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Dianas terapéuticas frenta a malaria y proteómica redox en "Plasmodium falciparum"

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
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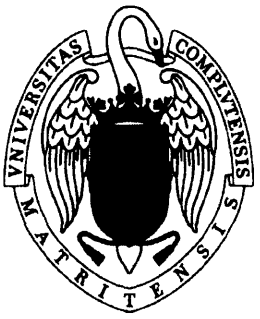

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Memoria presentada por **AZAR RADFAR** para optar al grado de Doctor por la Universidad Complutense de Madrid

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Dedicated to my parents,
My husband Iraj,
and my daughter Parastou

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ABBREVIATIONS

ABRA	acid basic repeat antigen
AGEs	advanced glycation end-products (AGEs)
ALEs	advanced lipoxidation end-products
CBB	Coomassie Brilliant Blue
cDNA	complementary deoxyribonucleic acid
CDP	citrate phosphate dextrose
CHAPS	3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate
CQ	chloroquine
C_T	threshold cycle
1-D	one-dimensional
2-DE	two-dimensional electrophoresis
dNTPs	deoxynucleotide triphosphate
DNPH	2,4 dinitrophenylhydrazine
DPAP1	dipeptidyl aminopeptidase 1
dsiRNA	double-stranded interference ribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMBL-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
FAM	6-carboxyfluorescein
FP IX	ferri/ferroprotoporphyrin IX
G6PD	Glucose-6-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine triphosphate
HAP	histoaspartic protease
Hbo-Fe^{+2}	oxy-haemoglobin containing ferriprotoporphyrin IX
HEPES	[4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid]
HNE	4-hydroxy-2-nonenal
HPLC	high-performance liquid chromatography
HSP	heat shock protein
IC_{50}	inhibitory concentration 50
IEF	isoelectrofocusing
Ig	immunoglobulin
IRBCs	infected red blood cells
Kb	kilobase
KDa	kilodalton (molecular mass)
MALDI-MS	matrix-assisted laser desorption/ionization–mass spectrometry
MALDI-TOF/TOF MS	matrix-assisted laser desorption/ionization time-of-flight/time -of-flight mass spectrometry
MDA	malondialdehyde
MetHb-Fe^{3+}	methaemoglobin containing ferroprotoporhpyrin IX
MIPS	Munich Information Centre for Protein Sequences
MS	mass spectrometry

MS/MS	tandem mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
PCI	potato carboxypeptidase inhibitor
PfEMP1	erythrocyte membrane protein 1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
6PGL	6-phosphogluconolactonase
PMF	Peptide Mass Fingerprinting
ppm	parts per million
qPCR	real-time quantitative PCR
qRT-PCR	real-time quantitative RT-PCR
RBM	Roll Back Malaria
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	reverse transcriptase-PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
SERA5	serine repeat antigen 5
Snrnp	small nuclear ribonucleoprotein
TAMRA	6-carboxytetramethylrhodamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
Tris	tris (hydroxymethyl) aminomethane
tubCP	tubulinyl-Tyr carboxypeptidase

ÍNDICE – CONTENTS

Resumen en Español

I. INTRODUCCIÓN

La malaria es una enfermedad parasitaria producida por un protozoo apicomplejo del género *Plasmodium* que infecta a un amplio rango de hospedadores, entre los que se encuentran mamíferos, aves y reptiles (Klemba and Goldberg, 2002) y que se transmite mediante la picadura de mosquito hembra del género *Anopheles* sp. infectada. La malaria es la enfermedad humana parasitaria más importante, que afecta a un gran número de poblaciones de las áreas tropicales y subtropicales del mundo, así como a un gran número de viajeros que visitan estas regiones. Aunque la malaria se produce en más de cien países, es en África donde ocurren un mayor número de casos, siendo causa de más del 90% de las muertes que tienen lugar en dicho continente (Williams *et al.*, 1999). La malaria humana puede estar producida por cuatro especies del género *Plasmodium*, siendo *P. falciparum* la que causa la forma más grave de la enfermedad y el responsable de la muerte de más de un millón de personas al año (Klemba and Goldberg, 2002).

El ciclo de vida del parásito de la malaria es complejo y requiere de dos etapas: una etapa de reproducción sexual, donde la multiplicación se produce en el tubo digestivo del mosquito y una etapa de reproducción asexual, donde la multiplicación se produce en el hospedador humano. En los estadios que se producen en el hombre se pueden distinguir dos fases importantes: la exoeritrocítica (fase hepática) y la eritrocítica (Frederich *et al.*, 2002).

