective in combination with quinine or artesunate at a dose of 200 mg for 7 days.

Table 1 Efficacy of doxycycline for prophylaxis against *P. falciparum* malaria

Year	Place	References	Pop	Number	Drug	Route	Dose/d	Other drug	Duration/d	Efficacity
1987	Thailand	Pang [54]	C	95	D	PO	50 or 100 mg[a]	/	35	94.7
1988	Thailand	Pang [55]	C	67	D	PO	50 or 100 mg[a]	/	97	97.0
1988	Thailand	Pang [55]	C	77	D	PO	25 or 50 mg[a]	/	107	97.4
1989	Thailand	Watanasook [56]	A	243	D	PO	50 mg	/	119	92.6
1989	Thailand	Watanasook [56]	A	243	D	PO	100 mg	/	119	84.4
1992	Thailand	Shanks [57]	A	77	D	PO	100 mg	/	80	96.1
1993	New Guinea	Rieckmann [47]	A	60	D	PO	100 mg	/	42	100
1993	New Guinea	Rieckmann [47]	A	69	D	PO	100 mg	PR	21	100
1993	New Guinea	Rieckmann [47]	A	125	D	PO	50 mg	CQ	91	100
1995	Kenya	Weiss [60]	C	32	D	PO	50 mg	/	77	84
1995	Somalia	Shanks [63]	A	900	D	PO	100 mg	/	135	99.9
1995	Cambodia	Shanks [63]	A	600	D	PO	100 mg	CQ	195	99.7
1995	New Guinea	Shanks [59]	A	53	D	PO	100 mg	PR	42	96.2
1997	Irian Jaya	Ohrt [103]	A	67	D	PO	100 mg	/	87	99
1998	Kenya	Andersen [104]	A	70	D	PO	100 mg	/	70	92.6
1999	Irian Jaya	Taylor [58]	A	75	D	PO	100 mg	/	140	96.3
1999	Gabon +CAR	Baudon [15]	A	171	D	PO	100 mg	/	150	97.1
1999	Ethiopia	Schwartz [105]	A	19	D	PO	100 mg	/	/	94.7
2002	Eastern Timor	Peragallo [106]	A	280	D	PO	100 mg	PR	168	98.4
2005	Afghanistan	Sonmez [19]	A	986	D	PO	100 mg	/	84	100

Pop population, *A* adults, *C* children, *D* doxycycline, *CQ* chloroquine, *PR* primaquine

[a] According to weight (< or >40 kg)

Table 2 Clinical trials of doxycycline monotherapy against *P. falciparum* malaria

Study demographic details					Regimen				
Year	Place	References	Population	Nb	Dosage/d	Nb doses/d	Route	Nb days	Efficacy (%)
1971	USA	Clyde [107]	A	4	200 mg	2	PO	4	NR
1971	USA	Clyde [107]	A	9	200 mg	2	PO	7	NR
1981	West Malaysia	Ponnampalam [68]	C	9	4 mg/kg	NR	PO	4	44.4
1981	West Malaysia	Ponnampalam [68]	C	26	4 mg/kg	NR	PO	7	84.6
2001	Indonesia	Taylor [108]	A	20	200 mg	2	PO	7	64.7

A adults, *C* children, *NR* not reported

Mechanism of resistance to doxycycline

The notion of *P. falciparum* resistance to doxycycline is a tricky concept to grasp. Treatment failures reported with quinine plus doxycycline are rare events. The only drug pressure with cycline on *Plasmodium* was performed in a murine model of *Plasmodium berghei* [75]. The administration of increasing doses of minocycline to mice infected with 1×10^7 parasites for 86 successive passages over 600 days made it possible to obtain a resistant *P. berghei* strain, with a median drug inhibitory concentration (IC_{50}) of 600 mg/kg/day, which is sixfold higher than that of the susceptible starting strain (100 mg/kg/day).

In addition, few studies have evaluated the *P. falciparum* in vitro susceptibility to doxycycline. However, several studies of isolates from different continents have established different groups of in vitro susceptibility based on IC_{50} doxycycline assessments. But, in the absence of standardized ex vivo and in vitro tests, it is difficult to compare data from different laboratories. Indeed, IC_{50} values and cut-off for in vitro resistance are specific to the methodology. For example, the in vitro effects and the IC_{50} values for doxycycline are dependent upon the time incubation conditions [52, 53], gas conditions (i.e., O_2 and CO_2 [76, 77] and methodology (i.e., an isotopic test versus an immunoenzymatic test) [78]. These differences in methodology must be taken account for comparing and analysing resistance data from different works.

A 2010 publication, with reported values of doxycycline IC_{50} on 747 isolates of *P. falciparum* in Africa over a period of 9 years (1996–2005), found a trimodal distribution of IC_{50} with three susceptibility levels identified [79]. Nine isolates (1.2 %) exceeded the threshold of 35 µM identifying isolates, with reduced susceptibility to doxycycline. Another evaluation on 484 isolates of imported *P. falciparum* parasites between 2006 and 2010, based on the same methodology, showed that 2.7 % had reduced susceptibility to doxycycline [80]. In a study published in 2013, on 113 isolates from Senegal, 9 (8.0 %) isolates exhibited IC_{50} over the limit of 35 µM [81]. In 2009–2010 and 2010–2011, 12 and 10.3 % of *P. falciparum* isolates collected in Dakar showed reduced susceptibility to doxycycline in comparable methodology (cut off of 37 µM) [78, 82]. A study in Kenya showed that 15 % of the isolates had an IC_{50} >35 µM [83]. A recent study on 620 Thai isolates found a bimodal distribution [84]. The two groups identified presented with a mean value of 13.15 µM for the group of 591 isolates with low IC_{50} and a mean value of 31.60 µM for the group of high IC_{50}, including 29 isolates. Only seven isolates of 620 (1.1 %) had doxycycline IC_{50} values that were superior to 35 µM. In 2008, a study performed in French Guiana investigated the prevalence of isolates with reduced susceptibility to doxycycline and found from 15 to 25 % of the isolates from 1996 to 2001, 51 % in 2002, to 61.5 % in 2003 and to more than 67 % in 2005 [50]. The low threshold of susceptibility of 9.6 µM chosen can explain this high level of in vitro resistance. As the methodology is the same as that subsequently used, the prevalence of reduced susceptibility can be recalculated with a cut off at 35 µM: the prevalences ranged from 0 to 4.8 % (0 % in 1997, 1999, 2000, 2003 and 2004, 1.8 % in 1998, 4.8 % in 2001, 2.2 % in 2002 and 1.9 % in 2005). A Ghanaian study performed in 2012 recorded a surprisingly high level of resistance (i.e., 23.7 %) for doxycycline [85], with a threshold IC_{50} value of 35 µM. This finding could be explained by the use of SYBR Green 1-based in vitro test applied to assess the susceptibility of clinical isolates. Indeed, Wein et al. demonstrated that doxycycline IC_{50} values were significantly higher in fluorescence-based SYBR green assays than in isotopic or HRP2-based tests [86]. However, despite the lack of standardization for the evaluation of doxycycline IC_{50}, the existence of a high IC_{50} group is indisputable.

The search on the potential mechanisms of the resistance of *P. falciparum* to doxycycline focuses on two ways: the exploration of plasmodial genes homologue to bacterial genes that are involved in bacterial resistance to doxycycline and the exploration of genes coding apicoplastic proteins which could be targets for doxycycline. Different hypotheses have been published

Table 3 Clinical trials of cycline plus other drug against *P. falciparum* malaria

Study demographic details					Regimen								Efficacy (%)
Year	Place	References	Pop	Nb	Cycline			Other Drug			Route	Days	
					Dosage/day	Drug	Nb doses/day	Drug	Dosage/day	Nb doses/day			
1972	Thailand	Colwell [109]	A	30	1000 mg	T	4	Q	1920 mg	3	PO	3 + 10	96.7
1973	Thailand	Chin [69]	A	12	NR	T	NR	Q	NR	NR	PO	NR	66.7
1973	Thailand	Chin [69]	A	13	NR	T	NR	QPy	NR	NR	PO	NR	66.7
1973	Thailand	Colwell [70]	A	32	NR	T	NR	Q	NR	NR	PO	NR	84
1983	Thailand	Noeypatimanond [71]	AC	51	NR	T	NR	Am	NR	NR	PO	NR	96
1988	Cambodia	Giboda [110]	A	22	1500 mg	T	3	Q	1500 mg	3	PO	7/10	100
1994	Thailand	Looreesuwan [111]	A	50	1000 mg	T	4	M	1250 mg	2	PO	7/1	94
1994	Thailand	Looreesuwan [111]	A	52	1000 mg	T	4	Q	1800 mg	3	PO	7	98
1994	Thailand	Looreesuwan [112]	A	54	200 mg	D	1	M	1250 mg	2	PO	7/1	96
1994	Thailand	Looreesuwan [112]	A	55	200 mg	D	1	A	100 mg	2	PO	7/2.5	80
1995	Gabon	Metzger [113]	A	35	4 mg/kg	D	2	Q	24 mg/kg	1	PO	3/1.5	91
1996	Thailand	Na-Bangchang [114]	A	30	200 mg	D	2	A	400 mg	2	PO	5/1	53.3
1996	Thailand	Looreesuwan [115]	A	25	1000 mg	T	4	At	2250 mg	3	PO	7/1.3	100
1996	Thailand	Looreesuwan [115]	A	22	200 mg	D	2	A	1000 mg	2	PO	3	91
1996	Brazil	Duarte [116]	AC	88	1500 mg	T	3	A	100 mg	2	PO	7	80
1996	Brazil	Duarte [116]	AC	88	1500 mg	T	3	Q	2000 mg	2	PO	7/3	77
1996	Thailand	Bunnag [117]	A	46	1000 mg	T	4	Q	1800 mg	3	PO	5	87
1996	Thailand	Bunnag [117]	A	40	1000 mg	T	4	Q	1800 mg	3	PO	7	100
2000	Thailand	Pukrittayakamee [118]	AC	68	16 mg/kg	T	4	Q	30 mg/kg	3	PO	7	98
2001	Indonesia	Taylor [108]	A	39	200 mg	D	2	CQ	STD	1	PO	7/3	90.9
2004	Thailand	Pukrittayakamee [72]	A	30	16 mg/kg	T	4	Q	30 mg/kg	3	PO	7	100
2006	Brazil	Alecrim [74]	A	31	200 mg	D	2	Q	1500 mg	3	PO	3 + 2	100
2007	Pakistan	Ejaz [119]	A	100	200 mg	D	2	Q	30 mg/kg	3	PO	3 + 4	100

Pop population, *A* adult, *C* children, *P* pregnant women, *A* artesunate, *Am* amodiaquine, *At* atovaquone, *C* chloroquine, *D* doxycydine, *M* mefloquine, *Q* quinine, *Py* pyrimethamine, *T* tetracycline, *STD* standard, i.e. 10 mg/kg on day 0 and 1 and 5 mg/kg on day 2, *NR* not reported

regarding the potential mechanisms of the resistance of *P. falciparum* to doxycycline correlated to the bacterial world. Several mechanisms of bacterial resistance to the cyclines have been identified [21]: (1) *tet* efflux protein genes encode for membrane-associated proteins that export tetracycline from the cell, reducing the intracellular drug concentration and thus protecting the ribosomes [87]; (2) TetX protein, a flavin-dependent monooxygenase, degrades tetracycline in vitro and in vivo [88]; and (3) ribosomal protection proteins in the cytoplasm protect ribosomes from the action of tetracycline in a GTP-dependent manner [89, 90]. Analogues of these proteins have been identified in *P. falciparum* [91]. Sequence analysis of 11 genes (*pftufA*, *pfEF-TS*, *pfmdt*, *pftetQ*, *pfrps3*, *pfrps7*, *pfrps8*, *pfrps9*, *pfrps11*, *pfrps14*, and *pfrps17*) and evaluation of *pfmdt* and *pftetQ* copy numbers were conducted using 90 isolates from 14 African countries [51]. It has been demonstrated that no polymorphism was found in a small subunit of apicoplastic ribosomal genes (*pfrps7*, *pfrps9*, and *pfrps17*, although S7, S9, and S17) and that the copy number increases of two genes, *P. falciparum* metabolite drug transporter gene (*pfmdt*, PFE0825w), a membrane transporter with similarities to the bacterial efflux pumps, and *P. falciparum* GTPase TetQ gene (*pfTetQ*, PFL1710c), similar to the bacterial ribosomal protein TetA involved in tetracycline resistance, were associated with reduced susceptibility to doxycycline in *P. falciparum* [51]. The number of parasites that is classed as in vitro resistant is very small, and unfortunately, that means that small random changes may be associated without being causal. However, this association was later confirmed using 89 African imported isolates [80]. In addition, PfTetQ KYNNNN motif repeats of <3 are predictive of in vitro resistant *P. falciparum* parasites with IC_{50} >35 µM (odds ratio 15) [83]. The involvement of the copy numbers of *pfmdt* and the PfTetQ KYNNNN motif repeats in reduced susceptibility to doxycycline was confirmed by the doxycycline prophylactic failure from the Central African Republic (i.e., the doxycycline failure in a compliant patient, as confirmed by a statement of correct intake of doxycycline and the presence of an expected plasmatic concentration of doxycycline), which was associated with two copies of the *Pfmdt* gene, as well as the two KYNNNN motif repeats [64]. However, these molecular markers were certainly not the only involved in cases of reduced susceptibility to doxycycline. A study of Senegalese isolates showed a lack of association between the number of copies of *pfmdt* and *pftetQ* and high IC_{50} for doxycycline, essentially because of an insufficient number of isolates with high IC_{50} [81]. There was an absence of association between the number of copies of *pfmdt* and *pftetQ* or the polymorphisms on *pftetQ* and susceptibility to doxycycline in *P. falciparum* isolates from Thailand and French Guiana [84, 92]. Copy number of *pfmdt* and *pftetQ* and polymorphisms on *pftetQ* are not sufficient to explain reduced susceptibility to doxycycline, which may be multigenic.

Other hypotheses were explored. Through homology with the bacterial world, the exploration of new apicoplast genes has been performed, and in particular, the association between the polymorphism of the small subunit ribosomal RNA gene, *pfssrRNA*, and in vitro susceptibility to doxycycline was investigated [93]. In *Helicobacter pylori*, tetracycline resistance has not been associated with efflux or ribosomal protection proteins; instead, it was attributed to mutations in the 16S rRNA-encoding genes that affect the binding site of tetracycline [94, 95]. Tetracycline resistance mediated by mutations in the 16S rRNA was first found in *Propionibacterium acnes*, and a mutation from G to C was reported at position 1058 (*Escherichia coli* numbering) in their 16S rRNA genes [96]. A triplet mutation in the same 16S rRNA domain (965–967; *E. coli* numbering) was also found [90, 95, 97, 98]. Because the apicoplast contains an independent genome, encoding prokaryote-like RNA polymerase subunits, 70S ribosomal subunits, tRNAs and a small number of proteins [99], it was interesting to investigate the mechanism of bacterial resistance of *P. falciparum* to doxycycline. Moreover, comparative analyses of the *P. falciparum* genome revealed that the nucleic acid sequence of a small subunit of ribosomal RNA gene belonging to the apicoplast shares 58 and 62 % of their identities with the 16S rRNA gene from *Propionibacterium acnes* and *Helicobacter pylori*, respectively. However, the sequencing of the small subunit ribosomal RNA gene (PFC10_API0057) in *P. falciparum* African and Thai isolates did not reveal any mutation, regardless of the determined IC_{50} values [93].

Another hypothesis to be explored is the role of plasmodial apicoplast genes, that bacterial homologues are not involved in bacterial resistance to doxycycline, such as *arps10*, could be involved in artemisinin resistance [100] by encoding the apicoplast ribosomal protein S10 precursor, as well as *fd*, by encoding the ferredoxin protein, a key component of the apicoplast electron transport chain. These apicoplast genes could also be involved in the decreased susceptibility of *P. falciparum* to doxycycline because of doxycycline mode of action.

However, the better way to identify the potential genes involved in reduced susceptibility to doxycycline is to create in vitro resistant parasites in cultivation by drug pressure and then to sequence and analyse the whole genome of the both original susceptible strain and resistant strain as it was successfully previously done for the artemisinin resistance [101, 102].

Conclusions

The emergence and rapid extension of *P. falciparum* resistance to principal anti-malarial drugs necessitates the search for new molecules. In addition, doxycycline (in combination with quinine) is an excellent candidate for the treatment of uncomplicated malaria and as prophylaxis in multi-resistant areas. The adequate tolerance and efficacy of cyclines have been demonstrated. A better comprehension of the mechanisms of action and resistance would facilitate the design of more effective structural analogues and the identification of molecular markers of resistance to predict and survey the emergence of resistance.

Authors' contributions

TG, MM and BP drafted the manuscript. All authors read and approved the final manuscript.

Author details

[1] Unité de Parasitologie, Département d'Infectiologie de Terrain, Institut de Recherche Biomédicale des Armées, Marseille, France. [2] Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Aix Marseille Université, Marseille, France. [3] Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, France. [4] Equipe Résidente de Recherche en Infectiologie Tropicale, Institut de Recherche Biomédicale des Armées, Hôpital d'Instruction des Armées, Marseille, France. [5] Centre National de Référence du Paludisme, Marseille, France. [6] Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France.

Competing interests

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REVUE II : MACROLIDES AND DERIVATIVES

Malaria Journal

Macrolides in malaria

--Manuscript Draft--

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Abstract:	Malaria, a parasite vector-borne disease, is one of the biggest health threats in tropical regions,despite the availability of malaria chemoprophylaxis. The emergence and rapid extension of Plasmodium falciparum resistance to various antimalarial drugs has gradually limited the potential malaria therapeutics available to clinicians. In this context, macrolides constitute an interesting alternative. These molecules, with an action spectrum similar to that of tetracyclines, have been administered to children and pregnant women. Azithromycin and clindamycin are effective and well tolerated in the treatment of uncomplicated malaria in combination with quinine. Until recently, few clinical failures resulting from resistance have been described. The effects of macrolides on parasites are not completely understood.
Corresponding Author:	Bruno Pradines, PhamrD, PhD Institut de recherche biomedicale des armees Brétigny sur Orge, FRANCE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Institut de recherche biomedicale des armees
Corresponding Author's Secondary Institution:	
First Author:	Thiphaine Gaillard, PharmD
First Author Secondary Information:	
Order of Authors:	Thiphaine Gaillard, PharmD
	Jérome Dormoi, PhD
	Marylin Madamet, PhD
	Bruno Pradines, PhamrD, PhD
Order of Authors Secondary Information:	

Background

Malaria, a parasite vector-borne disease, is one of the largest health threats in tropical regions, despite the availability of malaria chemoprophylaxis and the use of repellents and insecticide-treated nets. The prophylaxis and chemotherapy of malaria remains a major area of malaria research, and new molecules are constantly being developed prior to the emergence of resistant parasite strains. The use of antimalarial drugs is conditioned based on the level of resistance of *Plasmodium falciparum* in endemic areas and the contraindications, clinical tolerance and financial costs of these drugs. Among the compounds potentially used against *Plasmodium*, antibiotics have been examined *in vitro* or *in vivo*.

The second family of potential antibiotics in the fight against *Plasmodium* includes macrolides and macrolide derivatives, lincosamides and azalides. As the former molecules of this family are unattractive with respect to pharmacokinetic properties, recent studies have examine the effects of other compounds, azithromycin and clindamycin, on isolates from different continents. These molecules, whose action spectrum is similar to that of tetracyclines, are typically administered to children and pregnant women.

This literature review assesses the roles of macrolides and macrolide derivatives in the prophylaxis and treatment of malaria.

Classification of macrolides

The macrolide family comprises lincosamides and streptogramines, referred to as the MLSB group. These antibiotics, with a distinct chemical structure, are classified in the

same group based on comparable activity spectra and identical functional mechanism, based on the inhibition of protein synthesis. These antibiotics, with limited activity spectra, are particularly active against the intracellular germs and are currently administered to children and pregnant women, conferring a notable advantage compared with tetracyclines. Certain antibiotics of this family are useful as anti-malarial substances.

The classification of macrolides is based on the number of carbon links, which distinguishes macrolides with 14 atoms from carbon, and erythromycin is the oldest molecule (1952) identified, followed by roxithromycin, clarithromycin and dirithromycin. All second-generation macrolides are products of hemisynthesis are derived from erythromycin, synthesized through chemical modifications. The only azalide with 15 carbon atoms is azithromycin, produced through the introduction of a nitrogen atom inserted into the macrolide nucleus at C10. This modification improves the penetration of drugs into macrophages, fibroblasts and polymorphonuclear neutrophils, facilitating increased accumulation within acidified vacuoles and extending the half-life [1]. The antibiotics with 16 carbon links are spiramycin and josamycin. Chemical modifications are constantly being developed to optimize this family [2].

Lincosamides are antibiotics associated with the macrolides based on a similar action spectrum, although the structure of these compounds differs. Lincomycin is a sugar isolated in 1962 from fermentation through *Streptomyces lincolnensis*. Substitution of the C7 bearing a hydroxyl function with a chlorine atom generated a semi-synthetic derivative, such as 7-chloro-7-deoxy-lincomycin or clindamycin, with a higher antibiotic activity and digestive absorption.

Pharmacological properties

Macrolides exhibit poor digestive absorption of less than 60%, and this effect is strongly influenced by food. The half-life of these drugs is variable, from a short half-life for erythromycin (2 hours) and clarithromycin (4 hours) to long half-life for azithromycin (68 hours) [3]. Efforts for development of molecules of this family were first employed to improve pharmacological properties, but not antimicrobial activities.

With the exception of cerebrospinal fluid and brain, macrolides show excellent tissue distribution in tissues, such as bone and liver. Erythromycin and clarithromycin are highly metabolized in the liver through interactions with cytochrome P450 CYP3A4, which has been implicated in many drug interactions. Azithromycin is a slightly metabolized molecule, with 37% bioavailability after oral administration [4]. This drug accumulates in hepatic, renal, pulmonary and splenic tissues and gradually leaches into the bloodstream over a period of one week [5]. The elimination of macrolide and macrolide derivatives is primarily biliary. Indeed, liver failure severely disrupts pharmacokinetics. Concerning azithromycin, mild renal dysfunction and mild-to-moderate hepatic dysfunction do not significantly affect excretion.

Among lincosamides, clindamycin has a 90% digestive absorption. Unlike lincomycin, clindamycin absorption is not reduced, but only delayed, through food intake. The half-life of these molecules remains short, on the order of 4 to 6 hours. These compounds exhibit good tissue penetration, and contrary to macrolides, pass the blood-brain barrier. Clindamycin metabolism occurs in the liver, with elimination and high bile concentration. In hepatic insufficiency, the half-life can be extended twice and doses should be reduced accordingly.

With respect to the effect on *Plasmodium*, clindamycin slowly accumulates in parasites [6]. The accumulated intracytoplasmic concentration reaches a level beyond 0.01 g/ml after exposure for at least 72 hours [7], reflecting the delayed onset of activity *in vivo*.

Tolerance

Macrolides are generally well tolerated. The adverse events most frequently reported are gastrointestinal disorders: nausea, vomiting, and episgastralgia associated with the administered dose. Other side effects, such as neurosensory in the type of headache and dizziness disorders, skin allergies and rare cases of cholestatic hepatitis, have been reported, but these effects are rare. The "old" molecules typically present more side effects than the newer molecules, but gastrointestinal disorders are frequently reported in patients receiving azithromycin. Doses of azithromycin between 500 and 2,000 mg have been used in all trimesters of human pregnancy for the treatment of upper and lower respiratory tract infections, skin diseases, and infections with *Chlamydia trachomatis*, *Mycoplasma* and group B streptococci among women allergic to other antibiotics [1]. Macrolides are largely a problem of drug interference, reflecting the role of cytochrome. Notably, although the risk of drug interactions is high, the risk of interactions with new molecules is much less important.Nevertheless, azithromycin delays cardiac polarization [8,9], although preliminary studies concerning the combination of azithromycin with chloroquine for QT prolongation indicate that cardiac instability is not increased under this combination [10].

The oral forms (esophagitis) of lincosamides exhibit a more irritative effect than the parenteral forms (chemically induced phlebitis). Systemic reactions, including allergies, skin reactions and anaphylactic shock, have been reported. Diarrhoea and digestive

disorders primarily occur with the oral forms. Moreover, the appearance of pseudomembranous colitis resulting from *Clostridium difficile* toxin selection is characterized by profuse watery diarrhoea, fever, and occasional bleeding, requiring the discontinuation of treatment. Moreover, rapid intravenous administration might reflect the electrocardiographic changes and even collapse of cardiac arrest observed in response to lincosamide treatments. Haematologic disorders, such as leukopenia, neutropenia, and thrombocytopenia have been reported.

Drug interactions are less frequently observed with lincosamides than with macrolides.

Mechanism of antiplasmodial action

Macrolides inhibit the synthesis of cell proteins through binding to the 50S subunit of the ribosome. The inhibition of protein synthesis through the inhibition of transpeptidation explains the postantibiotic effects of this drug, measured after 3-4 hours. The macrolide antibacterial spectrum is similar to that of erythromycin. This spectrum is limited to Gram-positive bacteria, and Gram-negative bacilli remain impermeable to these molecules; however, because of the intracellular concentration of these drugs, macrolides are active against intracellular bacteria development [11].

The macrolide antibiotic azithromycin exhibits the best antiplasmodial properties. This molecule targets the 70S ribosomal subunit from the apical complex [5], comprising 50S and 30S subunits. Once fixed, macrolide prevents the synthesis of the polypeptide, which is subsequently prematurely released [1]. The synthesis of a nonfunctional apicoplast, resulting from exposure to azithromycin, is at the origin of the delayed effect of the molecule. The apicoplast is limited by four membranes and located within parasitic cells;

this structure contains a circular DNA equivalent to the bacterial molecular machinery [5] for DNA replication, RNA transcription and RNA-protein translation [12]. Thus, similar to tetracyclines, the antiplasmodial action of this macrolide is therefore delayed [13,14]; the parasite completes a full cycle before achieving growth inhibition, a phenomenon referred to as "shifted cell death". Shifted cell death is a strategy for examining whether an antibiotic acts on the apicoplast, and unlike antiparasitic molecules with immediate effects, the activity of antiparasitic compounds on some functions of the apicoplast is measurable beyond cell division. Several studies have also identified the immediate activity of azithromycin [15-17], well above that of older macrolides. The mechanism responsible for this activity has not been elucidated [2].

The target of clindamycin has been recently demonstrated in *Plasmodium*. This drug was originally extrapolated from *Toxoplasma gondii*, frequently used as a model based on structural similarities [18]. In *T. gondii*, clindamycin and the three major clindamycin metabolites are fixed to the large subunit ribosomal RNA of the apicoplast [19]. Several studies have shown a lethal effect of clindamycin on potentiated parasites after 72 hours of exposure [20], although the antibiotic concentrations were reduced 3 to 4 factors less than the IC_{50}.

It has also been suggested that parasites exposed to clindamycin divide and invade new host cells, but at this point, the cells are unable to grow and eventually perish. These results, prior to an in-depth study of the apicoplast, revealed the toxicity of clindamycin on a structure involved in the translation of plasmodial ribosomal RNA into protein [21]. These findings contributed to the antiplasmodial action of clindamycin after 3 days of administration [7]. In 2005, the target of clindamycin was identified [22]. Clindamycin binds the 50S subunit, comprising ribosomal 23S, 5S and ribosomal proteins L4 and L22.

The same mode of action was described for azithromycin two years later. *P. falciparum* ribosomal protein L4 (PfRpl4) has been demonstrated to associate with the nuclear genome-encoded *P. falciparum* ribosomal protein L22 (PfRpl22) and the large subunit rRNA 23S to form the 50S ribosome polypeptide exit tunnel, which could be occupied by azithromycin [5].

Clinical effectiveness

Because these molecules present a short half-life, the use of the oldest macrolides is limited for antimalarial treatment. The best-studied antiplasmodial molecules include azithromycin, for which chemical modifications significantly increase the half-life, and clindamycin.

The antiplasmodial action of azithromycin was first described *in vitro* at the beginning of the 90's [23,24]. At the end of the 90's, the mass distribution of azithromycin through the World Health Organization (WHO) trachoma elimination programme was shown to reduce malarial parasitaemia [25]. Several studies concerning the antiparasitic properties of antibiotics showed the delayed action of the molecule [5,13,14]. Only one clinical multicentre study of azithromycin for the treatment of acute uncomplicated *P. falciparum* malaria was conducted in India on 15 participants. In this study, patients were randomly assigned to groups treated with either azithromycin or chloroquine alone, or azithromycin associated with chloroquine [26]. The resolution of parasitaemia was inadequate with monotherapy with either azithromycin or chloroquine, but combination therapy provided substantially improved clinical and parasitological outcomes. The delayed resolution of parasitaemia, including the lack of the penetration of azithromycin into the parasite

confirmed that this drug was unsuitable for monotherapy treatment. In addition, different associations were tested *in vitro* (Table 1).

The effects of associations, such as azithromycin-chloroquine and azithromycin-quinine, were additive on sensitive chloroquine strains and synergistic on resistant strains [27]. Other associations were examined, showing effectiveness, associating azithromycin with a rapidly acting schizonticidal compounds, such as halofantrine or artemisinin [24,28]. Two *in vitro* studies [27,29] suggested that the dihydroartemisinin-azithromycin combination had antagonistic effects and should be avoided. An *in vivo* study conducted in Thailand [30], a geographic area with high levels of resistance to antimalarial drugs, showed that azithromycin-artesunate, even when administered only once daily for 3 days, and azithromycin-quinine, administered 3 times daily, are safe and efficacious combination treatments for uncomplicated falciparum malaria. A randomized controlled trial performed in Tanzanian children did not support the use of azithromycin-artesunate as treatment for malaria; indeed, the 58% parasitological failure rate observed after day 28 clearly showed that this treatment could not be an appropriate first line treatment for malaria [31]. One clinical trial conducted in Bangladesh performed on 152 patients suggested that this combination was an efficacious and well-tolerated treatment for patients with uncomplicated falciparum malaria compared with the artemether-lumefantrine combination [32]. This study did not consider the re-emergence of parasites in the peripheral blood as a failure of the treatment, although the mean time was 31.5 ± 5 days. Moreover, these authors did not distinguish the study group according to the age of the patients and mixed children and adults for the data integration.

The efficacy of the azithromycin-quinine combination was confirmed in 2006 [33] when 100% of the patients were cured through high azithromycin regimens (combination of

quinine with 1000 mg of azithromycin per day for 5 days or 1500 mg of azithromycin for 3 days).

A longitudinal trial comparing the effects of chloroquine as a monotherapy or in combination with other drugs, including azithromycin, on children with repeated malaria infections in Malawi demonstrated a high efficacy of the repeated administration of different regimens and showed a significantly higher haemoglobin concentration in children in the chloroquine-azithromycin group. This result might reflect the prevention or treatment of bacterial infections [34]. This combination, chloroquine-azithromycin was recently confirmed as highly efficient and well tolerated in African adults [35].

Another combination treatment comprising azithromycin with sulphadoxine-pyrimethamine was tested in pregnant women from Malawi [36]. Sulphadoxine-pyrimethamine has been adopted in many sub-Saharan Africa countries as the drug of choice for intermittent preventive therapy to reduce placental malaria and low-birth weight. The azithromycin-sulphadoxine-pyrimethamine combination might have several advantages: first, although the parasite clearance rate was slow compared with sulphadoxine-pyrimethamine-artesunate, the rate of recrudescence was low and markedly similar between the two groups. Secondly, azithromycin has an adequate safety profile, as this molecule has often been used in pregnant women to treat STIs. In contrast, there has been concern about the use of artemisinin derivatives during the first trimester based on animal studies [37]. Thirdly, azithromycin has a relatively long half-life compared with artesunate. The azithromycin-sulphadoxine-pyrimethamine combination protects the longer-acting drug (sulphadoxine-pyrimethamine), increasing the probability of parasites encountering sub-therapeutic drug levels and promoting the development of resistance [38].

The clindamycin is a major antibiotic for the treatment of anaerobic bacterial infections [39]. This drug also presents antimicrobial activity against *Plasmodium*, *Toxoplasma*, *Babesia* and *Pneumocystis spp*. Moreover, clindamycin is the drug of choice for treatment against toxoplasmic chorioretinitis in newborns and one of the treatments recommended in the babesiosis with *B. microti* and *B. divergens* [40]. Associated with pyrimethamine or primaquine, clindamycin is a treatment of second intention against toxoplasmosis and pneumocystosis [41].

The antiplasmodial indication of clindamycin was managed according to various therapeutic regimens. The effectiveness of clindamycin in monotherapy in this indication was initially reported in 1975 [42]. The WHO repeated this protocol in several studies conducted on different continents, and several sightings have been reported (Table 2), including the effectiveness of clindamycin in monotherapy against malaria. This efficiency is however conditioned through treatment for 5 days, with twice-daily administration, as this molecule acts slowly. Clindamycin is well tolerated, and minor side effects have been reported during treatment. The occurrence of diarrhoea resulting from *Clostridium difficile* has often been reported after treatment with clindamycin, and this side effect might progress to pseudomembranous colitis, as a result of lengthy treatment with antibiotics [43]. The potential problem of severe diarrhoea, observed in patients receiving a prolonged and high dose of clindamycin therapy, is not observed with a low dose and short duration of therapy to treat malaria [44]. WHO did not ultimately recommend clindamycin treatment when used alone as an anti-malarial treatment, as parasite clearance might be deleterious in cases of significant parasitaemia in fragile subjects (children and pregnant woman) [18].

The combination of clindamycin with other rapidly acting drugs is essential for the optimization of treatment. Clinically documented associations essentially involve the combination of clindamycin with quinine or chloroquine.

Quinine, showing a rapid onset and short half-life, is the ideal partner. *In vitro* studies have also shown a synergistic effect when the two molecules are associated [7,45]. The bioavailability of the two drugs, when co-administered, remains unchanged [46]. A methodology and satisfactory post-treatment follow-up in approximately ten clinical trials with a wide number of patients have been published (Table 3) [18]. The duration of combination therapy remains controversial. While most studies consider that the administration of quinine for at least seven days and clindamycin for at least five days is needed, treatments conducted for three days in African studies were effective [44,47]. Short-duration treatment is justified for obtaining adequate compliance and fear of side effects with quinine. Parasite clearance has been correlated with parasitaemia in children treated for four days [48,49]. In areas of multidrug resistance, such as Thailand, 5 to 7 days are needed to cure malaria.

The second well-studied combination is clindamycin with chloroquine. *P. falciparum* is highly resistant to chloroquine in most malarial regions. However, this drug is still widely used and remains a first-line treatment in Africa. The clindamycin-chloroquine combination has been studied in Gabon [44], where chloroquine resistance is markedly high. Clindamycin was administered every 12 hours for 3 days, and success rates ranged from 70% in children to 97% in adults, depending on the study [50]. The success rate in children was estimated as 94% with chloroquine administered at a dose of 45 mg/kg versus 25 mg/kg. Although these findings favour the effectiveness of the combined administration

of chloroquine with clindamycin for three days, this treatment has not been widely adopted in practice.

Fosmidomycin, a phosphonic acid derivative, is a new antimalarial drug with a novel mechanism of action that inhibits the synthesis of isoprenoid in *P. falciparum* and suppresses the growth of multidrug-resistant strains *in vitro* [51]. Studies in Africa evaluating fosmidomycin as a monotherapeutic agent demonstrated that the drug is well tolerated in humans. A randomized, controlled, open-label study was conducted in 2003 in children to evaluate the efficacy and safety of treatment with fosmidomycin combined with clindamycin (30 and 5 mg/kg body weight every 12 h for 5 days, respectively) compared with treatment with either fosmidomycin or clindamycin alone. The combined treatment with the two molecules was superior to that with either agent alone [52].

The WHO advocates artemisinin-based combination therapy (ACT) as the mainstay in combating drug-resistant malaria in Africa [53]. Various drugs have been studied in combination with artemisinin derivatives, according to the underlying principle to combine artemisinins with drugs that have long plasma elimination half-lives. This treatment is inappropriate for patients from areas with a high rate of malaria transmission because of the increased risk of drug-resistant mutants resulting from prolonged exposure to subtherapeutic levels of the slowly eliminated drug in the combination [54-56]. Combination therapy with drugs that have a rapid elimination time reduces the selection of resistant isolates [57]. One clinical trial combining artesunate with clindamycin for the treatment of uncomplicated *P. falciparum* malaria in Gabonese children was reported in 2005 [54]. In this trial, clindamycin was selected based on promising results from animal models, *in vitro* studies of *P. falciparum* and the use of sequential treatment with

artesunate and clindamycin on Brazilian children [58]. An open-labelled, randomized, controlled clinical trial to evaluate the efficacy and tolerance of oral artesunate-clindamycin therapy (2 and 7 mg/kg) administered twice daily for 3 days compared with a standard quinine-clindamycin regimen administered twice daily for 100 days to treat uncomplicated falciparum malaria in 100 children. The results showed that the artesunate-clindamycin combination was consistent with that of quinine-clindamycin with respect to the cure rates (87% versus 94% at day 28 of follow up). The decreased fever and parasites clearance were significantly shorter in the artesunate-clindamycin treatment group. Based on the results of this study, clindamycin associated with artemisinin-based combination therapy is a candidate for studies in areas with a high rate of malaria transmission.

Another *in vivo* study was conducted to evaluate the efficacy and drug interactions of clindamycin in combination with other antimalarial drugs in populations from endemic areas. Some artemisinin derivatives have been tested on mice, such as the novel semi-synthetic endoperoxide artemisone [59]. This compound is synthetized from dihydroartemisin in a one-step process and in combination with clindamycin, exhibited increased antiplasmodial activity, improved *in vivo* half-life, improved oral bioavailability and metabolic stability, and presented tolerance and no neurotoxicity in humans compared with artesunate. Because this drug is a good candidate, clinical studies must be performed to assess the effect of artemisone in combination with other antimalarials.

Resistance mechanisms

Resistance to macrolides and lincosamides has been increasingly reported in clinical isolates of Gram-positive bacteria. One aspect of this resistance is the multiplicity of

mechanisms and the diversity in phenotypic expression of several of these mechanisms. Bacteria resist macrolides and lincosamides antibiotics in 3 ways, including target-site modification through methylation or mutation to prevent the binding of the antibiotics to ribosomal targets, which confers broad-spectrum resistance to macrolides and lincosamides, antibiotic efflux, and drug inactivation. However, these last two mechanisms only affect some molecules [60].

Ribosomal methylation remains the most widespread mechanism of resistance. Resistance to erythromycin has been observed in staphylococci since 1956. Biochemical studies indicated that resistance resulted from the methylation of the ribosomal target of the antibiotics, leading to cross-resistance to macrolides, lincosamides and streptogramin B. Subsequently, the MLS_B phenotype encoded by a variety of *erm* (erythromycin ribosome methylase) genes was reported in a large number of microorganisms [61]. Erm proteins facilitate the dimethylation of a single adenine in nascent 23S rRNA, as a part of the large (50S) ribosomal subunit [62]. A wide range of microorganisms, including spirochetes and anaerobes, which express Erm methylases, are targets for macrolides and lincosamides. The target mutation was first described for *Escherichia coli* mutants highly resistant to erythromycin. Mutations in domain V of rRNA were identified in 2001 [63]. Depending on the species, bacteria possess 1 to several *rrn* operons encoding 23S rRNA. In general, these mutations were observed in pathogens with 1 or 2 *rrn* copies, often with each copy carrying the mutation. This mechanism is responsible for the clarithromycin resistance of some *Mycobacterium avium*, *Helicobacter pylori* and *Treponema pallidum* strains [60]. Mutations in ribosomal proteins L4 and L22, which confer erythromycin resistance, have been documented for *Streptococcus pneumoniae*.

The antibiotic efflux is the second mechanism of resistance described for macrolides. In Gram-negative bacteria, chromosomally encoded pumps contribute to intrinsic resistance to hydrophobic compounds, such as macrolides. These pumps often belong to a family comprising proteins with 12 membrane-spanning regions. In Gram-positive organisms, the acquisition of macrolide resistance through active efflux is mediated through two classes of pumps: the ATP-binding-cassette (ABC) transporter superfamily and the major facilitator superfamily (MFS). The genes encoding these pumps are variable depending on the bacterial genus. The efflux system is multicomponent in nature, involving plasmidic and chromosomal genes that constitute a fully operational efflux pump with specificity for 14- and 15-membered macrolides and type B streptogramines (the MS_B phenotype).

The last mechanism of bacterial resistance is the inactivation of antibiotics. Esterases and phosphotransferases reported in enterobacteria confer resistance to erythromycin and other 14- and 15-membered macrolides but not to lincosamides. These resistance mechanisms have not been considered of major clinical importance because enterobacteria are not targets for macrolides. Some clinical isolates of *S. aureus* produce phosphotransferases, but this event remains rare [64-66].

In pathogenic microorganisms, the impact of the 3 mechanisms is unequal in terms of incidence and clinical implications.

Mechanisms underlying the resistance of *Plasmodium* against molecules from the MLSB family are not clearly identified, although experimental models of resistant *Plasmodium berghei* isolates are typically developed under selection pressure [5]. Mutations on A1875 (corresponding to the *E. coli* A2058 nucleotide in the peptidyltransferase centre of domain V) and A706 (corresponding to the E coli A754 in domain II) in the *P. falciparum*

apicoplast LSU rRNA (bearing 70% identity to the 23S rRNA [67]) did not confer *in vitro* resistance to macrolide in *P. falciparum* as observed in bacterial species [5]. The G1878 mutation, which confers resistance to clindamycin and azithromycin in *T gondii* [68], remained unchanged in azithromycin-resistant *P. falciparum* parasites [5]. A mutation was identified at nucleotide position 438 (T438C) after azithromycin-resistance selection. A single point mutation was also identified at codon 76 (G76V) in the *Pfrpl4* gene in azithromycin-resistant parasites.

Conclusions

The emergence and rapid extension of *P. falciparum* resistance to principal antimalarial drugs necessitates the search for new molecules. In addition, macrolides constitute excellent candidates for the treatment of uncomplicated malaria in areas of combined multi-resistance. The adequate tolerance and efficacy of macrolides have been demonstrated. These molecules, whose activity spectrum is similar to that of tetracyclines, are typically administered to children and pregnant women. A better comprehension of the mechanisms of action and resistance would facilitate the design of more effective structural analogues and identification of molecular markers of resistance to predict and survey the emergence of resistance.

Competing interests

The authors declared that there are no competing interests.

Author contributions

All authors drafted and approved the final manuscript.

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Table 1. Clinical trials of azithromycin plus other drug against *P. falciparum* malaria

Study demographic details					Regimen								
					Azithromycin			Other Drug					
Year	Place	Reference	Pop	Nb	Dosage/d	Route	Nb doses/d	Drug*	Dosage/d	Nb doses/d	Route	Days	Efficacy (%) d28
1996	Thailand	NaBangchang [69]	A	30	500 mg	PO	1	Ath	300mg	1	PO	3/1	14.8
2001	India	Dunne 2005 [26]	A	64	1000mg	PO	2	C	600mg	2	PO	3	90
2006	Thailand	Noedl 2006 [30]	A	27	1500mg	PO	2	A	200mg	2	PO	3	92
			A	27	1000mg	PO	1	A	200mg	1	PO	3	89
			A	16	1500mg	PO	2	Q	20mg/kg	2	PO	3	73
			A	27	1500mg	PO	3	Q	30mg/kg	3	PO	3	92
2006	Thailand	Miller 2006 [33]	A	10	1000mg	PO	2	Q	30mg/kg	3	PO	3	90
			A	20	1000mg	PO	2	Q	30mg/kg	3	PO	5	100
			A	20	1500mg	PO	3	Q	30mg/kg	3	PO	3	100
2007	Malawi	Kalilani 2007 [36]	P	47	1000mg	PO	2	SP	1500/75mg	3	PO	2	91
2008	Tanzania	Sykes 2009 [31]	C	129	20mg/kg	PO	1	A	4mg/kg	1	PO	3	42
2009	Bangladesh	Thriemer 2010 [32]	A		1500mg	PO	1	A	200mg	1	PO	3	95
			or	152	or				or				
			C		30mg/kg	PO	1	A	4mg/kg	1	PO	3	95
2007-8	Malawi	Laufer 2012 [34]	C	160	30mg/kg	PO	1	C	10mg/kg	1	PO	2	99
2004-6	Africa	Sagara 2014 [35]	A	227	1000mg	PO	1	C	600mg	1	PO	3	99

- Randomized controlled trial
- Pop = population: A: adult, C: children, P: pregnant women.
- * A: artesunate, Ath: arthemeter, C: chloroquine, F: fosmidomycin, Q: quinine, SP: sulfadoxine-pyriméthamine
- Only trials with adequate dosing, i.e. clindamycin given at least twice daily are mentioned in this table.

Table 2. Clinical trials of clindamycin monotherapy against *P. falciparum* malaria

Study demographic details					Regimen					
Year	Place	Reference	Pop	Nb	Dosage	Form	Nb doses/d	Route	Nb days	Efficacy (%)
1975	USA	Clyde, 1975 [42]	A	3	450 mg	Salt	3	PO	3	100
1975	Thailand	Hall, 1975 [70]	A	10	450 mg	Salt	3	PO	3	50
1981	Brazil	Alecrim, 1981 [71]	A	17	10 mg/kg	Salt	2	IV	3	65
1981	Brazil	Alecrim, 1981 [71]	A	14	10 mg/kg	Salt	2	IV + PO	7	100
1982	Brazil	Alecrim, 1982 [72]	A	26	10 mg/kg	Salt	2	IV, PO	5	100
1982	Philippines	Rivera, 1982 [73]	A	24	300 mg	Salt	2	IV + PO	7	100
1982	Philippines	Rivera, 1982 [73]	A	12	600 mg	Salt	2	IV + PO	7	100
1982	Philippines	Cabrera, 1982 [74]	A	12	10 mg/kg	Salt	2	IV + PO	7	100
1982	Philippines	Cabrera, 1982 [74]	A	19	20 mg/kg	Salt	2	IV	3	89
1984	Columbia	Restrepo, 1984 [75]	A	6	20 mg/kg	Salt	2	IV	3	100
1984	Columbia	Restrepo, 1984 [75]	A	9	10 mg/kg	Salt	2	IV + PO	7	100
1984	Columbia	Restrepo, 1984 [75]	A	5	20 mg/kg	Salt	2	IV	7	100
1984	Columbia	Restrepo, 1984 [75]	A	10	20 mg/kg	Salt	1	IV	7	100
1985	Sudan	El Wakeel, 1985 [76]	A	20	5 mg/kg	Salt	2	PO	5	90
1988	Brazil	Meira, 1988 [77]	A,C	129	10 mg/kg	Salt	2	PO, IV	5-7	97
1988	Brazil	Meira, 1988 [77]	A,C	16	10 mg/kg	Salt	1	PO, IV	5-7	50
1988	Brazil	Meira, 1988 [77]	A,C	35	2.5mg/kg	Salt	1	PO	5	80
1989	Brazil	Kremsner, 1988 [78]	A	35	5 mg/kg	Base	2	PO	5	100
1990	Philippines	Salazar, 1990 [79]	A	31	300 mg	Salt	2	PO	5	100
1990	Philippines	Salazar, 1990 [79]	A	10	600 mg	Salt	2	PO	5	100
1993	Gabon	Salazar, 1990 [79]	A	38	5 mg/kg	Base	2	PO	5	97
1994	East Timor	Oemijati, 1994 [80]	A	30	300 mg	Salt	2	PO	5	100

A: adults, C: children

Table 3. Clinical trials of clindamycin plus other drug against *P. falciparum* malaria

Study demographic details					Regimen								
					Clindamycin			Other Drug					
Year	Place	Reference	Pop	Nb	Dosage	Form	Nb doses/d	Drug*	Dosage/d	Nb doses/d	Route	Days	Efficacy (%)
1974	USA	Miller, 1973 [46]	A	5	450 mg	Salt	4	Q	650 mg	3	PO	3	100
1975	USA	Clyde, 1975 [42]	A	5	450 mg	Salt	3	Q	560 mg	3	PO	3	60
1975	USA	Clyde, 1975 [42]	A	2	600 mg	Salt	1	Q	560 mg	3	PO	3	50
1975	Thailand	Hall, 1975 [70]	A	4	450 mg	Salt	3	Q	540 mg	3	PO	3	100
1975	Thailand	Hall, 1975 [70]	A	5	150 mg	Salt	3	Q	270 mg	3	PO	3	60
1987	Brazil	Kremsner, 1988 [78]	A	40	15mg/kg	Base	2	Q	10mg/kg	2	PO	3	90
1992	Gabon	Kremsner, 1994 [44]	C	34	5 mg/kg	Base	2	Q	12mg/kg	2	PO	3	88
1993	Gabon	Metzger, 1995 [47]	C	33	5 mg/kg	Base	2	CQ	25mg/kg	3	PO	3	70
1995	Gabon	Kremsner, 1995[49]	C	50	5 mg/kg	Base	3	Q	8 mg/kg	3	IV	4	96
1995	Gabon	Metzger, 1995 [81]	A	40	5 mg/kg	Base	2	Q	12mg/kg	2	PO	3	92
1996	France	Parola, 2001 [82]	A	53	5 mg/kg	Salt	3	Q	8 mg/kg	3	IV	3	100
1997	Gabon	Vaillant, 1997 [83]	C	161	8 mg/kg	Salt	2	Q	8 mg/kg	2	PO	3	97
2000	Thailand	Pukrittayakamee [84]	A	68	5 mg/kg	Base	4	Q	8 mg/kg	3	PO	7	100
2001	Thailand	McGready, 2001 [85]	P	65	5 mg/kg	Salt	3	Q	8 mg/kg	3	PO	7	100
2004	Gabon	Bormann, 2004 [52]	C	12	10mg/kg	Salt	2	F	60 mg/kg	2	PO	5	100
2004	Gabon	Ramharter, 2005 [54]	C	100	7 mg/kg	Salt	2	A	2 mg/kg	2	PO	3	87

- Randomized controlled trial
- Pop = population; A: adult, C: children, P: pregnant women.
- *C : chloroquine, Q: quinine, F: fosmidomycin, A: artesunate
- Only trials with adequate dosing, i.e. clindamycin given at least twice daily are mentioned in this table.

REVUE III : OTHER ANTI-MALARIAL ANTIBIOTICS

ANTIBIOTICS IN MALARIA

Tiphaine Gaillard, Marylin Madamet, Jérome Dormoi, Bruno Pradines

Abstract

Despite the availability of individual protection by chemoprophylaxis, malaria, a vector-borne parasitic disease, is one of the greatest health concerns in tropical regions. Indeed, prophylaxis and chemotherapy for malaria remains a major area of research, and new molecules are constantly being developed in anticipation of the emergence of resistant parasite strains. The use of antimalarial drugs depends on the local level of resistance of *Plasmodium falciparum* in the destination country, contraindications, clinical tolerance and financial cost. Some of the antibiotics potentially useful in *Plasmodium* infection have been studied *in vitro* or *in vivo*; in particular, two families, tetracyclines and macrolides, as well as their derivatives, have been evaluated in recent years. In addition, other antibiotics have also been used or tested against malaria, some of which belong to older families, such as quinolones, co-trimoxazole or fusidic acid, with others being new molecules. These antibiotics could be a useful against malaria in the future. This review provides an overview of the use of these antibiotics against malaria.

Keywords

Antibiotics – Malaria – Prophylaxis – Treatment

Introduction

Malaria, a vector-borne parasitic disease, is among the major threats to public health in tropical regions, even though individual protection through malaria chemoprophylaxis is available. The prophylaxis and chemotherapy of malaria remains a major area of research, and new molecules are constantly being developed to counter the emergence of resistant parasite strains. The use of antimalarial drugs is dependent on the local level of resistance of *Plasmodium falciparum* in the country of destination, contraindications, clinical tolerance and financial cost. Among the compounds potentially useful in *Plasmodium*, certain antibiotics have been studied *in vitro* or *in vivo*.

A literature review was conducted to assess the role of antibiotics in the prophylaxis and the treatment of malaria. In particular, two families, tetracyclines and macrolides, and their derivatives, have been studied in the past 30 years, though other antibiotics could also be important anti-malaria agents in the future. This review is devoted to antibiotics used in malaria, including established and novel molecules that are known to act against *Plasmodium*. However, tetracyclines and macrolides are not discussed in this review because their use has already been presented [1,2].

Cotrimoxazole

Cotrimoxazole is a drug combination therapy consisting of trimethoprim and sulfamethoxazole. Trimethoprim, derived from pyrimidine, belongs to a group of compounds that are well documented for their antibacterial activity. Trimethoprim inhibits dihydrofolate reductase and has been shown to act as a sulfonamide potentiator [3]. In 1971, a 1:5 combination of trimethoprim and sulfamethoxazole was used to treat malaria infections in semi-immune Nigerian children [4] and was also effective for treating

chloroquine-resistant *P. falciparum* infections. Cotrimoxazole prophylaxis is currently recommended by the WHO to prevent opportunistic infections in AIDS patients [5]. Considering the reports on the effect of cotrimoxazole on malaria, both in HIV-infected and healthy individuals, some teams have studied the efficacy of this drug as an antimalarial for both preventive and curative use [6]. It appears that cotrimoxazole could constitute an alternative for malaria treatment and prophylaxis in different target groups, including children and adults, pregnant women, HIV-positive or -negative patients [7-10]. Despite its long-term use, cotrimoxazole is not associated with a higher prevalence of mutations related to antifolate resistance [11,12]. Cotrimoxazole is also inexpensive, almost universally available, and has a wide clinical spectrum of activity against bacterial, fungal and protozoan infections [13]. The greatest amount of information is from Africa, and more randomized controlled trials, including from other areas, are needed to evaluate the efficacy and safety of cotrimoxazole.

Anti-malarial quinolones

Quinolones are synthetic compounds containing a 4-oxo-1,4-dihydroquinoline skeleton that are primarily used as antibiotics. The first anti-bacterial quinolone, nalidixic acid, was discovered as a by-product during the synthesis of chloroquine [14]. In addition to possessing bactericidal properties, the quinolone scaffold present in the structure of compounds that display anti-malarial activity most likely functions by targeting the parasite's gyrase enzyme [15]. Compared to reports on their anti-bacterial properties, reports on the anti-malarial properties of quinolones are relatively limited. Nonetheless, recent research indicates that these compounds demonstrate promising potential, showing very good efficacy and targeting more that one stage of the malaria parasite's life cycle,

including the blood, liver and gametocyte stages. Quinolones also present novel modes of action that are different from those of most of the current, clinically used drugs. The subsequent discovery of an apicoplastic prokaryotic-like organelle in *Plasmodium* has increased research interest in further elucidating the mode of action of fluoroquinolones [16]. Within this context, it has been shown that ciprofloxacin affects *P. falciparum* by causing the formation of abnormal apicoplasts and "delayed death" in treated parasites [17,18]. Derivatization of ciprofloxacin by combining biorganometallic chemistry and a prodrug approach has been achieved, greatly increasing its malarial activity [17]. Recently, the addition of a ferrocenic moiety to ciprofloxacin led to enhancement of its activity, which could be attributed to (1) oxidative stress due to the redox properties of ferrocene/ferricenium and/or (2) the high lipophilicity of ferrocene, which may help to transport the drug across membranes [15].

The compounds derived from quinolones studied can be divided into different families: endochin and its analogues, acridinones, haloalkoxyacridinones and carboxyquinolones.

The anti-malarial properties of endochin, synthesized from the quinolone nucleus, were demonstrated at the end of the 1940s [20], and further studies on this molecule have established its activity against both the liver and blood stages of the parasite. Regardless, endochin has proven to be ineffective *in vivo* against human malaria due to its metabolization to inactive products via cytochrome P450 [21].

Acridinones are tricyclic compounds comprising the 4-oxo-1,4-dihydroquinolone skeleton and are structurally closely related to quinolones. Their anti-malarial activity was first reported in 1947 [22]. Other molecules with improved therapeutic properties were discovered later; however, because of their poor aqueous solubility and metabolic instability, they were not further evaluated for some time. Recent studies aimed at re-

evaluating the acridinone scaffold and some of these molecules highlight their potential [23].

Haloalkoxyacridinones constitute a new class of acridinone [24], some of which exhibit extraordinarily strong anti-malarial activity *in vitro*, with favourable IC_{50} values of 1 pM. Studies have shown that these compounds are active *in vitro* against chloroquine-susceptible and chloroquine-resistant *P. falciparum* strains [25,26]. Further work concerning this new class of molecules is in process.

Since the discovery of the anti-malarial activity of ICI56-780, a carboxyl derivative of quinolones, much research has focused on this family. Due to the need for inexpensive drugs that have novel mechanisms of action, screening has led to the discovery of various 3-carboxyquinolones that are progressively being tested against reference strains of *P. falciparum*. More research is required to optimize the pharmacodynamic properties of all of these quinolone derivatives [27].

Tigecycline

Tigecycline is the first member of a new class of antimicrobials: glycylcyclines. It is a semisynthetic derivate containing a glycyl amino substitution at position 9 [28], with an expanded spectrum of *in vitro* and *in vivo* activity against gram-positive, gram-negative, atypical, anaerobic, and other difficult–to-treat pathogens. However, this tetracycline analogue is not recommended for pregnant women and children. Tigecycline is specifically designed to overcome two common mechanisms of tetracycline resistance, namely, resistance mediated by acquired efflux pumps and/or ribosomal protection [29]. Tigecycline has a twice-daily dosing regimen and is generally well tolerated; because it must be administered intravenously, its use in malaria treatment should be reserved for

patients with severe and complicated malaria. The antimalarial activity of tigecycline was first tested in *in vitro* isolates from Blangadesh [29], whereby fresh *P. falciparum* isolates were cultured in the presence of threefold serial dilutions of tigecycline and other antimalarial drugs: doxycycline, azithromycin, dihydroartemisinin, chloroquine, quinine and mefloquine. The aim of the study was to quantify the inhibition of parasite growth using a highly sensitive HRP2 ELISA assay. Tigecycline showed a significant activity correlation only with doxycycline. The results suggested that tigecycline may have a delayed action on malaria parasites, similar to doxycycline; it also appeared to be one of the best antibiotics against *P. falciparum*, with an IC_{50} in the nanomolar range and a relatively steep dose-response curve.

The *in vitro* activity of this molecule was then studied in clinical isolates of *P. falciparum* from Gabon [30]. As in the previous study, the activity was compared with clindamycin and doxycycline, and the results demonstrated a substantial *in vitro* activity of tigecycline against *P. falciparum*: it appeared to act faster that any of the other tetracyclines, with the highest activity at day 3. This study emphasized the limited clinical use of tigecycline due to its pharmacokinetic properties [31], with the risk of exposing parasite populations to a prolonged period of subtherapeutic concentrations, thus increasing the risk of resistance.

All these results were confirmed in the Americas with the evaluation of the *in vitro* antimalarial activity of tigecycline against culture-adapted reference strains and clinical isolates from the Brazilian Amazon [32]. However, all of these *in vitro* studies were performed on a limiter number of isolates, and *in vivo* assays and randomized clinical trials are needed to establish the clinical applicability of tigecycline. Moreover, the co-administration of tigecycline with schizonticidal drugs that have a short elimination half-life should be performed to assess any potential synergistic effects.

A recent report demonstrated the *in vivo* non-clinical anti-malarial activity of tigecycline [33] based on the treatment of mice infected with *P. berghei*. The same report showed the complete cure of malaria in *P. berghei*-infected mice through the administration of tigecycline in combination with a subcurative dose of chloroquine. These results indicate the promising anti-malarial action of glycylcyclines in combination with chloroquine and further support *in vivo* assays and randomized clinical trials.

Mirincamycin

Mirincamycin is a synthetically produced lincosamide antibiotic, similar to clindamycin. This first-generation molecule was studied in 2009, and one study was performed on *P. falciparum* isolates from Gabon, in which the inhibitory activities of *cis*- and *trans*-mirincamycin were compared with the activities of doxycycline and clindamycin [34]. The study reported high *in vitro* activity against clinical *P. falciparum* isolates, and the IC_{50} values of both isomers were substantially lower than those of any other antibiotic tested thus far, including the lincosamide comparator clindamycin. Preclinical studies on mice [35] and monkeys [36] demonstrated the *in vivo* activity of mirincamycin against plasmodia. for *Plasmodium cynomolgi* infections in rhesus monkeys, mirincamycin was found to be curative as a monotherapeutic regimen, showing an addictive effect when administered together with primaquine. Mirincamycin also showed activity in *Plasmodium berghei*-infected mice. In another report, a hypnozoiticidal effect was observed in monkeys [37]. In these studies, toxicity was reported to be similar to that of clindamycin. After several years, further clinical development of mirincamycin is being pursued, and the molecule appears to be an interesting combination partner for fast-acting antimalarials.

Fusidic acid

One study [38] attempted to test the effect of the elongation factor-G (EF-G) inhibitor fusidic acid on the translation apparatus of two organelles of the malaria parasite: the mitochondrion and the apicoplast. Fusidic acid stalls the EF-G/GDP complex by binding to it immediately after GTP hydrolysis and by inhibiting the conformational change required for the release of the factor from the ribosome. The effect of fusidic acid was investigated on recombinant *P. falciparum* apicoplasts and mitochondria, and the results showed that fusidic acid-mediated inhibition of *P. falciparum* growth in erythrocytes does not exhibit the classic "delayed death" phenotype observed for apicoplast-targeted proteins. Indeed, inhibition of the parasite occurred both in the first as well as the second cycle of infection of *P. falciparum* parasites in blood culture. In addition, fusidic acid presented a greater inhibitory effect on apicoplastic EF-G compared to mitochondrial EF-G. Although there are no clinical trial actually evaluating the efficacy of fusidic acid on *P. falciparum* infections, research on the precise target of such antibiotics is useful for designing future anti-malarial molecules and derivatives.

Thiostrepton

Thiostrepton, a fusidic acid, is an antibiotic that inhibits elongation factor-G (EF-G) but in different ways. Thiostrepton is known to inhibit bacterial protein biosynthesis by binding to the 50S subunit and dually targeting apicoplast translation and the proteasome in *Plasmodium*. Although the *P. falciparum* apicoplast 23S rRNA is reported to be the preferred interaction site for thiostrepton [39], some authors [38] have suggested additional actions of this drug on mitochondrial EF-G. As this drug could be a candidate against malaria in the future, its mechanism of action should be better understood.

Nocathiacins

Nocathiacins are a group of thiazolyl peptide antibiotics structurally related to thiostrepton [40,41]. These compounds exhibit potent activity against a wide spectrum of multidrug-resistant gram-positive bacteria and inhibit protein synthesis. A study evaluating the potential anti-malarial activity of nocathiacin derivatives against asexual blood stages of *Plasmodium falciparum* [42] found that in comparison to thiostrepton, one nocathiacin derivative was water soluble and therefore biologically accessible for parasiticidal activity. The derivative caused irreversible growth inhibition within the first growth cycle and was immediately effective. These results revealed potent *in vitro* antimalarial activity in the nano-molar range against chloroquine-susceptible and resistant *P. falciparum* strains, warranting further investigation.

Conclusions

Although tetracyclines and macrolides are now well known as acting against malaria, other antibiotics should also be considered. Major discoveries could originate from the chemical modification of early molecules with anti-plasmodial properties.

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DEUXIEME PARTIE : TRAVAIL EXPERIMENTAL : VALIDATION DE MARQUEURS MOLECULAIRES DE DIMINUTION DE SENSIBILITE A LA DOXYCYCLINE CHEZ *PLASMODIUM FALCIPARUM*

CHAPITRE I : Validation de l'utilisation des gènes *pfmdt* et *pftetQ* comme marqueurs moléculaires de diminution de sensibilité à la doxycycline sur des isolats de *Plasmodium falciparum*

Les forces armées françaises utilisent en chimioprophylaxie antipaludique des comprimés de doxycycline à raison de 100 mg par jour, poursuivie 4 semaines après le retour. Malgré la disponibilité de moyens de protection individuels et collectifs, les armées doivent faire face à un nombre non négligeable de cas de paludisme. Les deux-tiers d'entre eux sont attribués à une mauvaise observance de la chimioprophylaxie, le pourcentage restant pourrait être le fait d'une résistance du parasite. Peu d'échecs dus à une résistance à la doxycycline, qu'ils soient prophylactiques ou thérapeutiques, ont été rapportés. La possibilité d'une pression de sélection par la doxycycline a néanmoins été mise en évidence sur un modèle murin de *Plasmodium berghei.* D'autre part, l'étude de la sensibilité à la doxycycline de plusieurs centaines d'isolats originaires de différents continents a permis de distinguer différents groupes de sensibilité *in vitro,* sur la base de l'évaluation des sensibilités à la doxycycline (DOX CI_{50}). Dans une étude de 2009, l'analyse de la distribution des CI_{50} de la doxycycline par une modélisation statistique bayésienne mixte de 747 isolats africains de *P. falciparum* avait permis de mettre en évidence trois phénotypes distincts, stables dans le temps et d'estimer à 35 µM le seuil de diminution de sensibilité *in vitro* à la doxycycline [60]. L'objectif d'une deuxième étude parue en 2010 était de mettre en évidence une association potentielle entre les trois phénotypes et certains génotypes ; l'étude de onze gènes candidats sur 90 isolats appartenant aux différents groupes phénotypiques et provenant de 14 pays africains avait suggéré que le nombre de copies des gènes *pfmdt* et *pftetQ* étaient des marqueurs moléculaires potentiels de diminution de sensibilité à la doxycycline chez les isolats africains de *P. falciparum* [61]. Cette étude concluait à la nécessité de valider le modèle sur davantage d'isolats, et si possible en provenance de différents continents.

L'objectif de ce premier travail était d'évaluer la distribution des CI_{50} pour la doxycycline sur une série d'isolats de *P. falciparum* Africains indépendante des précédentes, avec un nombre conséquent d'isolats. Il s'agissait ensuite d'en déterminer les nombres de copies

des gènes *pfmdt* et *pftetQ*. Les isolats choisis étaient les 484 isolats d'importation d'origine africaine, collectés entre janvier 2006 et décembre 2010 au Centre National de Référence du Paludisme situé à l'Hôpital Bichat – Claude Bernard (Paris). Chaque isolat avait bénéficié d'une évaluation initiale par technique isotopique de la CI_{50} à la doxycycline. L'analyse bayésienne des CI_{50} de la doxycycline des 484 isolats a permis de distinguer trois groupes de résistance, tels que précédemment publiés [60]. L'évaluation du nombre de copies des gènes *pfmdt* et *pftetQ* parmi 89 isolats choisis après randomisation dans chaque groupe phénotypique a permis de démontrer que la CI_{50} à la doxycycline était significativement plus élevée dans le groupe avec un nombre de copies du gène *pftetQ* > 1 ou *pfmdt* >1 par comparaison au groupe ne possédant qu'une copie du gène *pftetQ* ou *pfmdt*.

Article : **Gaillard T, Briolant S, Houzé S, Baragatti M, Wurtz N, Hubert V, Lavina M, Pascual A, Travaillé C, Le Bras J, Pradines B, French National Reference Centre for Imported Malaria Study Group: *PftetQ* and *pfmdt* copy numbers as predictive molecular markers of decreased *ex vivo* doxycycline susceptibility in imported *Plasmodium falciparum* malaria. Malar J 2013, 12:414.**

Dans un deuxième temps, nous avons montré pour la première fois l'association d'un nombre de copies du gène *pftetQ* > 1 ou du gène *pfmdt* >1 à un échec prophylactique avéré à la doxycycline en République Centrafricaine (accès palustre en présence d'un taux plasmatique de doxycycline considéré comme protecteur).

Briolant et Coll. ont également montré en 2010 que le nombre de répétitions du motif d'acides aminés KYNNNN < 3 sur le gène *pftetQ* était associé à une diminution de sensibilité à la doxycycline (Odd Ratio = 3.00 [1.02-8.86], *P* = 0.046) [61]. Cette association a été confirmée par une autre équipe sur des isolats de *P. falciparum* du Kenya [62]. Nous avons également montré que ce cas d'échec prophylactique à la doxycycline était associé à une répétition du motif KYNNNN sur le gène *pftetQ* < 3.

Article : **Madamet M, Gaillard T, Velut G, Ficko C Houzé P, Bilicky C, Houzé S, Taudon N, Michel R, Rapp C, Pradines B. Malaria prophylaxis failure with**

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Ces deux articles ont montré l'intérêt des gènes *pfmdt* et *pftetQ* comme marqueurs prédictifs de la diminution de sensibilité *in vitro* à la doxycycline chez des isolats de *P. falciparum* Africains mais aussi *in vivo*. Cependant, les résultats de ces travaux nécessitaient d'être confirmés sur des isolats originaires d'autres continents, avec un support génomique distinct.

Gaillard T, Briolant S, Houzé S, Baragatti M, Wurtz N, Hubert V, Lavina M, Pascual A, Travaillé C, Le Bras J, Pradines B, French National Reference Centre for Imported Malaria Study Group.

PftetQ and _pfmdt_ copy numbers as predictive molecular markers of decreased _ex vivo_ doxycycline susceptibility in imported _Plasmodium falciparum_ malaria.

Malar J 2013, 12:414.

Gaillard et al. Malaria Journal 2013, 12:414
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PftetQ and pfmdt copy numbers as predictive molecular markers of decreased ex vivo doxycycline susceptibility in imported Plasmodium falciparum malaria

Tiphaine Gaillard[1,2,3], Sébastien Briolant[1,2], Sandrine Houzé[4,5,6], Meïli Baragatti[7], Nathalie Wurtz[1,2], Véronique Hubert[4,6], Morgane Lavina[1,2], Aurélie Pascual[1,2,6], Christelle Travaillé[8], Jacques Le Bras[4,5,6], Bruno Pradines[1,2,6*] and The French National Reference Centre for Imported Malaria Study Group

Abstract

Background: The objective of this study was to evaluate the distribution of a series of independent doxycycline inhibitory concentration 50% (IC_{50}) values to validate the trimodal distribution previously described and to validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* doxycycline susceptibility in *Plasmodium falciparum* malaria.

Methods: Doxycycline IC_{50} values, from 484 isolates obtained at the French National Reference Centre for Imported Malaria (Paris) between January 2006 and December 2010, were analysed for the first time by a Bayesian mixture modelling approach to distinguish the different *in vitro* phenotypic groups by their IC_{50} values. Quantitative real-time polymerase chain reaction was used to evaluate the *pftetQ* and *pfmdt* copy numbers of 89 African *P. falciparum* isolates that were randomly chosen from the phenotypic groups.

Results: The existence of at least three doxycycline phenotypes was demonstrated. The mean doxycycline IC_{50} was significantly higher in the group with a *pftetQ* copy number >1 compared to the group with a *pftetQ* copy number = 1 (33.17 μM *versus* 17.23 μM) and the group with a *pfmdt* copy number >1 (28.28 μM *versus* 16.11 μM). There was a significant difference between the combined low and medium doxycycline IC_{50} group and the high IC_{50} group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%) or *pfmdt* gene (8.33% *versus* 37.93%). In the logistic regression model, the *pfmdt* and *pftetQ* copy numbers >1 (odds ratio = 4.65 and 11.47) were independently associated with the high IC_{50} group.

Conclusions: Copy numbers of *pftetQ* and *pfmdt* are potential predictive molecular markers of decreased susceptibility to doxycycline.

Keywords: Malaria, *Plasmodium falciparum*, Anti-malarial, *In vitro*, Resistance, Molecular marker, Doxycycline

*Correspondence: bruno.pradines@free.fr
[1]Unité de Parasitologie, Institut de Recherche Biomédicale des Armées, Marseille, France
[2]Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Aix Marseille Université, Marseille, France
Full list of author information is available at the end of the article

Background

Daily administration of doxycycline is currently a recommended chemoprophylactic regimen for travellers visiting malaria-endemic areas with high prevalence of chloroquine or multidrug resistance [1]. In addition, the French malaria consensus recommends quinine and doxycycline for the first-line treatment of *Plasmodium falciparum* severe malaria in Asia and South America. In combination with artesunate or quinine, doxycycline remains the recommendation as the second-line treatment of uncomplicated falciparum malaria or for the treatment of severe malaria as a seven-day course [2]; however, its use is limited. Prophylactic failure of doxycycline against *P. falciparum* has been associated with both inadequate doses [3] and poor compliance [4].

Since September 2002, French troops have participated in the peace-keeping operation, Operation Licorne, in the Ivory Coast. Soldiers had been prescribed doxycycline (100 mg) daily for prophylaxis. Many cases of malaria have been reported, but most of these cases are believed to be the result of poor compliance [5,6]. From 2002 to 2006, 1,787 falciparum malaria cases were observed in French soldiers who were expected to take doxycycline. A surge in the number of malaria cases within three weeks after doxycycline prophylaxis discontinuation is often observed after return [7,8]. Therefore, it is recommended that doxycycline be taken for four weeks after returning from an endemic area. However, resistance can also explain failures of prophylactic doxycycline.

The ability to maximize the efficacy and longevity of anti-malarial drugs for malaria control will depend critically on intensive research to identify *in vitro* markers along with ex vivo and *in vivo* surveillance programmes. It is necessary to identify molecular markers that predict doxycycline resistance or decreased susceptibility in order that active surveillance can monitor temporal trends in parasite susceptibility [9]. Although there have been no reported clinical failures for the treatment of falciparum malaria with doxycycline, a Bayesian mixture modelling approach has distinguished three different *in vitro* phenotypic groups: low, medium and high doxycycline IC_{50} values, among 747 *P. falciparum* isolates obtained from 14 African countries over a nine-year period [10]. The sequences of 11 *P. falciparum* genes that are analogous to those involved in bacterial resistance to doxycycline were obtained from 30 isolates from each phenotypic group. The data suggested that the copy numbers of a *tet*Q GTPase family gene, *pftetQ* (PFL1710c), and a metabolic drug transporter gene, *pfmdt* (PFE0825w), were potential molecular markers of decreased *in vitro* susceptibility to doxycycline in African isolates [11].

The objective of this study was first to evaluate the distribution of a new series of independent doxycycline IC_{50} values assessed by another group for goodness of fit with the trimodal compartment model of doxycycline response previously proposed [10] and then to validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline. This was performed by assessing the gene copy numbers in *P. falciparum* clinical isolates that were randomly chosen from the phenotypic groups with different doxycycline IC_{50} values.

Methods

Patients and sample collection

Between January 2006 and December 2010, 484 fresh *P. falciparum* isolates were obtained at the French National Reference Centre for Imported Malaria (Paris) from patients hospitalized with malaria after having returned to France. These samples were successfully assessed for doxycycline susceptibility. Ex vivo testing of doxycycline susceptibility was performed as previously described by a standard 42-hour ^{3}H-hypoxanthine uptake inhibition assay [12]. Batches of plates were tested and validated on the chloroquine-susceptible 3D7 strain and the chloroquine-resistant W2 strain.

The drug concentration that inhibited 50% parasite growth (IC_{50}) was calculated with the inhibitory sigmoid Emax model, with estimation of the IC_{50} through non-linear regression using a standard function of the R software (ICEstimator) [13].

Quantification of pftetQ and pfmdt copy numbers

pfmdt (PFE0825w) and *pftetQ* (PFL1710c) copy numbers were estimated by TaqMan real-time PCR (7900HT Fast Real-Time PCR system, Applied Biosystems) relative to the single-copy gene, *pfβtubulin* (PF10_0084). The following oligonucleotide primers and probes were designed using the Primer Express software v2.0 (Applied Biosystems) for use in the polymerase chain reactions (PCRs): 5′-TTATGCAAACATTTCAAGCTTCCT-3′, 5′- ACCC ATTCCATAACTTAGATTTAGATAACC-3′ and 5′-VIC-TAAAAACAAATTTCGACAAAAGGACAGGAGCC-TAMRA-3′ for *pfmdt*, 5′-ACCCCTTTTTTATCTTACGAAAG-3′, 5′-ATGGTTGTACGTTATATCATATGG-3′ and 5′-VIC-AAAAATGTGGCAACAATTCAGACATGTATCA-TAMRA-3′ for *pftetQ* and 5′-TGATGTGCGCAAGTGATCC-3′, 5′-TCCTTTGTG GACATTCTTCCTC-3′ and 5′-FAM-TAGCACATGCCGTTAAATATCTTCCATGTCT-TAMRA-3′ for *pfβtubulin* (Eurogentec). Individual PCRs were performed using 1 X TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 250 nM TaqMan probe and 5 μL template DNA in a final volume of 25 μL The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min and

50 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was assayed in triplicate and analysed with the SDS software 2.2.1 (Applied Biosystems). The PCR efficiencies of all the primer pairs were evaluated on a dilution series of *P. falciparum* 3D7 genomic DNA. The efficiencies were found to be sufficiently close to obviate the need for any correction factor. Therefore, the $2^{-\Delta\Delta Ct}$ method of relative quantification was used and adapted to estimate the number of copies of the *pfmdt* and *pftetQ* genes [14,15] with the formula $\Delta\Delta Ct = (Ct_{pfmdt} - Ct_{pf\beta tubulin})_{\text{sample}} - (Ct_{pfmdt} - Ct_{pf\beta tubulin})_{\text{calibrator}}$. Genomic DNA extracted from 3D7 *P. falciparum*, which has a single copy of each gene, was used for calibration, whereas *pfβtubulin* served as the control housekeeping gene in all the experiments.

Genetic diversity of Plasmodium falciparum isolates with pftetQ and pfmdt multicopies

Mixed infection could influence read-out in Taqman real-time PCR, potentially leading to false positive results of gene copy number. The genomic DNA of *P. falciparum* isolates with at least two copies of *pfmdt* or *pftetQ* were investigated for genetic diversity at highly polymorphic loci, merozoite surface proteins 1 and 2 (MSP1 and MSP2). The *msp1* and *msp2* loci were genotyped using the nested PCR strategy and conditions previously described [16].

Statistical analysis

The statistical analysis has been designed to answer the specific question of whether *P. falciparum* has different doxycycline susceptibility phenotypes. A heterogeneous population of IC_{50} values was observed; therefore, the data were assumed to represent a univariate Gaussian mixture with k components. Each observation was assumed to originate from one of the k components, and the label of the group from which each observation arose was unknown. The unknowns of the model were the number of components, the means, variances and weights of the different components, and the vector of allocations of the observations. The analysis was performed in two steps. First, reversible jump Monte Carlo Markov Chains (RJMCMC) [17] samplers were used to choose a suitable number of components k, and the present algorithm followed the recommendations of Cappé et al. [18]. After a relevant number of components was chosen, standard Gibbs samplers were run to obtain estimates of the model parameters and to classify the observations [19]. Because of the'label-switching' problem, due to the symmetry in the likelihood of the model parameters, the mixture components should be labelled before making an inference on the parameters [20]. The classical ordering constraint, which was biologically relevant here, was used. The algorithms were run for 5,000 burn-in iterations and 20,000 post-burn-in iterations. These numbers were assumed to be sufficient to obtain reliable results. Moreover, each algorithm was run three times to check that the results between two different runs were similar and that there was no convergence problem [17].

The data were analysed using the R software* (version 2.10.1). The differences in the *pfmdt* and *pftetQ* copy numbers between the phenotypic groups were tested using the Mann Whitney test and the Kruskal-Wallis test. The genotype proportions were compared using the Fisher exact test. The risk of the high doxycycline IC_{50} was analysed using a logistic regression model (univariate and multivariate analysis).

Ethics

Informed consent was not required for this study because the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

Results

The doxycycline IC_{50} values ranged from 0.49 to 65.1 μM. The mean was 11.64 μM (95% confidence interval, 10.96-12.33). The average parameter estimates for the IC_{50} values by year are given in Table 1.

The triple normal distribution model is represented in Figure 1. The parameter estimates for the three-component mixture model, including the number of isolates in each normal distribution, the mean of the IC_{50} values and the standard deviation for each distribution, are summarised in Table 2. A double normal distribution model and a quadruple normal distribution model were also fitted to the data to assess the validity of considering a three-component mixture (data not shown). These two models fit the data worse than the triple normal distribution model.

Eighty-nine *P. falciparum* isolates (30, 30 and 29) were randomly chosen from the three phenotypic groups, A, B and C, that differed in their doxycycline IC_{50} values. These isolates were classified as follows: low doxycycline IC_{50} group from component A [mean, 4.33 μM (95% CI, 3.39-4.37 μM)], medium doxycycline IC_{50} group from component B [mean, 16.97 μM (95% CI, 16.45-17.49 μM)]

Table 1 Statistical analysis of the 484 doxycycline IC_{50} values by year

Year	IC_{50} number	Mean (μM)	95% CI	IC_{50} min	IC_{50} max
2006	119	10.05	9.14-10.96	0.63	43.7
2007	172	11.91	10.72-13.11	2.34	44.8
2008	59	9.45	8.56-10.35	4.27	23.1
2009	40	12.21	8.01-16.41	0.49	65.1
2010	94	14.3	12.63-15.97	4.55	46.2
Total	484	11.64	10.96-12.33	0.49	65.1

95% CI: 95% confidence interval.

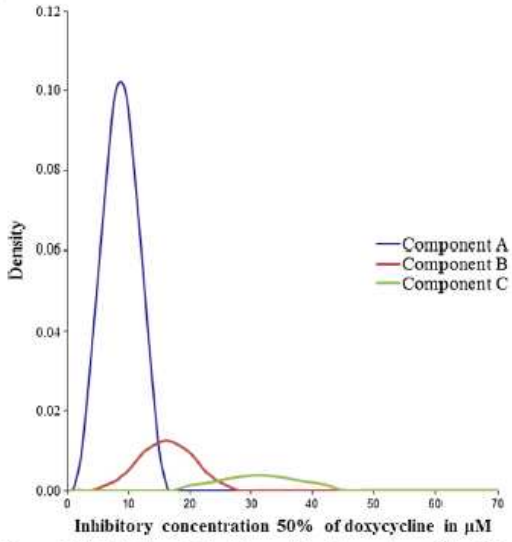

Figure 1 Distribution of the doxycycline IC_{50} values of the 484 *Plasmodium falciparum* isolates (2006 to 2010) in the three-component mixture model (Bayesian mixture modelling approach). The dotted lines represent the three fitted mixtures.

and high doxycycline IC_{50} group from component C [mean, 34.60 µM (95% CI, 31.30-37.90 µM)].

Only one or two copies of *pfmdt* and *pftetQ* were identified in the 89 isolates. All of the isolates with two copies *pfmdt* or *pftetQ* had 1 allelic family for each of the two genes (*msp1* and *msp2*), confirming that these infections were single and not mixed. The mean doxycycline IC_{50} was significantly higher in the group with a *pftetQ* copy number >1 compared to the group with a *pftetQ* copy number = 1 (33.17 µM *versus* 17.23 µM; $P = 0.0041$, Mann Whitney test) (Table 3). The mean doxycycline IC_{50} was significantly higher in the group with a *pfmdt* copy number >1 (28.28 µM *versus* 16.11 µM; $P = 0.0025$, Mann–Whitney test).

The number of *pftetQ* copies was significantly higher in the high doxycycline IC_{50} group compared to the low and medium doxycycline IC_{50} groups (1.21 *versus* 1.0 and 1.0; $P = 0.0014$, Kruskal-Wallis test). The number of *pfmdt* copies was significantly higher in the high doxycycline IC_{50} group compared to the low and medium doxycycline IC_{50} groups (1.38 *versus* 1.13 and 1.03, respectively; $P = 0.0019$, Kruskal-Wallis test).

There was no significant difference between the low and medium doxycycline IC_{50} groups for the *pfmdt* and *pftetQ* copy numbers. Therefore, these two phenotypic groups were combined. There was a statistically significant difference between the low and medium doxycycline IC_{50} combined group and the high doxycycline group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%; $P = 0.0008$, Fisher's exact test) or *pfmdt* gene (8.33% *versus* 37.93%; $P = 0.0021$, Fisher's exact test) (Table 4).

In the logistic regression model (Table 5), the *pfmdt* copy number >1 (adjusted OR = 4.65 [1.31-16.51], $P = 0.0176$) and *pftetQ* copy number >1 (adjusted OR = 11.47 [1.23-106.98], $P = 0.0322$) were independently associated with the high IC_{50} phenotypic group.

Table 2 Parameter estimates for the three-component mixture model for the 484 *Plasmodium falciparum* isolates

Component	Isolates number	Proportion (%)	IC_{50} mean (µM)	Standard deviation
A	393	81.1	8.70	2.87
B	60	12.4	16.18	4.86
C	31	6.5	31.23	10.32

Table 3 Statistical analysis of the doxycycline IC_{50} values based on the *pftetQ* and *pfmdt* copy numbers in 89 *Plasmodium falciparum* isolates

	pftetQ copy number		*pfmdt* copy number	
	= 1	> 1	= 1	> 1
Number of values	83	6	73	16
IC_{50} mean (µM)	17.23	33.17	16.11	28.28
Standard deviation	13.32	6.85	12.53	14.03
95% Confidence interval	14.32-20.13	25.98-40.37	13.19-19.04	20.81-35.76
Minimal IC_{50}	0.49	25.16	0.49	4.62
Maximal IC_{50}	65.11	43.7	44.82	65.11

Discussion

Most prophylactic failures of doxycycline against *P. falciparum* are associated with the use of standard doses resulting in lower than expected serum drug levels [21], inadequate low doses [3], or poor compliance [4,22]. Moreover, doxycycline pharmacokinetic parameters could explain some of these cases. Doxycycline has a short elimination half-life (16 hours) compared to proguanil (24 hours), atovaquone (31–73 hours), chloroquine (two to

Table 4 Statistical analysis of *pftetQ* and *pfmdt* copy numbers in 89 *Plasmodium falciparum* isolates (Fisher's exact test)

	PftetQ		*Pfmdt*	
	Low and medium IC_{50}	High IC_{50}	Low and medium IC_{50}	High IC_{50}
Copy number >1	0	6	5	11
Copy number = 1	60	23	55	18
%	0.00	20.69	8.33	37.93
Fisher's exact test	*P* value	0.0008	*P* value	0.0021

Table 5 Multivariate regression model

	Doxycycline IC_{50} group, number					
Molecular marker	Low or medium	High	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
pftetQ copy number						
1	60	23	1.00 (reference)		1.00 (reference)	
> 1	0	6	18.77 (2.18-161.43)	0.0076	11.47 (1.23-106.98)	0.0322
pfmdt copy number						
1	55	5	1.00 (reference)		1.00 (reference)	
> 1	18	11	6.72 (2.06-21.96)	0.0016	4.65 (1.31-16.51)	0.0176

three days), or mefloquine (six to 41 days), and a short mean residence time (63% of the administered dose is eliminated in 27 hours) [8]. In addition, its slow action *in vitro* has a delayed effect upon growth and requires the prolonged incubation of parasites [23]. Determination of the IC_{50} after two generations of parasite growth decreases the 42-hour IC_{50} from ten- to 20-fold [24,25]. However, in practice, the standard 42-hour test remains the method of monitoring doxycycline ex vivo susceptibility.

Maximizing the efficacy and longevity of anti-malarial drugs to control malaria will critically depend on intensive research to identify *in vitro* markers along with the implementation of ex vivo and *in vivo* surveillance programmes, such as those championed by the WorldWide Antimalarial Resistance Network [26]. Therefore, there is a need to identify molecular markers that predict doxycycline resistance, which can provide an active surveillance method to monitor temporal trends in parasite susceptibility [9]. In addition, the early detection of resistance or decreased susceptibility to doxycycline will require that the baseline parasite chemosusceptibility of current isolates from endemic regions is established.

To validate the trimodal distribution model of doxycycline IC_{50} values previously described for *P. falciparum* African isolates [10], the distribution of a new series of independent doxycycline IC_{50} values that were assessed by a separate group under the same technical conditions [12] was evaluated. This analysis was performed with a Bayesian mixture modelling approach. Again, the demonstration of the existence of at least three doxycycline phenotypes was confirmed. All 484 values were classified into three components: component A (IC_{50} mean 8.7 μM), component B (IC_{50} mean 16.2 μM), and component C (IC_{50} mean 31.2 μM). This trimodal distribution model of doxycycline IC_{50} values from imported *P. falciparum* isolates obtained from 2006 to 2010 confirms the previous data [10]. However, the level of the IC_{50} value in each component is different between the series of imported *P. falciparum* isolates obtained from 2006 to 2010 and those obtained from 1999 to 2006 [10]. The IC_{50} doxycycline values from the isolates obtained from 1999 to 2006 were classified into three components: component A (IC_{50} mean 4.9 μM), component B (IC_{50} mean 7.7 μM), and component C (IC_{50} mean 17.9 μM). It appears that components A and B (IC_{50} means 4.9 μM and 7.7 μM, respectively) for the values obtained from 1999 to 2006 have merged into a single component A (IC_{50} mean 8.7 μM) for the values obtained from 2006 to 2010. In addition, the percentage of isolates (78%) in the two components, A and B, for the values obtained from 1999 to 2006 is similar to the percentage of isolates (81%) for component A for the values obtained from 2006 to 2010. The component B (IC_{50} mean 16.2 μM) values obtained from 2006 to 2010 correspond to the component C (IC_{50} mean 17.9 μM) values obtained from 1999 to 2006. A new component, component C (IC_{50} mean 31.2 μM, proportion 6.4%), emerged for the values obtained from 2006 to 2010. These data suggest the emergence of strains with decreased susceptibility to doxycycline. In addition, based on the previously defined cut-off for reduced susceptibility to doxycycline (35 μM) [10], 1.2% of the 747 *P. falciparum* isolates tested from 1999 to 2006 were considered to have decreased susceptibility to doxycycline *versus* 2.7% for the 484 isolates tested from 2006 to 2010.

Plasmodium falciparum possesses a *tetQ* GTPase family gene analogue of the genes that encode bacterial ribosomal protection proteins. These genes are the *pftetQ*, which is involved in bacterial resistance to the cycline drugs, and a multidrug transporter gene, *pfmdt*, which shares a high sequence identity with efflux pumps. In a multivariate logistic regression model, an increased *pfmdt* copy number was associated with high doxycycline IC_{50} values with an adjusted odds ratio (OR) of 7.09 ($p = 0.011$), and an increased *pftetQ* copy number was associated with an adjusted OR of 5.23 ($p = 0.042$) [11]. To validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline by assessing the gene copy numbers, 89 (30, 30 and 29) *P. falciparum* clinical isolates were randomly chosen from the three phenotypic groups (A, B and C) with different doxycycline IC_{50} values. These isolates were classified as follows: low doxycycline IC_{50} group from component A [mean, 4.33 μM (95% CI, 3.39-4.37 μM)],

medium doxycycline IC_{50} group from component B [mean, 16.97 μM (95% CI, 16.45-17.49 μM)] and high doxycycline IC_{50} group from component C [mean, 34.60 μM (95% CI, 31.30-37.90 μM)]. These isolates were obtained from patients hospitalized with malaria after travel in Cameroon (n = 18), Ivory Coast (n = 14), Mali (n = 11), Niger (n = 5), Burundi (n = 4), Burkina Faso (n = 4), Djibouti (n = 4), Madagascar (n = 4), Congo (n = 5), Ghana (n = 3), Sudan (n = 2), Central African Republic (n = 2), Zambia (n = 1), Rwanda (n = 1), Togo (n = 1), Guinea (n = 1), and nine from Africa without specificity regarding the country.

The mean doxycycline IC_{50} value is significantly higher in the groups with *pftetQ* or *pfmdt* copy numbers >1, suggesting that *pftetQ* and *pfmdt* could be involved in the reduced susceptibility to doxycycline.

The number of *pftetQ* and *pfmdt* gene copies is significantly higher in the high doxycycline IC_{50} group than the low and medium doxycycline IC_{50} groups. However, there is no significant difference between the low and the medium doxycycline IC_{50} groups for the *pfmdt* and *pftetQ* copy numbers. These two phenotypic groups were, therefore, combined. There is a statistically significant difference between the low and medium doxycycline IC_{50} combined group and the high doxycycline group in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (0% *versus* 20.69%; $P = 0.0008$) or *pfmdt* gene (8.33% *versus* 37.93%; $P = 0.0021$). In addition, in the multivariate logistic regression model, an increased *pfmdt* copy number is associated with high doxycycline IC_{50} values with an adjusted OR of 4.65 ($P = 0.0176$), and an increased *pftetQ* copy number is associated with an adjusted OR of 11.47 ($P = 0.0322$). These results are consistent with previous data [11] and confirm the potential use of *pftetQ* and *pfmdt* as predictive molecular markers for decreased *P. falciparum* susceptibility to doxycycline in Africa.

In a study on fresh *P. falciparum* clinical isolates from Dakar, Senegal, it was shown that there was no statistically significant difference between a group with a doxycycline IC_{50} <25 μM and a group with an IC_{50} >25 μM in terms of the per cent of isolates with one or more copy numbers of the *pftetQ* gene (p = 0.079) or *pfmdt* gene (p = 0.066) [27]. However, the significance levels of these associations were just above the *P* value threshold (0.05). It seems that the number of isolates from the high doxycycline IC_{50} group (15.9%) was most likely too low to obtain statistically significant differences, indicating the necessity of assessing the gene copy numbers with more isolates. Another possibility is that over-expression of *pftetQ* or *pfmdt* could confer *in vitro* reduced susceptibility to doxycycline in association with other contributing determinants, which could modulate the *in vitro* response to doxycycline.

In summary, this study demonstrates that copy numbers of the *pftetQ* and *pfmdt* genes are potential predictive molecular markers of decreased *P. falciparum* susceptibility to doxycycline in Africa. Epidemiological studies using large numbers of parasites with reduced susceptibility to doxycycline are now required to determine whether *pftetQ* and *pfmdt* can be used as markers of reduced *in vitro* doxycycline susceptibility.

Competing interests

The authors have declared that they have no competing interests.

Authors' contributions

TG, NW, ML and AP carried out the molecular genetic studies. SH, VH and JLB carried out the ex vivo evaluation of doxycycline susceptibility. The French National Reference Centre for Imported Malaria Study Group supervised, carried out and coordinated the field collections of patient isolates. BP and SB conceived and coordinated the study. SB, MB, CT and BP analysed the data. TG, SB, SH, MB, JLB and BP drafted the manuscript. All the authors read and approved the final manuscript.

Authors' information

French National Reference Centre for Imported Malaria Study Group: A Aboubacar (CHU Strasbourg), Agnamey P. (CHU Amiens), Ajana F. (CH Tourcoing), Amal C. (CH Mourier, Colombes), Amira R. (CHG Saint Denis), Argy N. (CHU Bichat-Claude Bernard, Paris), Baumard S. (CHRU Reims), Bellanger A. P. (CHU Minjoz, Besancon), Bemba D. (CH Verdier, Bondy), Beytout J. (CHRU Clermont Ferrand), Bigel M.L. (CH Quesnay, Mante la Jolie), Bloch M. (CH Mourier, Colombes), Bonnet R. (CHRU Clermont Ferrand), Borel A. (CHU Amiens), Bouchaud O. (CH Avicenne, Bobigny), Branger C. (CH Mourier, Colombes), Bruneel F. (CH Mignot, Versailles), Cambon M. (CHRU Clermont Ferrand), Camus D. (CH Lille), Casalino E. (CHU Bichat-Claude Bernard, Paris), Clain J. (CHU Bichat-Claude Bernard, Paris), Cojean S. (CHU Bichat-Claude Bernard, Paris), Cuisenier B. (CHU Dijon), De Gentile L. (CHU Angers), Delarbre J. M. (CH Moenchsberg, Mulhouse), Delaval A. (CH Balanger, Aulnay sous Bois), Durand R. (CH Avicenne, Bobigny), Dutoit E. (CH Lille), Eloy O. (CH Mignot, Versailles), Faucher J. F. (CHU Minjoz, Besancon), Faye A. (CH Debre, Paris), Fenneteau O. (CH Debre, Paris), Filisetti D. (CHU Strasbourg), Fulleda C. (CHU Lariboisiere, Paris), Godineau N. (CHG Saint Denis), Grenouillet F. (CHU Minjoz, Besancon), Hurst J. P. (CH Monod, Le Havre), Ichou H. (CH Mourier, Colombes), Klein E. (CHU Lariboisiere, Paris), Lariven S. (CHU Bichat-Claude Bernard, Paris), Lefevre M. (CH Laennec, Creil), Lemoine M. (CHU Bichat-Claude Bernard, Paris), Lesens O. (CHRU Clermont Ferrand), Lohmann C. (CH Moenchsberg, Mulhouse), Lusina D. (CH Balanger, Aulnay sous Bois), Machouart M. C. (CHR Nancy), Mary R. (CHG Saint Denis), Matheron S. (CHU Bichat-Claude Bernard, Paris), Mechali D. (CHG Saint Denis), Merrens A. (HIA Begin, Saint Mande), Millon L. (CHU Minjoz, Besancon), Monnier S. (CH Mignot, Versailles), Mortier E. (CH Mourier, Colombes), Moussel F. (CH Quesnay, Mante la Jolie), Pageot L. (CHU Bichat-Claude Bernard, Paris), Parez N. (CH Mourier, Colombes), Patoz P. (CH Tourcoing), Pfaff A. (CHU Strasbourg), Pihet M. (CHU Angers), Pilo J. E. (HIA Begin, Saint Mande), Poilane I. (CH Verdier, Bondy), Pons D. (CHRU Clermont Ferrand), Poupart M. (CHG Saint Denis), Prevel M. (CHG Saint Denis), Pull L. (CH Debre, Paris), Rapp C. (HIA Begin, Saint Mande), Rivier A. (CHR Nancy), Ronez E. (CHU Lariboisiere, Paris), Rotten D. (CHG Saint Denis), Sarrasin V. (CHU Bichat-Claude Bernard, Paris), Silva M. (CH Monod, Le Havre), Simonet A. L. (CHU Dijon), Siriez J. Y. (CH Debre, Paris), Strady C. (CH Debre, Reims), Therby A. (CH Mignot, Versailles), Thibault M. (CH Dubos, Cergy Pontoise), Thouvenin M. (CH Troyes), Toubas D. (CHRU Reims).

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Author details

[1]Unité de Parasitologie, Institut de Recherche Biomédicale des Armées, Marseille, France. [2]Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Aix Marseille Université, Marseille, France. [3]Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, France. [4]Laboratoire de Parasitologie-Mycologie, Hôpital Bichat-Claude Bernard, Paris, France. [5]Unité

Mixte de Recherche 216 IRD, Université Paris Descartes, Paris, France. [6]Centre National de Référence du Paludisme, Paris, France. [7]Unité de Recherche Mixte Sup-Agro-Inra MISTEA, SupAgro, Montpellier, France. [8]Unité de Recherche Mixte MD3, Institut de Recherche Biomédicale des Armées, Marseille, France.

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Address for correspondence: Nobumichi Kobayashi, Department of Hygiene, Sapporo Medical University School of Medicine, S-1 W-17, Chuo-ku, Sapporo 060-8556, Japan; email: nkobayas@sapmed.ac.jp

Malaria Prophylaxis Failure with Doxycycline, Central African Republic, 2014

Marylin Madamet, Tiphaine Gaillard, Guillaume Velut, Cecile Ficko, Pascal Houzé, Claire Bylicki, Stéphane Mérat, Sandrine Houzé, Nicolas Taudon, Rémy Michel, Pierre Pasquier, Christophe Rapp, Bruno Pradines

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Author affiliations: Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France (M. Madamet, N. Taudon, B. Pradines); Aix Marseille Université, Marseille, France (M. Madamet, N. Taudon, B. Pradines); Centre National de Référence du Paludisme, Marseille (M. Madamet, N. Taudon, B. Pradines); Hôpital d'Instruction des Armées Saint Anne, Toulon, France (T. Gaillard); Centre d'Epidémiologie et de Santé Publique des Armées, Marseille, France (G. Velut, R. Michel); Hôpital d'Instruction des Armées Begin, Saint Mandé, France (C. Ficko, S. Mérat, P. Pasquier, C. Rapp); Hôpital Saint-Louis, Paris, France (P. Houzé); Hôpital Bichat Claude Bernard, Paris (S. Houzé); Institut pour la Recherche et le Développement, Paris (S. Houzé), Université Paris Descartes, Paris (S. Houzé); Centre National de Référence du Paludisme, Paris (S. Houzé); Antenne Médicale de Fontevraud, Fontevraud, France (C. Bylicki); Ecole du Val-de-Grâce, Paris (R. Michel, C. Rapp)

To the Editor: Doxycycline is an effective antimalarial prophylactic drug when administered as a monotherapy 1 day before, daily during, and for 4 weeks after travel to an area where malaria is endemic (*1*). Doxycycline is currently a recommended chemoprophylactic regimen for travelers visiting areas where malaria is endemic and has a high prevalence of chloroquine or multidrug resistance (*2*). The World Health Organization also recommends doxycycline in combination with quinine or artesunate as the second-line treatment for uncomplicated Plasmodium falciparum malaria (*3*).

Prophylactic and clinical failures of doxycycline against *P. falciparum* have been associated with both inadequate doses (*4*) and poor patient compliance (*5*). However, resistance can also explain failures of prophylaxis. Cycline resistance in *Plasmodium* spp. has been documented as a consequence of selective drug pressure in a *P. berghei* murine malaria model (*6*). The administration of increasing doses of minocycline to mice infected with 1×10^7 parasites for 86 successive passages over 600 days made it possible to obtain a resistant *P. berghei* strain with a median drug inhibitory concentration (IC_{50}) of 600 mg/kg/d, which is 6-fold higher than that of the susceptible starting strain (100 mg/kg/d) (*6*). A Bayesian mixture modeling approach identified 3 different phenotypes (low, medium, and high doxycycline IC_{50} phenotypic groups) among *P. falciparum* clinical isolates (*7,8*). Using 90 isolates from 14 countries, we demonstrated that increases in copy numbers of *P. falciparum* metabolite drug transporter gene (*Pfmdt*, PFE0825w) and *P. falciparum* GTPase TetQ gene (*PfTetQ*, PFL1710c) are associated with reduced susceptibility to doxycycline (*9*); this association was later confirmed (*7*). In addition, isolates with *PfTetQ* KYNNNN motif repeats are associated with in vitro reduced susceptibility to doxycycline and with a significantly higher probability of having an IC_{50} above the doxycycline resistance threshold of 35 mM (*9,10*).

We report a case of documented malaria prophylactic failure with doxycycline in a 26-year-old soldier from France who was infected during a 6-week peacekeeping mission in the Central African Republic in 2014. According to his colleagues and the collective prophylaxis intake, the patient had been compliant with doxycycline prophylaxis. On admission to a hospital in Bangui, Central African Republic, the patient had fever (temperature 40°C), alteration of consciousness, and hypotension. The diagnosis of severe *P. falciparum* malaria was made on the basis of a rapid diagnostic test confirmed by a blood smear test (parasitemia 8% on day 0). Intravenous artesunate was immediately started, in accordance with World Health Organization recommendations (*3*). The patient's clinical condition worsened, and kidney failure developed. Twenty-four hours later (day 1), he was transported by airplane to Bégin Military Teaching Hospital (Saint-Mandé, France). On admission, he had

cerebral edema and a *P. falciparum* parasitemia level of 0.7%. The patient died 1 day later (day 2).

A blood sample obtained from the patient on day 1 in Bangui showed a doxycycline concentration of 195 μg/mL plasma. This concentration, which was determined by liquid chromatography coupled with tandem mass spectrometry, was compatible with a last doxycycline uptake 1 day before diagnosis (day -1). The finding of the expected doxycycline plasma concentration, together with assurances (colleague's statements and collective intake of doxycycline) that the patient had followed the drug regimen, was sufficient to suggest prophylaxis failure in a treatment-compliant patient.

The *P. falciparum* sample obtained from the patient on arrival in France was evaluated for in vitro susceptibility to doxycycline, but the evaluation was unsuccessful. The number of copies of *PfTetQ* and *Pfmdt* genes were evaluated relative to the single-copy *P. falciparum b-tubulin* gene (*pf*β*tubulin*), as previously described (*7,8*). The sample was assayed in triplicate. The $2^{-\Delta\Delta Ct}$ method (where C_t indicates cycle threshold) of relative quantification was used and adapted to estimate the number of copies of *Pfmdt* and *PfTetQ* by using the formula $DDC_t = (C_{t\ (PfTetQ\ or\ Pfmdt)} - C_{t\ (Pf\beta tubulin)})_{Sample} - (C_{t\ (PfTetQ\ or\ Pfmdt)} - C_{t\ (Pf\beta tubulin)})_{Calibrator}$. Genomic DNA extracted from 3D7 *P. falciparum*, which has a single copy of each gene, was used for calibrator sample; *Pf*β*tubulin* served as the control housekeeping gene. The experiment was assayed twice. The sample had 2 copies of *PfTetQ* and *Pfmdt* genes, which suggested decreased in vitro susceptibility of the sample to doxycycline (*8,9*). The genotyping of *PfTetQ* sequence polymorphisms was done by using conventional methods with the primers *PfTetQ* forward (5'-TCACGACAAATGTGCTAGATAC-3') and *PfTetQ* reverse (5'-ATCATCATTTGTGGTGGATAT-3'), as previously described (*10*). Two *PfTetQ* KYNNNN motif repeats were found in the sample; <3 KYNNNN motif repeats are predictive of in vitro *P. falciparum*–resistant parasites with an IC_{50} of >35 mM (odds ratio 15) (*10*). The 2 copies of *Pfmdt* and the 2 KYNNNN motif repeats have been shown to be associated with parasites with in vitro resistance to doxycycline (*9,10*). The association of doxycycline resistance (prophylactic failure with statement of correct intake and the presence of an expected concentration) with increased *Pfmdt* copies and decreased *PfTetQ* KYNNNN motif repeats suggest that these molecular markers are predictive markers of doxycycline resistance that can be used for resistance surveillance.

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Address for correspondence: Bruno Pradines, Unité de parasitologie et d'entomologie, Institut de recherche biomédicale des Armées, BP 73, 91223 Brétigny sur Orge, France; email: bruno.pradines@free.fr

Avian Gyrovirus 2 DNA in Fowl from Live Poultry Markets and in Healthy Humans, China

Jianqiang Ye,[1] Xiaoyan Tian,[1] Quan Xie, Yu Zhang, Yuanzhao Sheng, Zhenwen Zhang, Chengming Wang, Hong Zhu, Yumeng Wang, Hongxia Shao, Aijian Qin

Author affiliations: Ministry of Education Key Laboratory for Avian Preventive Medicine and Key Laboratory of Jiangsu Preventive Veterinary Medicine, Yangzhou University, Yangzhou, China (J. Ye, X. Tian, Q. Xie, Y. Zhang, Y. Sheng, H. Zhu, Y. Wang, H. Shao, A. Qin); Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou (J. Ye, Z. Zhang, C. Wang, H. Shao, A. Qin); College of Medicine, Yangzhou University, Yangzhou (Z. Zhang)

[1]These authors contributed equally to this article.

CHAPITRE II : Limites de l'utilisation des gènes *pfmdt* et *pftetQ* comme marqueurs moléculaires de diminution de sensibilité à la doxycycline

L'objectif de ce nouveau travail était de valider l'utilisation des gènes *pfmdt* et *pftetQ* comme marqueurs prédictifs de la diminution de sensibilité à la doxycycline sur des isolats Sénégalais de *P. falciparum*. Les valeurs de CI_{50} de la doxycycline s'échelonnaient entre 0.43 à 43.54 µM. Sur les 113 isolats étudiés, 18 (15.9 %) appartenaient au phénotype à forte CI_{50} défini pour ce groupe par des valeurs supérieures à 25 µM. Après évaluation du nombre de copies des gènes *pfmdt* et *pftetQ*, les résultats n'étaient pas en faveur d'une différence statistique significative entre le groupe à faible CI_{50} (< 25 µM) et le groupe à forte CI_{50} (> 25 µM). Cependant, l'étude concluait à la nécessité de reproduire l'étude sur davantage d'isolats, une tendance pouvant être observée mais le nombre d'isolats à fortes CI_{50} relativement faible ne permettant pas de valider le modèle.

Article : **Gaillard T, Fall B, Tall A, Wurtz N, Diatta B, Lavina M, Fall KB, Sarr FD, Baret E, Diémé Y, Wade B, Bercion R, Briolant S, Pradines B. Absence of association between *ex vivo* susceptibility to doxycycline and *pftetQ* and *pfmdt* copy numbers in *Plasmodium falciparum* isolates from Dakar, Senegal. Clin Microbiol Infect 2012, 18:E238–240.**

Nous avons identifié un deuxième cas d'échec prophylactique à la doxycycline en République Centrafricaine, non du à une résistance à la doxycycline mais à un retard d'apparition des symptômes (accès survenant trois mois après le retour en France et deux mois après l'arrêt de la prophylaxie par doxycycline). L'isolat de *P. falciparum* présentait une CI_{50} faible à la doxycycline (< 10 µM) associée à une copie des gènes *pfmdt* et *pftetQ* avec une répétition du motif KYNNNN sur le gène *pftetQ* < 3 (prédictive d'une souche résistante *in vitro* avec une CI_{50} > 35 µM). Ce résultat montre que l'association entre le nombre de répétitions du motif KYNNNN sur le gène *pftetQ* et une diminution de sensibilité à la doxycycline n'est pas satisfaisante.

Article : **Javelle E, Madamet M, Gaillard T, Velut G, Surcouf C, Michel R, Garnotel E, Simon F, Pradines B. Delayed *P. falciparum* Malaria after doxycycline Prophylaxis, Central African Republic, 2015. Antimicrob Agents Chemother 2015 (submitted).**

L'objectif de cette nouvelle étude était d'évaluer pour la première fois les distributions de CI_{50} pour la doxycycline pour des isolats asiatiques de *P. falciparum* et de valider les gènes *pfmdt* et *pftetQ* comme marqueurs moléculaires de résistance à la doxycycline. Le travail portait sur 620 isolats cliniques obtenus entre 2001 et 2010 au Centre de Recherche sur le Malaria de Shoklo (SMRU) situé à la frontière birmano-thaï. Les valeurs de CI_{50} de doxycycline obtenues s'échelonnaient entre 0.21 to 55.44 μM. Seuls onze isolats sur 620 présentaient une CI_{50} supérieure à 35 μM, chiffre particulièrement bas révélateur d'une faible incidence de la résistance de *P. falciparum* à la doxycycline en Thaïlande, zone de mulirésistance. D'autre part, l'étude bayésienne de la distribution des CI_{50} de doxycycline permettait d'observer deux niveaux de sensibilité à la doxycycline, un groupe majoritaire de 590 isolats à faible CI_{50} et un groupe de 30 isolats à forte CI_{50}. Ces résulats différaient de ceux précédemment obtenus avec les isolats d'origine africaine.

Dans un deuxième temps était réalisée la quantification du nombre de copies des gènes *pfmdt* et *pftetQ* selon le protocole préalablement appliqué sur les isolats africains ; aucune association du nombre de copies de ces deux gènes ne pouvait être établie avec les fortes DOX CI_{50}. Il en était de même pour l'association entre le nombre de répétitions du motif KYNNNN sur le gène *pftetQ* et une diminution de sensibilité à la doxycycline. Ces gènes ne semblent pas être impliqués dans la diminution de sensibilité à la doxycycline en Thailande.

Article : **Gaillard T, Sriprawat K, Briolant S, Wangsing C, Wurtz N, Baragatti M, Lavina M, Pascual A, Nosten F, Pradines B. Molecular markers and *in vitro* susceptibility to doxycycline in *Plasmodium falciparum* isolates from Thailand. Antimicrob Agents Chemother 2015, 59(8): 5080-5083.**

Le modèle expérimental révéle ainsi ses limites, avec peut-être une composante géographique. Cette absence de corrélation entre données *in vitro* et polymorphisme des gènes *pfmdt* et *pftetQ* vient d'être récemment montré sur des isolats de Guyane [63]. D'autres mécanismes se doivent d'être évoqués. La résistance à la doxycycline semble être multigénique. Il est primordial d'identifier de nouveaux gènes pouvant être impliqués dans la diminution de sensibilité à la doxycycline.

Gaillard T, Fall B, Tall A, Wurtz N, Diatta B, Lavina M, Fall KB, Sarr FD, Baret E, Diémé Y, Wade B, Bercion R, Briolant S, Pradines B.

Absence of association between *ex vivo* susceptibility to doxycycline and *pftetQ* and *pfmdt* copy numbers in *Plasmodium falciparum* isolates from Dakar, Senegal.

Clin Microbiol Infect 2012, 18:E238–240.

Absence of association between *ex vivo* susceptibility to doxycycline and *pftetQ* and *pfmdt* copy numbers in *Plasmodium falciparum* isolates from Dakar, Senegal

T. Gaillard[1,2], B. Fall[3], A. Tall[4], N. Wurtz[1], B. Diatta[5], M. Lavina[1], K. B. Fall[6], F. D. Sarr[4], E. Baret[1], Y. Diémé[3], B. Wade[7], R. Bercion[3], S. Briolant[1] and B. Pradines[1,8]

1) Unité de Parasitologie – Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes – UMR 6236, Institut de Recherche Biomédicale des Armées, Marseille, 2) Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, 3) Fédérations des Laboratoires, Hôpital Principal de Dakar, Dakar, Sénégal, 4) Unité d'Epidémiologie des Maladies Infectieuses, Institut Pasteur de Dakar, Dakar, Sénégal, 5) Service de Réanimation Médicale, Hôpital Principal de Dakar, Dakar, Sénégal, 6) Service de Pathologie Infectieuse, Hôpital Principal de Dakar, Dakar, Sénégal, 7) Chefferie, Hôpital Principal de Dakar, Dakar, Sénégal and 8) Centre National de Référence du Paludisme, Marseille, France

Abstract

The objective of this study was to validate the use of *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline in 113 *Plasmodium falciparum* isolates from Dakar, Senegal. The results show that copy numbers of *pftetQ* and *pfmdt*, estimated by TaqMan real-time PCR, are not significantly associated with reduced susceptibility to doxycycline *in vitro*; however, the number of samples with a high doxycycline IC_{50} was likely to be too low to derive statistically significant results. Thus, no definitive conclusions could be drawn. The markers should be further tested by analysing more isolates.

Keywords: Antimalarial drug, *in vitro*, malaria, *Plasmodium*, resistance

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Corresponding author: B. Pradines, Unité de Parasitologie – Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes – UMR 6236, Institut de Recherche Biomédicale des Armées, Marseille, France
E-mail: bruno.pradines@free.fr

Daily administration of doxycycline (DOX) is now one of the recommended chemoprophylactic regimens for travellers visiting malaria-endemic areas with high prevalence of resistance to chloroquine or multidrug resistance [1]. In addition, the French malaria consensus recommends quinine associated with DOX for Asian and South-American *P. falciparum*. DOX remains recommended in the second-line treatment of uncomplicated *falciparum* malaria or in the treatment of severe malaria in combination with artesunate or quinine for a 7-day course [2], but its use is limited. Prophylactic failure of DOX against *P. falciparum* has been associated with inadequate doses [3] or poor compliance [4]. Failures with prophylactic DOX could be also explained by resistance.

Our ability to maximize the efficacy and longevity of antimalarials as tools for malaria control will depend critically on intensive research (to identify *in vitro* markers) and on *in vitro* and *in vivo* surveillance programmes. It is necessary to identify molecular markers that predict DOX resistance so that active surveillance can monitor temporal trends in parasite susceptibility [5]. While no clinical failure in treating *falciparum* malaria with DOX has yet been reported, a Bayesian mixture modelling approach has distinguished three different *in vitro* phenotypic groups (low, medium and high DOX IC_{50}) among 747 *P. falciparum* isolates obtained from 14 African countries over a 9-year period [6]. The sequences of 11 *P. falciparum* genes analogous to those involved in bacterial resistance to DOX were obtained from 30 isolates from each phenotypic group. Data suggested that copy numbers of a tetQ GTPase family gene, *pftetQ* (PFL1710c), and a metabolic drug transporter gene, *pfmdt* (PFE0825w), were potential molecular markers of decreased *in vitro* susceptibility to DOX in African isolates [7].

The objective of this study was to validate the use of *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to DOX in field isolates from Dakar, Senegal.

In total, 113 patients with malaria were recruited between October 2009 and October 2010 at the Hôpital Principal de Dakar and at Centre de Santé Elisabeth Diouf in Dakar. Venous blood samples were collected in Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ, USA) prior to patient treatment; blood samples were used to test for drug susceptibility within 24 h of collection.

Informed oral consent was obtained from patients and/or their parents before blood collection. The study was reviewed and approved by the ethical commission of Hôpital Principal de Dakar.

Ex vivo testing of DOX susceptibility was performed as previously described by a 42-h culture test revealed by the *Plasmodium* lactate dehydrogenase (pLDH) ELISA method [8].

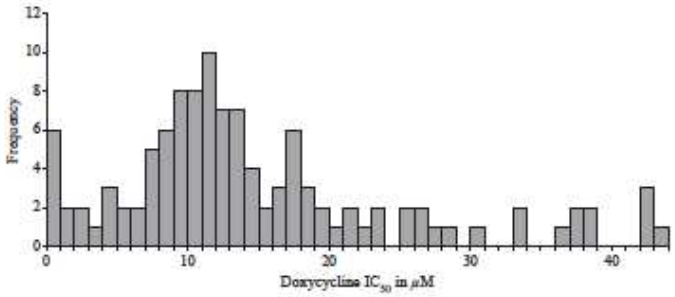

FIG. 1. Distribution of the doxycycline IC_{50} for the 113 *P. falciparum* isolates.

pfmdt and *pftetQ* copy numbers were estimated by TaqMan real-time PCR as previously described [7].

Data were analysed using R software (version 2.10.1.). Differences in *pfmdt* and *pftetQ* copy numbers across phenotypic groups were tested using the Mann–Whitney *U*-test. Genotype proportions were compared using the Fisher's exact test. The Spearman's rank correlation coefficient (rho) was evaluated between *pfmdt* and *pftetQ* copy numbers. Statistical differences were considered significant when $p < 0.05$.

The IC_{50} values were distributed from 0.43 to 43.54 μM (Fig. 1). Of the 113 Senegalese isolates, 18 (15.9%) belong to the high-DOX-IC_{50} phenotype ($IC_{50} > 25$ μM). There was no statistically significant difference between the group with $IC_{50} < 25$ μM and the group with $IC_{50} > 25$ μM in terms of the percentage of isolates with one or more copies of the *pftetQ* gene (p 0.079, Fisher's exact test) or *pfmdt* gene (p 0.066, Fisher's exact test) (Table 1).

No statistically significant difference was found between DOX IC_{50} medians of isolates with one copy of the *pftetQ* gene (mean = 14.41 μM) and more than one copy of the gene (mean = 20.64 μM) (p 0.16, Mann–Whitney *U*-test) (Table 2). Similarly, no statistically significant difference was found between DOX IC_{50} medians of isolates with one copy of the *pfmdt* gene (mean = 14.49 μM) and more than one copy of the gene (mean = 26.04 μM) (p 0.30, Mann–Whitney *U*-test) (Table 2). *pfmdt* and *pftetQ* copy numbers were significantly correlated ($\rho = 0.30$, CI 95% (0.19–0.40), Spearman's test, $p < 0.0001$).

Most DOX prophylactic failures against *P. falciparum* are associated with the use of standard doses that result in lower-than-expected serum drug levels [9], inadequate doses [3] or poor compliance [4,10]. Moreover, DOX pharmacokinetic parameters could explain some of these cases. DOX has a short elimination half-life (16 h) and a short mean residence time (63% of the administered dose is eliminated in 27 h) [11]. In addition, its slow action *in vitro* has a delayed effect upon growth and requires prolonged incubation of parasites [12]. Determination of IC_{50} after two generations of parasite growth decreases the 42-h IC_{50} by a factor from 10 to 20 [13,14]. However, in practice, the standard 42-h test is still used to monitor DOX *in vitro* susceptibility surveillance.

pftetQ is a *tetQ* GTPase-gene-family analogue of bacterial ribosomal protection protein genes, which are involved in bacterial resistance to cyclines. A metabolic drug transporter gene, *pfmdt*, shares high sequence identity with efflux pumps. In a multivariate logistic regression model, an increase in the *pfmdt* copy number was associated with high DOX IC_{50}, with an adjusted odds ratio (OR) of 7.09 (p 0.011); similarly, an increase in the *pftetQ* copy number was associated with

TABLE 1. Statistical analysis of *pftetQ* and *pfmdt* copy numbers in 113 Senegalese isolates (Fisher's exact test)

	PftetQ		*Pfmdt*	
	IC_{50} < 25 µM	IC_{50} > 25 µM	IC_{50} < 25 µM	IC_{50} > 25 µM
Copy number > 1	4	3	1	2
Copy number = 1	91	15	94	16
Percentage	4.40	20.00	1.06	12.50
Fisher's exact test	p value	0.0793	p value	0.0656

TABLE 2. Statistical analysis of *pftetQ* and *pfmdt* copy numbers in Senegalese isolates (Mann–Whitney *U*-test)

	pftetQ		*pfmdt*	
	Copy number = 1	Copy number > 1	Copy number = 1	Copy number > 1
Number of values	106	7	110	3
Minimum	0.43	5.57	0.43	6.72
25% percentile	8.72	10.43	8.935	6.72
Median	11.79	17.2	12.06	33.38
75% percentile	18.06	33.38	18.06	38.02
Maximum	43.54	38.02	43.54	38.02
Mean	14.41	20.64	14.49	26.04
Standard deviation	10.11	12.13	10	16.89
Standard error	0.9819	4.585	0.9538	9.752
Lower 95% CI of mean	12.46	9.421	12.6	−15.92
Upper 95% CI of mean	16.36	31.86	16.38	68
Mann–Whitney *U*-test	p value	0.1617	p value	0.2961

an adjusted OR of 5.23 (p 0.042) [7]. In this study, there was no statistically significant difference between the group with an IC_{50} < 25 μM and the group with an IC_{50} > 25 μM in terms of the percentage of isolates with one or more copies of the *pfmdt* gene (p 0.079) or of the *pfmdt* gene (p 0.066). However, these data showed a tendency; the percentages of *pfmdt* and *pfmdt* with more than one copy were higher in the group with an IC_{50} > 25 μM (20% vs. 4.4% and 12.5% vs. 1.1%, respectively). The significance levels of these associations were just above the p-value threshold. These results suggest that the number of isolates from the group with high DOX IC_{50} (15.9%) was likely to be too low to yield statistically significant differences, thus showing the necessity of using more isolates to assess gene copy numbers. Epidemiological studies using large numbers of parasites with reduced susceptibility to DOX are required if *pfmdt* and *pfmdt* are to be used as markers of reduced *in vitro* DOX susceptibility.

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Transparency Declaration

All authors declare that they have no potential conflicts of interest.

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Delayed *P. falciparum* Malaria after doxycycline Prophylaxis, Central African Republic, 2015.

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Delayed *P. falciparum* Malaria after Doxycycline Prophylaxis, Central African Republic, 2015

Running title: Delayed *P. falciparum* Malaria after Doxycycline

Emilie Javelle,[a,b] Marylin Madamet,[b,c,d] Tiphaine Gaillard,[e] Guillaume Velut,[f] Corinne Surcouf,[g] Rémy Michel,[h,i] Eric Garnotel,[g,i] Fabrice Simon,[a,b,i] Bruno Pradines[c,d,j]

Service de Maladies Infectieuses et Tropicales, Hôpital d'Instruction des Armées Laveran, Marseille, France[a]; Aix Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Marseille, France[b]; Equipe Résidente de Recherche en Infectiologie Tropicale, Institut de Recherche Biomédicale des Armées, Hôpital d'Instruction des Armées Laveran, Marseille, France[c]; Centre National de Référence du Paludisme, Marseille, France[d]; Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, France[e]; Service de Santé Publique, Centre d'Epidémiologie et de Santé Publique des Armées, Marseille, France[f]; Fédération des Laboratoires, Hôpital d'Instruction des Armées Laveran, Marseille, France[g]; Service de Surveillance Epidémiologique, Centre d'Epidémiologie et de Santé Publique des Armées, Marseille, France[h]; Ecole du Val-de-Grâce, Paris, France[i]; Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France[j]

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More than 97% of imported *Plasmodium falciparum* malaria infections in France in 2014 were diagnosed within 2 months after returning from an endemic area (personnel data). The onset of malaria symptoms occurred a median of 6 days after arrival in France (25%-75% percentile [2-11]). Most of the delayed malaria cases appeared in patients with partial immunity in the absence of antimalarial prophylaxis (1,2). Doxycycline 100 mg, given one day before travel to an area with endemic malaria administered daily during travel and for four weeks after return from an endemic area is currently a recommended chemoprophylactic regimen for travelers visiting malaria-endemic areas with a high prevalence of chloroquine or multidrug resistance (3). Causes of prophylactic and clinical failures of doxycycline against *P. falciparum* are both inadequate doses and poor patient compliance due to simply forgetting and side effects/safety concerns (4). However, resistance can also explain failures of prophylaxis. Here, a case of *P. falciparum* malaria in a French soldier is reported. He was diagnosed 3 months after his last stay in an endemic area and after correct intake of chemoprophylaxis by doxycycline (self-reported nature of the compliance).

A 36-year-old French soldier without past medical history was admitted to Laveran Military Hospital in Marseille, France, in January 2015 with a high grade fever (temperature 39°C). Three months previously (November 2014), he had returned from a 4-month peace keeping mission in the Central African Republic and had stayed in metropolitan France since then. He had been compliant in taking doxycycline prophylaxis daily during the mission until the end of the fourth week after his return home. He used appropriate personal protective measures against mosquito bites, including bed net and mosquito repellant, during the mission. He didn't take medication that can interact with doxycycline pharmacokinetic. The diagnosis of uncomplicated *P. falciparum* malaria was made on the basis of thick smear and QBC® (Quantitative buffy coat) Malaria Test completed by a thin smear for determination of

parasitemia (0.7%). No other infection was documented. He was successfully treated with atovaquone-proguanil (1000 mg/400 mg/day for 3 days) (parasitemia <0.01% at Day 3).

The delayed malaria presentation despite appropriate prophylaxis and the absence of re-exposure suggested a 3-month period of sub-clinical and latent *P. falciparum* infection. A decreased susceptibility to doxycycline was investigated, particularly because in 2014 a fatal cerebral malaria due to *P. falciparum* isolate resistance to doxycycline occurred in a French soldier in the Central African Republic (expected plasmatic concentration of doxycycline with predictive molecular markers of *in vitro* resistance i.e., two copies of *pfmdt* and *pftetQ* and two PfTetQ KYNNNN motif repeats in the isolate) (*5*).

Indeed, to date, two studies suggest that the copy numbers >1 of a TetQ GTPase family gene, *pftetQ*, and a metabolic drug transporter gene, *pfmdt*, are potential molecular markers of decreased *in vitro* susceptibility to doxycycline in African isolates (6,7). In addition, isolates with PfTetQ KYNNNN motif repeats <3 are associated with *in vitro* reduced susceptibility to doxycycline and a significantly higher probability of having a half maximal inhibitory concentration (IC_{50}) above the doxycycline resistance threshold of 35 µM (odds ratio of 15) (6,8).

In our patient, the *in vitro* susceptibility to doxycycline and other antimalarial drugs was successfully evaluated by using the 72-hour histidine-rich protein 2 test in controlled atmospheric conditions that consisted of 10% O_2, 5% CO_2 and 85% N_2 (9). *In vitro*, the isolate was susceptible to doxycycline (8.7 ± 0.6 µM (standard deviation), no = 4) (10) and the majority of the antimalarial drugs (Table 1). The IC_{50} of mefloquine was relatively high compared with previous studies in Africa. The incubation time is one of the conditions that interferes significantly with the IC_{50} values for doxycycline. The IC_{50} values decrease by a factor between 10 to 100 in prolonged exposure to doxycycline (11,12). However, the current time incubation ranges from 48h to 72h for all of the studies that evaluated the *ex vivo* or *in*

vitro susceptibility of *P. falciparum* isolates to doxycycline. The number of copies of the *pfTetQ* and *pfmdt* genes was evaluated relative to the single-copy gene *pfβtubulin* as previously described (6,7). The genotyping of *pfTetQ* sequence polymorphisms was performed by a conventional method as previously described (5,8). The sample had only one copy of the *pfTetQ* and *pfmdt* genes; one PfTetQ KYNNNN motif repeat was also present but was not associated with $IC_{50} > 35$ µM as it was previously shown (8). In this case, the single copy number of *pfTetQ* and *pfmdt* suggested *in vitro* susceptibility of *P. falciparum* to doxycycline, and the single PfTetQ KYNNNN motif repeat was not predictive of *in vitro* resistance to doxycycline. The activity of doxycycline was demonstrated to be partially effective on the liver forms of *P. falciparum* (13). These findings justify the recommendation of the currently approved doxycycline regimen (i.e., 100 mg daily for four weeks after returning from an endemic area). However, it was not sufficient in this case. This prophylactic failure with doxycycline seems to be not due to inadequate doses, poor patient compliance or resistance but to much longer parasite liver stage development.

Thus, we report a delayed doxycycline prophylaxis failure in a non-immune compliant patient with a doxycycline susceptible *P. falciparum* isolate from the Central African Republic.

Competing interests

The authors have declared that they have no competing interests.

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144 **Table 1** *In vitro* susceptibility to standard antimalarial drugs of the *Plasmodium*
145 *falciparum* isolate in comparison with *P. falciparum* W2 clone tested with the same plate
146 batch
147

Drugs	Isolate IC_{50}	W2 IC_{50}	Ratio IC_{50} Isolate/W2	Resistance cut-off
Doxycycline	8.7 µM*	10.2 µM	0.85	35 µM
Chloroquine	24.7 nM	423 nM	0.06	100 nM
Quinine	35.4 nM	350 nM	0.10	800 nM
Desethylamodiaquine	20.8 nM	110 nM	0.19	80 nM
Lumefantrine	0.6 nM	0.6 nM	1.00	150 nM
Mefloquine	64.0 nM	26.9 nM	2.38	30 nM
Piperaquine	26.4 nM	57.3 nM	0.46	135 nM
Pyronaridine	20.7 nM	32.7 nM	0.63	60 nM
Dihydroartemisinin	1.5 nM	1.9 nM	0.79	10.5 nM
Artesunate	1.8 nM	1.6 nM	1.13	10.5 nM

148 IC_{50}: Inhibitory concentration 50%
149 *Average of 4 independent experiments
150

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Molecular Markers and *In Vitro* Susceptibility to Doxycycline in *Plasmodium falciparum* Isolates from Thailand

Tiphaine Gaillard,[a,b,c] Kanlaya Sriprawat,[d] Sébastien Briolant,[a,b,e,f] Chirapat Wangsing,[d] Nathalie Wurtz,[a,b] Maïli Baragatti,[g] Morgane Lavina,[a,b] Aurélie Pascual,[a,b,h] François Nosten,[d,i] Bruno Pradines[a,b,h,j]

Unité de Parasitologie, Département d'Infectiologie de Terrain, Institut de Recherche Biomédicale des Armées, Marseille, France[a]; Aix Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Marseille, France[b]; Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, France[c]; Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand[d]; Direction Inter-Armées du Service de Santé, Cayenne, French Guiana[e]; Laboratoire de Parasitologie, Institut Pasteur de la Guyane, Cayenne, French Guiana[f]; Unité de Recherche Mixte Sup-Agro-Inra MISTEA, SupAgro, Montpellier, France[g]; Centre National de Référence du Paludisme, Marseille, France[h]; Centre for Tropical Medicine, University of Oxford, Oxford, United Kingdom[i]; Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France[j]

Determinations of doxycycline 50% inhibitory concentrations (IC_{50}) for 620 isolates from northwest Thailand were performed via the isotopic method, and the data were analyzed by the Bayesian method and distributed into two populations (mean IC_{50}s of 13.15 μM and 31.60 μM). There was no significant difference between the group with low IC_{50}s versus the group with high IC_{50}s with regard to copy numbers of the *Plasmodium falciparum tetQ* (*pfrtetQ*) gene ($P = 0.11$) or *pfmdt* gene ($P = 0.87$) or the number of PfTetQ KYNNNN repeats ($P = 0.72$).

The World Health Organization (WHO) recommends doxycycline in combination with quinine or artesunate as the second-line treatment for uncomplicated *Plasmodium falciparum* malaria (1). Doxycycline is currently one of the recommended chemoprophylactic regimens for travelers visiting areas of malaria endemicity, particularly in countries with multiple-drug resistance. The prophylactic failure of doxycycline against *P. falciparum* may be explained by drug resistance, but this has not yet been documented. Indeed, -cycline resistance in *Plasmodium* has been documented only as a consequence of drug pressure in a *P. berghei* murine malaria model (2).

Recent studies have suggested that *P. falciparum mdt* (*pfmdt*) and *pfrtetQ* copy numbers are potential molecular markers of decreased *in vitro* susceptibility to doxycycline in African *P. falciparum* isolates (3, 4). In addition, isolates with PfTetQ KYNNNN motif repeats have been associated with reduced *in vitro* susceptibility to doxycycline and with a significantly greater probability of a 50% inhibitory concentration (IC_{50}) greater than the doxycycline resistance threshold of 35 μM (3, 5).

The objective of this study was to evaluate for the first time the distribution of doxycycline IC_{50}s for *P. falciparum* isolates collected in Asian patients and to validate the use of the *pfrtetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline.

Clinical isolates were obtained from patients with acute *P. falciparum* malaria attending Shoklo Malaria Research Unit (SMRU) clinics between 2001 and 2010. The SMRU clinics are all located along the Thai-Myanmar border. Isolates were collected from primary infections with a parasite density of at least 5 parasites/1,000 red blood cells. Samples were kept at room temperature before being transported to the main laboratory, where they were immediately tested *in vitro*. The fresh parasite isolate samples were obtained as part of prospective clinical evaluations of antimalarial drug therapy. Written informed consent translated into the patient's own language was obtained from each participant, whose consent signature was witnessed. The studies were approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University, and Oxford University. All cases were microscopically confirmed to be *falciparum* malaria.

In vitro drug susceptibility was determined by the hypoxanthine uptake inhibition assay, which has been described previously (6). The reproducibility of the IC_{50} measurements was assessed regularly using cloned *P. falciparum* strain K1 (Table 1). There was a significant reduction in the doxycycline median IC_{50} for strain K1 in 2003 ($P < 0.0001$). The doxycycline IC_{50}s for the 620 isolates ranged from 0.21 to 55.44 μM, with a mean of 14.0 μM ± 6.5 μM. The average parameter estimates for the IC_{50}s and their distribution by year are given in Table 1 and in Fig. 1. There were significant differences in the doxycycline median IC_{50}s for the sample isolates collected during the study period of 2001 to 2010. The reduction in the doxycycline median IC_{50} in sample isolates collected in 2003 can be explained only by a bias in methodology. Considering the reduction in the doxycycline median IC_{50} for strain K1 in 2003, only 7 isolates of 620 (1.1%) had a doxycycline IC_{50} greater than 35 μM, which was the threshold determined for reduced susceptibility to doxycycline (7), demonstrating that isolates with reduced susceptibility to doxycycline (according to the IC_{50} values) were rare even in Thailand, a geographic area known for multiple drug resistance. This cutoff value of 35 μM was determined for an exposure to doxycycline from 42 h to 48 h (7). A cutoff for *in vitro* resistance is defined for a specific methodology. For example, the *in*

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Address correspondence to Bruno Pradines, bruno.pradines@hnsch.

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TABLE 1 Statistical analysis of the 620 *P. falciparum* Thai isolates and K1 strain *in vitro* responses (IC_{50} in μM) to doxycycline by year

Parameter	Value						
	2001	2002	2003	2007	2008	2009	2010
Plasmodium falciparum clinical isolates							
No. of isolates	173	244	91	31	24	45	12
Minimum IC_{50}	2.37	0.83	0.21	7.89	9.04	7.08	14.42
25% percentile	11.99	9.66	5.13	12.05	11.66	15.91	17.41
Median	14.56	12.45	8.93	14.77	14.56	17.79	19.86
75% percentile	18.44	16.01	12.71	17.82	19.13	20.16	21.17
Maximum IC_{50}	29.28	55.44	30.48	21.5	27.33	33.36	29.26
Mean	15.16	13.44	9.61	15.08	15.89	18.37	19.93
SD	5.20	7.27	6.04	3.60	5.10	5.05	3.67
SE	0.40	0.47	0.63	0.64	1.042	0.75	1.06
Lower 95% CI of mean	14.38	12.52	8.36	13.76	13.73	16.86	17.6
Upper 95% CI of mean	15.94	14.36	10.87	16.4	19.89	16.86	22.26
Plasmodium falciparum K1 clone							
No. of isolates	6	6	7	4	4	10	6
25% percentile	15.82	13.81	8.59	11.79	8.54	15.89	17.52
Median	17.47	14.37	8.9	12.04	11.33	18.60	18.60
75% percentile	18.85	15.06	9.54	12.28	13.76	21.93	19.31

vitro effects and the IC_{50}s for doxycycline are dependent on the duration of incubation (8–10), on gas conditions, i.e., O_2 and CO_2 levels (11, 12), and on methodology, i.e., isotopic test versus immunoenzymatic or SYBR green test (13, 14). The incubation time is one of the conditions that interferes significantly with the IC_{50}s of antibiotics (10, 15). In the present study, the *in vitro* testing conditions (i.e., parasitemia, hematocrit, serum use, incubation time in the presence of doxycycline, isotopic test) were the same as those used in the previous works (3, 4, 7).

The distribution of doxycycline IC_{50}s for 620 *P. falciparum* isolates was analyzed by the Bayesian method to identify the presence of subpopulations with different levels of doxycycline chemosusceptibility as previously described for doxycycline (4) and for pyronaridine and piperaquine (16). Two distributions were

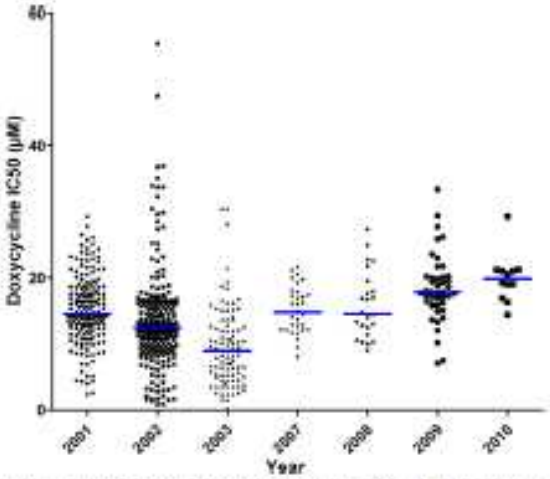

FIG 1 Doxycycline 50% inhibitory concentrations (IC_{50}s) during the 2001-to-2010 period. The horizontal bars indicate the medians.

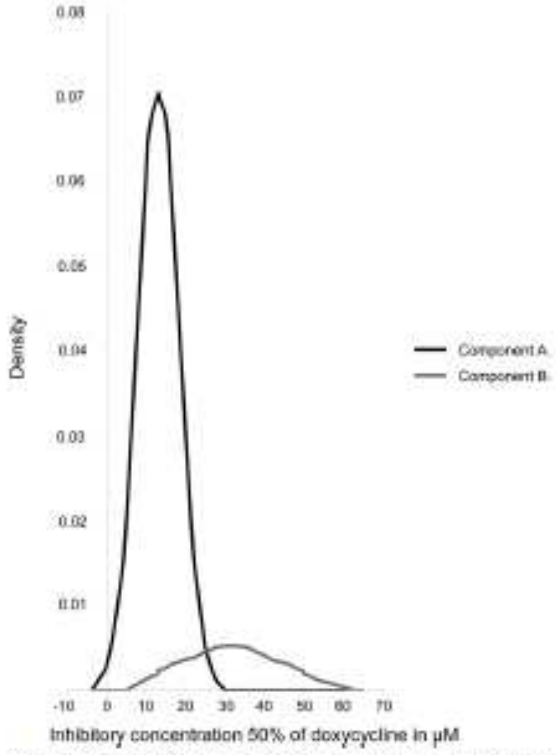

FIG 2 Distribution of the doxycycline IC_{50}s of the 620 *Plasmodium falciparum* isolates from Thailand in the two-component mixture model (Bayesian mixture modeling approach).

TABLE 2 Statistical analysis of *pfierQ* and *pfmdr* gene polymorphism in 89 *Plasmodium falciparum* isolates (Fisher's exact test)

Gene copy no. and repeat frequency	No. (%) of isolates		
	Component A	Component B	*P* value
No. of *pfmdr* copies			
>1	7 (12.1)	4 (13.3)	
1	51 (87.9)	26 (86.7)	1
No. of *pTetQ* copies			
>1	1 (1.7)	2 (6.7)	
1	58 (98.3)	28 (93.3)	0.26
No. of PfTetQ KYNNNN repeats			
<3	11 (18.6)	7 (23.3)	
3	48 (81.4)	23 (76.7)	0.59

identified with, respectively, a mean value (± standard deviation) of 13.15 ± 5.25 μM for phenotypic group A, including 590 isolates (95.3%), and a mean value of 31.60 ± 9.39 μM for phenotypic group B, including 30 isolates (4.7%) (Fig. 2). This differs from the three doxycycline phenotypes observed in *P. falciparum* African isolates under the same laboratory conditions (3, 4).

Two recent studies have demonstrated that there was an association between the *pfmdr* and *pfierQ* copy numbers and the level of doxycycline susceptibility in Africa (3, 4). In addition, in Kenya, Achieng et al. showed that isolates with one copy of *pfmdr* had a median IC_{50} lower than those with two or more *pfmdr* copies (5). The quantification of *pfmdr* and *pfierQ* copy numbers of 59 *P. falciparum* Thai isolates randomly chosen from phenotypic group A with low or moderate doxycycline IC_{50}s (mean, 9.41 μM [95% confidence interval [CI], 3.61 to 15.21 μM]) and all 30 isolates from group B with high doxycycline IC_{50}s (mean, 29.16 μM [95% CI, 22.76 to 35.56 μM]) was performed by TaqMan real-time PCR under the same conditions as previously described for African isolates (3, 4, 17). Two isolates possessed two copies of *pfierQ* and one copy of *pfmdr*. Ten isolates had one copy of *pfierQ* and two copies of *pfmdr*. Only one isolate had two copies of both *pfierQ* and *pfmdr*. All isolates with two copies of *pfmdr* or *pfierQ* had one allelic family for each of the two genes (*msp1* and *msp2*) as determined by the use of the nested PCR strategy previously described (18), confirming that these infections were clonal. Mixed-clone infections could influence TaqMan real-time PCR readouts, potentially leading to false-positive gene copy number data.

In Thai isolates, there was no association between *pfmdr* or *pfierQ* copy numbers and the level of susceptibility to doxycycline (Table 2 and Table 3). This result differs from data from previous studies in African isolates but is similar to data from a recent study from Senegal that found no significant association between doxycycline *in vitro* susceptibility and increased copy numbers of *pfierQ* ($P = 0.08$) or *pfmdr* ($P = 0.07$) (17).

In addition, reduced *in vitro* doxycycline susceptibility was found associated with PfTetQ KYNNNN sequence polymorphism: a total of <3 KYNNNN motif repeats is predictive of *P. falciparum* parasites with resistance *in vitro*, with IC_{50}s of >35 μM (odds ratio of 15) (5). There was no association between doxycycline *in vitro* susceptibility and the number of PfTetQ KYNNNN repeats in Thai isolates (Table 2 and Table 3). This result differs from those obtained in Kenyan isolates (5). The difference in these data might be explained by the differences in the methods used for parasite IC_{50} evaluation (isotopic assay versus SYBR green I-based assay), data interpretation, sample size, parasite genetic background, population structure, and much more.

These results may also indicate that the molecular mechanisms of resistance to doxycycline are more complex than anticipated. The overexpression of *pfierQ* or *pfmdr* and the PfTetQ KYNNNN sequence polymorphism could confer reduced *in vitro* susceptibility to doxycycline in association with other contributing determinants which could modulate the *in vitro* response to doxycycline. Some genes which encoded apicoplast proteins such as apicoplast ribosomal protein S10 (*arps10* gene; PF3D7_1460900.1) or ferredoxin (*fd* gene; PF3D7_1318100), a key component of the apicoplast electron transport chain, might be involved in doxycycline resistance. These two genes might be also involved in *P. falciparum* artemisinin resistance (19). Thus, further studies are needed to better characterize the genetics of doxycycline resistance in *P. falciparum*.

TABLE 3 Statistical analysis of the doxycycline IC_{50}s (in μM) based on the *pfierQ* and *pfmdr* copy numbers and PfTetQ KYNNNN repeat numbers in 89 *Plasmodium falciparum* isolates

Parameter	Value					
	No. of *pfierQ* copies		No. of *pfmdr* copies		No. of PfTetQ KYNNNN repeats	
	1	>1	1	>1	<3	3
No. of isolates	86	3	77	11	18	71
Minimum IC_{50}	2.17	15.20	2.17	2.58	3.03	2.17
25% percentile	4.34	15.20	4.33	4.44	4.59	4.44
Median IC_{50}	14.62	25.88	14.68	14.9	14.64	14.68
75% percentile	24.15	29.38	25.11	25.88	27.08	23.73
Maximum IC_{50}	55.44	29.38	55.44	36.92	36.92	55.44
Mean	15.66	23.49	15.85	16.49	16.99	15.65
SD	11.08	7.39	11.21	10.87	11.86	10.89
SE	1.20	4.27	1.28	3.28	2.79	1.29
Lower 95% CI of mean	13.28	5.14	13.30	9.19	13.92	14.87
Upper 95% CI of mean	18.03	41.84	18.39	23.79	20.71	16.43
Mann-Whitney test *P* value		0.11		0.87		0.72

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We declare that we have no competing interests.

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CHAPITRE III : Identification de nouveaux marqueurs impliqués dans la diminution de sensibilité à la doxycycline : Etude du gène *pfssrRNA*

Les investigations relatives aux gènes *pfmdt* et *pftetQ* n'ayant pas permis d'affirmer le rôle de ces marqueurs moléculaires dans la résistance de *P. falciparum* à la doxycycline au moins en Asie, il importait de rechercher d'autres gènes potentiellement impliqués.

Chez les bactéries, les tétracyclines agissent en se fixant à différentes protéines de la petite sous-unité ribosomale 30S et à différents sites de l'ARN 16S. Dans certaines espèces bactériennes telles que *Helicobacter pylori* et *Propionibacterium acnes*, la résistance aux tétracyclines n'a pas pour origine l'expression de protéines d'efflux mais la présence de mutations sur les gènes codés par l'ARN 16S. Chez *Plasmodium*, le mécanisme d'action des tétracyclines est bien moins connu. L'apicoplaste, une organelle héritée du chloroplaste des cellules végétales, longtemps sous-estimée, apparaît dorénavant comme un des acteurs majeurs de la capacité d'adaptation de *Plasmodium* à son environnement. L'apicoplaste, structure possédant son propre génome, code entre autres pour des ribosomes. Elle serait à l'origine d'un effet toxique tardif caractéristique de la doxycycline sur *Plasmodium* (comme sur d'autres protozaires tels que *Toxoplasma*), à l'origine d'une mort cellulaire inéluctable. Compte tenu des homologies entre l'ARN 16S bactérien et la petite sous-unité ribosomale apicoplastique, nous avons entrepris de rechercher d'éventuelles mutations sur le gène *PfssrRNA* (PFC10_API0057) codant cette petite sous-unité ribosomale. Cette recherche a été effectuée sur les isolats Africains et Thaï, précédemment investigués pour *pfmdt* et *pftetQ* en fonction de leur CI_{50} à la doxycycline. Malheureusement, aucune mutation sur le gène *PfssrRNA* pouvant expliquer des baisses de sensibilité à la doxyxycline n'a été mise en évidence.

Article : **Gaillard T, Wurtz N, Houzé S, Wangsing C, Hubert V, Lebras J, Nosten F, Briolant S, Pradines B. Absence of association between *Plasmodium falciparum* small subunit ribosomal RNA gene mutations and *in vitro* decreased susceptibility to doxycycline. Malar J 2015, 14 : 348.**

Une hypothèse récemment publiée est celle de gènes apicoplastiques qui pourraient être impliqués dans la résistance à l'artémisinine, tels qu'*arps 10*, codant pour le précurseur de la protéine ribosomale S10, et *fd*, codant pour la ferredoxine, un élément essentiel de la chaîne électronique de transport apicoplastique. Ces marqueurs doivent être investigués pour en déterminer un rôle éventuel dans la diminution de sensibilité à la doxycycline.

Gaillard T, Wurtz N, Houzé S, Wangsing C, Hubert V, Lebras J, Nosten F, Briolant S, Pradines B.

Absence of association between *Plasmodium falciparum* small subunit ribosomal RNA gene mutations and *in vitro* decreased susceptibility to doxycycline.

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Absence of association between *Plasmodium falciparum* small sub-unit ribosomal RNA gene mutations and in vitro decreased susceptibility to doxycycline

Tiphaine Gaillard[1,2,3], Nathalie Wurtz[1,2,4], Sandrine Houzé[5,6,7], Kanlaya Sriprawat[8], Chirapat Wangsing[8], Véronique Hubert[5,7], Jacques Lebras[5,6,7], François Nosten[8,9], Sébastien Briolant[1,2,10,11], Bruno Pradines[1,2,4,12*] and The French National Reference Centre for Imported Malaria Study Group

Abstract

Background: Doxycycline is an antibiotic used in combination with quinine or artesunate for malaria treatment or alone for malaria chemoprophylaxis. Recently, one prophylactic failure has been reported, and several studies have highlighted in vitro doxycycline decreased susceptibility in *Plasmodium falciparum* isolates from different areas. The genetic markers that contribute to detecting and monitoring the susceptibility of *P. falciparum* to doxycycline, the *pfmdt* and *pftetQ* genes, have recently been identified. However, these markers are not sufficient to explain in vitro decreased susceptibility of *P. falciparum* to doxycycline. In this paper, the association between polymorphism of the small sub-unit ribosomal RNA apicoplastic gene *pfssrRNA* (PFC10_API0057) and in vitro susceptibilities of *P. falciparum* isolates to doxycycline were investigated.

Methods: Doxycycline IC_{50} determinations using the hypoxanthine uptake inhibition assay were performed on 178 African and Thai *P. falciparum* isolates. The polymorphism of *pfssrRNA* was investigated in these samples by standard PCR followed by sequencing.

Results: No point mutations were found in *pfssrRNA* in the Thai or African isolates, regardless of the determined IC_{50} values.

Conclusions: The *pfssrRNA* gene is not associated with in vitro decreased susceptibility of *P. falciparum* to doxycycline. Identifying new in vitro molecular markers associated with reduced susceptibility is needed, to survey the emergence of doxycycline resistance.

Keywords: Malaria, *Plasmodium falciparum*, In vitro, Anti-malarial, Molecular marker, Doxycycline, Small ribosomal sub-unit RNA gene, *pfssrRNA*, 16S rRNA

Background

Doxycycline is an effective anti-malarial prophylactic drug when administered as a monotherapy 1 day before, daily during, and for 4 weeks after return from travel to an area where malaria is endemic. Doxycycline is currently a recommended chemoprophylactic regimen for travellers visiting areas where malaria is endemic and has a high prevalence of chloroquine or multidrug resistance [1–3]. The World Health Organization also recommends doxycycline in combination with quinine or artesunate as the second-line treatment for uncomplicated *Plasmodium falciparum* malaria [2].

Most prophylactic failures of doxycycline against *P. falciparum* were associated with the use of inadequate, low doses or poor compliance [4–6]. However, resistance

*Correspondence: bruno.pradines@ttee.fr
[1] Unité de Parasitologie, Département d'Infectiologie de Terrain, Institut de Recherche Biomédicale des Armées, Marseille, France
Full list of author information is available at the end of the article

could also explain prophylactic failures with doxycycline. Cyclines resistance has been documented in *Plasmodium berghei* as a consequence of minocycline drug pressure in a *P. berghei* murine malaria model [7]. Recently, one prophylactic failure has been reported [8].

A Bayesian mixture modelling approach identified three different phenotypes (low, medium, and high doxycycline IC_{50} phenotypic groups) among *P. falciparum* African clinical isolates [9, 10]. Using 90 isolates from 14 African countries, it was demonstrated that increases in copy numbers of *P. falciparum* metabolite drug transporter gene (*Pfmdt*, PFE0825w) and *P. falciparum* GTPase TetQ gene (*PfTetQ*, PFL1710c) are associated with reduced susceptibility to doxycycline [11], and this association was later confirmed in African *P. falciparum* isolates [9]. In addition, isolates with PfTetQ KYNNNN motif repeats <3 are associated with in vitro reduced susceptibility to doxycycline and with a significantly higher probability of having an IC_{50} above the doxycycline resistance threshold of 35 µM (odds ratio of 15) [11, 12]. The isolate obtained from the patient with prophylactic resistance to doxycycline harboured two copies of *pfmdt* and two PfTetQ KYNNNN motif repeats [8], consistent with previous in vitro data [12].

However, some recent publications have demonstrated that these molecular markers were certainly not only encountered in cases of reduced susceptibility to doxycycline [13, 14] and were not associated with resistance in Thai isolates [14]. Therefore, it is necessary to investigate other hypotheses. Based on bacterial world, proteins homologue to those implicated in doxycycline resistance in bacteria were identified in silico in *P. falciparum*.

Indeed, cyclines bind to proteins S4, S7, S9, and S17 of the 30S small ribosomal sub-unit and various ribonucleic acids of the 16S ribosomal RNA, preventing the binding of aminoacyl-transfer RNA to site A of the ribosome and thus blocking the elongation step of translation in bacteria [15]. Specific mutations in genes coding these targets can confer resistance to tetracyclines in bacteria. However, no point mutation was found in small sub-unit plastid ribosomal homologue plasmodial genes in African isolates (*pfrps7*, *pfrps9*, and *pfrps17*, although S7, S9, and S17) [11]. It has been also shown that resistance to tetracycline was mediated by mutations in the 16S rRNA gene, particularly in *Helicobacter pylori* or in *Propionibacterium acnes* [16–18]. An analogue of this gene exists in *P. falciparum* apicoplast, the small sub-unit ribosomal RNA gene, the *pfssrRNA* gene, (PFC10_API0057) [19–22]. First, the *pfssrRNA* gene shares 58 and 62 % identities with the 16S rRNA gene of *Propionibacterium acnes* and *Helicobacter pylori*, respectively. Secondly, this gene belongs to the apicoplast, an organelle related to the chloroplast of plant cells that contains its own genome-encoding, prokaryote-like, ribosomal RNAs, tRNAs and some proteins [23]. Three studies confirmed the specific action of cyclines on the apicoplast of *P. falciparum* [24–26]. A parasite exposed to 1 µM of doxycycline for 20 h presented during the next cycle (72 h), the inhibition of apicoplastic replication visualized by confocal fluorescence microscopy, electron microscopy and an analysis of the parasite transcriptome [24]. The most recently published study confirms the action of doxycycline on the apicoplast but in two stages, with an immediate toxic effect and a toxic effect measurable after cell division [25]. A proteomic approach confirmed the specific deregulation of proteins involved in apicoplast metabolism after doxycycline treatment [27].

Thus, the aim of this study was to identify specific point mutations in this plasmodial ribosomal gene, according to what is observed in other species, to determine whether this gene could be involved in reduced susceptibility to doxycycline. For this purpose, the apicoplastic *pfssrRNA* gene from the 89 African and 89 Thai *P. falciparum* isolates, belonging to phenotypic groups differing in doxycycline IC_{50} values and already analysed for *pftetQ* and *pfmdt* genes, was sequenced and analysed [9, 14].

Methods

Plasmodium falciparum isolates

A total of 89 African *P. falciparum* isolates, obtained at the French National Reference Centre for Imported Malaria, Hôpital Bichat, Paris, from patients hospitalized with malaria after having returned to France between January 2006 and December 2010, and 89 isolates obtained from the Shoklo Malaria Research Unit (Mae Sot, Thailand) from patients infected with *P. falciparum* from 2001 to 2010, were used. These isolates were previously tested to evaluate their *pfmdt* and *pftetQ* genes copy numbers [9, 14].

Consent

Informed consent was not required as the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

Concerning the Thai isolates, written informed consent translated into the patient's own language was obtained from each participant, whose signature was witnessed. The studies were approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University and Oxford University.

Amplification and sequencing of *pfssrRNA* gene

PfssrRNA (PFC10_API0057) was amplified by polymerase chain reaction (PCR) using the following primers: 5′-AGCTAATGGTGAGATTTGAACTCA-3′ (forward) and 5′-CGTCGTGAGACAGTTCGGTC-3′ (reverse)

(Eurogentec, Angers, France), designed with the NCBI/Primer-BLAST online tool.

The reaction mixture included 2 µl of genomic DNA, 2.5 µl of 10× reaction buffer (Eurogentec), 0.5 µM of each primer, 200 µM of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP and dCTP) (Euromedex, Souffelweyersheim, France), and 1.5 mM of $MgCl_2$ and 1.25 units of RedGoldStar® DNA polymerase (Eurogentec) in a final volume of 25 µL. The thermal cycler (T3 Biometra, Archamps, France) was programmed as follows: an initial 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 60 °C for 2 min, and a final extension step of 60 °C for 5 min. The PCR products were loaded on 1 % agarose gel containing 0.5 µg/mL ethidium bromide. Amplicons were purified using the QIAquick 96 PCR BioRobot Kit and an automated protocol on the BioRobot 8000 workstation (Qiagen, Courtaboeuf, France). The purified fragments were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the following primers: 5′-ACTAGTG TATTTCGGTTAACAGCCG-3′ (forward), 5′-ACCCT TATCAAGAGTATGTTTTAACCAT-3′ (reverse) and Pf_SSU_rRNA_R1481 CTTAAGAACTTATTCACCG CTA (reverse). The sequence reaction products were purified using the BigDye XTerminator® Purification Kit (Applied Biosystems), in accordance with the manufacturer's instructions. The purified products were sequenced using an ABI Prism 3100 analyser (Applied Biosystems), and the sequences were analysed using Vector NTI advance (TM) software (version 11, Invitrogen, Cergy Pontoise, France).

Results

In *Helicobacter pylori*, tetracycline resistance has not been associated with efflux or ribosomal protection proteins but rather attributed to mutations in the 16S rRNA-encoding genes that affect the binding site of tetracycline [16–18]. Tetracycline resistance mediated by mutations in the 16S rRNA was first found in *Propionibacterium acnes*, and a mutation from G to C was reported at position 1058 (*Escherichia coli* numbering) in their 16S rRNA genes [17]. A triplet mutation in the same 16S rRNA domain (965–967; *E. coli* numbering) was also found [24, 28–30] and is located in the primary tetracycline-binding site [1, 15]. However, the sequencing of *pfssrRNA* did not reveal a polymorphism in *P. falciparum*. There was no single nucleotide polymorphism in the *pfssrRNA* gene in either the 89 African isolates, regardless of the phenotypic group for doxycycline (group A of low doxycycline IC_{50} [mean IC_{50} = 3.88 µM; confident interval 95 % (CI 95 %) [3.39–4.37], no = 30], group B of moderate IC_{50} [mean IC_{50} = 16.97 µM; CI 95 % [16.45–17.49]; no = 30]) and group C of high IC_{50} [mean IC_{50} = 34.60 µM, CI 95 % [31.3–37.9], no = 29), or the 89 Thai isolates (group A [mean IC_{50} = 3.64 µM, CI 95 % [3.29–3.99], no = 30], group B [mean IC_{50} = 14.73 µM, CI 95 % [14.6–14.85], no = 30] and group C [mean IC50 = 28.94 µM, CI 95 % [26.51–31.37], no = 29]). No sequence polymorphism in the *pfssrRNA* gene was observed by comparison with the reference strain 3D7. This gene was not associated with reduced susceptibility to doxycycline in either African or Thai *P. falciparum* isolates and the small sub-unit ribosomal RNA seemed to be not a target for doxycycline.

Conclusions

The decreased susceptibility of *P. falciparum* to doxycycline is certainly multigenic. *Pfmdt* and *pftetQ* genes polymorphism and number of copies are involved partly to the decreased susceptibility. Intensive research into identifying in vitro markers associated with decreased susceptibility should allow survey of the emergence of doxycycline resistance. Another hypothesis to be explored is some apicoplastic genes, which could be involved in artemisinin resistance [31], such as *arps10*, encoding the apicoplast ribosomal protein S10 precursor, and *fd*, encoding the ferredoxin protein, a key component of the apicoplast electron transport chain.

Authors' contributions

SB, FN, JL, and BP conceived and designed the experiments. KS and CW performed the evaluation of doxycycline IC_{50} in Mae Sot City, Thailand, using the isotopic method. SH and VH performed the evaluation of doxycycline IC_{50} in Paris, France, using the isotopic method. TG and NW performed the PCR, sequencing and sequence analyses of the *pfssrRNA* gene. TG, FN, SB and BP wrote the paper. All authors read and approved the final manuscript.

Author details

[1] Unité de Parasitologie, Département d'Infectiologie de Terrain, Institut de Recherche Biomédicale des Armées, Marseille, France. [2] Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Aix Marseille Université, UM 63, CNRS 7278, IRD 198, Inserm 1095, Marseille, France. [3] Fédération des Laboratoires, Hôpital d'Instruction des Armées Saint Anne, Toulon, France. [4] Centre National de Référence du Paludisme, Marseille, France. [5] Laboratoire de Parasitologie-Mycologie, Centre National de Référence du Paludisme, APHP, Hôpital Bichat-Claude Bernard, Paris, France. [6] IRD UMR216, Mère et enfant face aux infections tropicales, Paris, France. [7] PRES Sorbonne Paris Cité, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Paris, France. [8] Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sod, Thailand. [9] Centre for Tropical Medicine, University of Oxford, Oxford, UK. [10] Direction Inter-Armées du Service de Santé, Cayenne, French Guiana. [11] Laboratoire de Parasitologie, Institut Pasteur de la Guyane, Cayenne, French Guiana. [12] Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France.

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French National Reference Centre for Imported Malaria Study Group

Ahmed Aboubacar, Patrice Agnamey, Faïza Ajana, Roger Amira, Nicolas Argy, Sonia Baumard, Pauline Bellanger, Dieudonné Bemba, Jean Beytout, Marie-Laure Bigel, Martine Bloch, Richard Bonnet, Alice Borel, Olivier Bouchaud,

Catherine Branger, Fabrice Bruneel, Monique Cambon, Daniel Camus, Enrique Casalino, Jérome Clain, Sandrine Cojean, Bernadette Cuisenier B, Ludovic De Gentile, Jean-Marie Delarbre, Anne Delaval, Rémy Durand, Emmanuel Dutoit, Odile Eloy, Jean-François Faucher, Albert Faye, Odile Fenneteau, Denis Filisetti, Christian Fulleda, Nadine Godineau, Frédéric Grenouillet, Jean-Pierre Hurst, Houria Ichou, Elizabeth Klein E, Sylvie Lariven, Magalie Lefevre, Monique Lemoine, Olivier Lesens, Caroline Lohmann, Daniel Lusina, Marie-Claude Machouart, Robert Mary, Sophie Matheron, Denis Mechali, Audrey Merrens, Laurence Millon, Sébastien Monnier, Emmanuel Mortier, François Moussel, Olivier Pageot, Nathalie Parez, Pierre Patoz, Alexander Pfaff, Marc Pihet, Jean-Etienne Pilo, Isabelle Poliane, Denis Pons, Marie Poupart, Marc Prevel, Lauren Pull, Christophe Rapp, Alexandre Rivier, Emily Ronez, Daniel Rotten, Anne-Laure Simonet, Jean-Yves Sirlez, Christophe Strady, Audrey Therby, Michel Thibault, Maxime Thouvenin, Dominique Toubas.

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The authors have declared that they have no competing interests.

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TROISIEME PARTIE :
SYNTHESE, CONCLUSIONS ET PERSPECTIVES

Un des problèmes majeurs dans la prévention et le traitement du paludisme est la résistance croissante du parasite à des substances disponibles à moindre coût. Les résistances à la chloroquine et à l'association sulfadoxine-pyriméthamine sont observées aujourd'hui dans tous les continents dans lesquels sévit *P. falciparum*. Les recommandations internationales relatives au traitement du paludisme reposent actuellement sur les associations à base d'artémisinine. Il est envisageable que ces associations perdent petit à petit de leur efficacité, l'observation de souches de *P. falciparum* ayant des clairances diminuées à l'artémisinine ayant été montrée en Asie du Sud-Est. Dans le domaine de la chimioprophylaxie anti-paludique, il existe un regain d'intérêt pour des substances dont les propriétés thérapeutiques ne s'appliquent pas seulement au domaine des infections parasitaires; cependant, des facteurs rendent inappropriées ces molécules pour un certain nombre de voyageurs non immuns : l'émergence de résistances à ces molécules (les folates par exemple), les difficultés à évaluer les posologies adaptées, la mauvaise tolérance (méfloquine), voire le coût excessif de ces molécules (association atovaquone-proguanil). Les tétracyclines, dont l'usage n'est pas autorisé chez le jeune enfant ni chez la femme enceinte, font partie de ces molécules à usage élargi. En dépit d'un nombre réduit d'études expérimentales, la tétracycline et la doxycycline ont été préconisées dans le traitement du paludisme peu de temps après leur avènement en tant qu'antibiotique, et elles sont devenues classiques dans les thérapies anti-malariques. Aux Etats-Unis, l'association thérapeutique usuelle pour le traitement du paludisme est l'association quinine ou quinidine plus doxycycline, et les preuves d'échec de prophylaxie à la doxycycline sont rares. La doxycycline est toujours recommandée par le CDC en chimioprophylaxie antipaludique pour les voyageurs en zone d'endémie, en particulier dans les zones de multirésistance.

Dans les études de sélection de la résistance aux tétracyclines sur modèle murin, le développement d'une résistance aux cyclines est bien plus lent qu'avec des molécules antimalariques spécifiques : chloroquine, quinine, pyriméthamine, suggérant que des tétracyclines améliorées auraient une longue durée d'utilisation. La synthèse de nouvelles tétracyclines a été ainsi encouragée pour tirer profit des propriétés pharmacologiques et antiparasitaires de ces molécules tout en contrant leurs effets indésirables. Et ce d'autant plus que des études récentes ont montré que certaines tétracyclines étaient dépourvues de

toxicité cutanée, dentaire ou gastro-intestinale, lorsque administrées à posologie et durée adaptées. Une équipe [36] a récemment procédé à la modification de tétracyclines et obtenu 13 composés dont l'activité antipaludique a été démontrée sur un modèle murin de *Plasmodium berghei*. La modification en particulier du carbone en position 7 de la sancycline aboutit à des composés actifs sur *Plasmodium,* à posologie moindre et administrables chez l'enfant.

La synthèse de nouveaux macrolides a également été entreprise selon un procédé original différent de celui appliqué pour les tétracyclines. La stratégie employée [64] a consisté à préparer des composés glycosylés selon une approche *in vivo* car synthétisés à partir d'un macrolide à 14 atomes de carbone, la mégalomicine, produite par l'actinomycète *Micromonospora megalomicea* [65]. La mégalomicine, en termes d'activité antibiotique, de spectre d'action et de pharmacocinétique est comparable à l'érythromycine. L'adjonction d'un sucre, la L-mégosamine, rend la mégalomicine antivirale et antiparasitaire, propriétés non observées chez l'érythromycine. Les macrolides mégosaminés obtenus par un procédé de bioconversion à partir de L-mégosamine produite par *Escherichia coli*, ensuite testés sur des isolats de *Plasmodium falciparum* et *Plasmodium berghei, ont* démontré une nette augmentation d'activité contre *P. falciparum* à des doses nanomolaires. L'effet observé après une courte exposition est en faveur d'une cible non apicoplastique de ces dérivés. Le développement de dérivés de macrolides avec, à la fois, une action rapide et prolongée est en cours d'investigation, ce qui constituerait une approche optimisée contre le développement de resistances par le parasite. De plus, cette démarche permettrait d'éviter toute pression de sélection sur le monde bactérien, par des molécules antibiotiques utilisées comme antiparasitaires.

Un certain nombre de travaux porte enfin sur les quinolones, en particulier la ciprofloxacine, des modifications chimiques permettant d'aboutir à un accroissement considérable des propriétés antiplasmodiales. En 2008, il avait déjà été démontré que l'incorporation d'un noyau ferrocène sur la structure de la chloroquine aboutissait à la formation de complexes présentant une forte activité antiplasmodiale, même sur des isolats chloroquino-résistants [66]. Cette approche structure-activité a été appliquée aux fluoroquinolones, chez lesquelles une augmentation de l'hydrophobicité conduit à une augmentation des propriétés antiplasmodiales. L'hydrophobicité a été obtenue dans une

étude [67] en incorporant à la ciprofloxacine et à d'autres fluoroquinolones un groupement ferrocenyl. D'autres composés ont été créés par la suite, reposant sur l'intégration de groupements phényl ou adamantanyl conférant des propriétés chimiques similaires au précédent [68]. Les activités antiplasmodiales des composés obtenus ont été évaluées *in vitro* sur les souches de références 3D7 (chloroquino-sensible) et W2 (chloroquino-résistante). Les résultats ont confirmé un abaissement des CI_{50} après contact avec les molécules synthétisées, obtenu après 48 heures d'exposition du parasite. Le caractère lipophile de ces dérivés expliquerait en partie le regain d'activité par rapport à la molécule source, puisqu'il augmente leur capacité à franchir les différentes parois membranaires pour parvenir à leur cible cellulaire. Ces résultats obtenus *in vitro* doivent faire l'objet d'études *in vivo*, afin d'en explorer la biodisponibilité et l'éventuelle toxicité.

Une autre voie d'optimisation de molécules antiplasmodiales par processus chimique consiste à utiliser des sidérophores. Les sidérophores sont des chélateurs de fer secrétés par des microorganismes qui, lorsqu'ils sont conjugués à une molécule potentialisent son activité. Ce modèle biochimique, assimilable à un « cheval de Troyes » a, à ce jour, été étudié dans la tuberculose et le paludisme [36]. Il pourrait être appliqué à des antibiotiques. L'hypothèse retenue est la réduction du conjugué ferrique en conjugué ferreux qui aboutirait à la formation d'un radical létal vis-à-vis des structures cellulaires cibles. La synthèse d'un conjugué d'artémisinine et d'un analogue de la mycobactine, sidérophore naturel pour *Mycobacterium tuberculosis*, a montré une activité antimicrobienne sélective portant à la fois sur *P. falciparum* et sur *M. tuberculosis*, avec pour *P. falciparum* des CI_{50} au moins équivalentes à celles obtenues avec l'artémisinine. D'autres conjugués ont été testés à ce jour, tels que l'artemisinine-desferrioxiamine, la desferrioxiamine étant un sidérophore naturel produit par *Streptomyces pilosus* [69]. Cette conjugaison s'est évérée beaucoup moins efficace que la précédente en termes de CI_{50} [36].

Les processi de synthèse chimique restent prometteurs pour la mise au point de molécules aux propriétés antiplasmodiales, mais ne peuvent être optimisés que si les mécanismes d'action complexes des différentes molécules sont connus et les mécanismes de résistance décrits. C'est toute la difficulté pour *P. falciparum* d'en déterminer des marqueurs moléculaires de résistance indiscutables pour une molécule donnée, la complexité du génome et les fortes variations géographiques [70] étant deux contraintes majeures à la

découverte de marqueurs de résistance universels. La solution d'approche « classique » consiste à déterminer les CI_{50} pour une molécule donnée, et de comparer la présence et le nombre de copies d'un gène donné entre des isolats catégorisés « sensibles » versus des « résistants ». C'est la méthode qui a été appliquée dans notre travail sur la doxycycline, avec la détermination du nombre de copies des gènes *pfmdt* et *pftetQ* [71][72][73]. Une autre approche consiste à rechercher des points de mutations éventuels dans le génome qui pourraient expliquer une élévation des CI_{50}. Cette démarche a été appliquée pour déterminer une mutation potentielle à l'origine d'une augmentation des CI_{50} à la doxycycline, par homologie avec les mécanismes de résistance déployés par les bactéries, sans succès. Des données sur la résistance aux dérivés de l'artémisinine (ART) au Cambodge suggèrent que ces phénotypes résistants seraient le fait de la survenue de divers évènements indépendants [74]. Une approche publiée récemment [70] afin de déterminer des marqueurs moléculaires de résistance aux ART a consisté à étudier l'acquisition de mutations induites spécifiquement en laboratoire pendant 3 ans par une technique de pression de sélection appliquée sur des isolats capables de survivre à de fortes doses d'ART. La mise en évidence des marqueurs moléculaires de résistance potentiels a été obtenue après séquençage du génome complet. Huit mutations sur 7 gènes ont été préalablement décrites. Il s'agissait ensuite de les mettre en évidence sur des isolats cliniques. Les polymorphismes du propeller K13 ont été retenus comme marqueurs moléculaires de résistance aux dérivés de l'artémisinine, dont l'évaluation doit être généralisée sur d'autres zones géographiques afin d'objectiver l'étendue de cette résistance. Cette démarche scientifique et épidémiologique, extrêmement chronophage et complexe, permet d'observer et de décrire de façon optimale les capacités d'adaptation et de réaction de *Plasmodium*. Généralisée à plusieurs molécules antiplasmodiales parmi lesquelles certains antibiotiques, elle doit permettre de mettre en évidence des marqueurs moléculaires de résistance incontestés, afin d'anticiper l'émergence de résistance.

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