En humanos, la infección comienza con la picadura de una hembra de mosquito *Anopheles* que transmite las formas parasitarias que alberga en sus glándulas salivales al hospedador. Los esporozoítos tras circular durante un corto período de tiempo en el torrente circulatorio, invaden las células del hígado, donde se desarrollan a esquizontes exoeritrocíticos en un período que varía entre 5 a 10 días. Un esquizonte hepático (exoeritrocítico) puede contener de 10000 a 30000 merozoítos, que se liberan e invaden los glóbulos rojos (Frederich *et al.*, 2002). Una vez que los merozoítos han invadido los eritrocitos, comienzan a reproducirse asexualmente, hasta la formación de esquizontes, pasando previamente por la formacion de anillos y trofozoítos (esquizogonia eritrocítica) (Frederich *et al.*, 2002). Los eritrocitos que contienen los esquizontes maduros se rompen y liberan los merozoítos los cuales pueden invadir otros glóbulos rojos (Frederich *et al.*, 2002), comenzando de nuevo el ciclo.

En 1996 se inició un proyecto internacional para secuenciar el genoma de *P. falciparum* con la esperanza de que se abrieran nuevos caminos para la investigación antimalárica. La secuencia del genoma de *P. falciparum* se obtuvo en el año 2002 (Gardner *et al.*, 2002). Los 22.8 Mb del genoma de *P. falciparum* están organizados en 14 cromosomas lineales (Gardner *et al.*, 2002), un genoma circular similar al de los cloroplastos y un genoma mitocondrial lineal (Bozdech *et al.*, 2003). Los 14 cromosomas

de *P. falciparum* 3D7 varían entre un tamaño aproximado de 0.643 a 3.29 Mb (Gardner *et al.*, 2002). La predicción de los genes codificantes de proteínas en el genoma ha permitido contabilizar aproximadamente 5300 genes de los que el 60% son proteínas hipotéticas de función desconocida hasta la fecha (Gardner *et al.*, 2002). Se debe resaltar el hecho de que se trata del genoma más rico en A+T secuenciado hasta la fecha, que llega a ser del 80.6% en las regiones codificantes (Gardner *et al.*, 2002).

Por otra parte, ha sido caracterizado el proteoma de los estadios de esporozoíto, merozoíto, trofozoíto y gametocito de *P. falciparum* (Florens *et al.*, 2002). Más recientemente también se han caracterizado dos proteomas más, el de los estadios de anillo y esquizonte (Le Roch *et al.*, 2004). De las 2415 proteínas identificadas en el primer estudio (más tarde ampliadas a 2904), solamente el 6% se expresa durante los cuatro estadios del ciclo, mientras que algo más de la mitad son únicas de una etapa. Prácticamente la mitad de las proteínas del esporozoíto son específicas de etapa, mientras que en los estadios sanguíneos este número variaba del 20 al 30% (Kooij *et al.*, 2006).

Estrategias de lucha contra la malaria

Todos los programas de control de malaria que se han llevado a cabo hasta la fecha han mostrado limitación en cuanto a la efectividad en erradicar la enfermedad. De hecho, la situación de malaria en el mundo ha empeorado considerablemente. Existen varios factores que explican esta escasa efectividad, entre ellos, la aparición de resistencias a los fármacos que se utilizan para combatirla, que aunque son relativamente recientes, son el factor más importante porque aumenta los reservorios de parásitos resistentes en las áreas endémicas. En general, los esfuerzos para controlar la malaria incluyen el desarrollo potencial de vacunas eficaces, la erradicación del vector, el desarrollo de nuevos fármacos (Oaks *et al.*, 1991; Olliaro *et al.*, 1996) además de las medidas higiénicas y sanitarias preventivas al uso. Sin embargo, las dificultades actuales para controlar al vector y desarrollar una vacuna eficaz, así como el rápido desarrollo de resistencias ante los fármacos existentes, subraya la necesidad de desarrollar nuevos agentes antimaláricos.

Fármacos Antimaláricos

La terapia mediante el empleo de fármacos sigue siendo el pilar fundamental del tratamiento y prevención de esta patología (Daily, 2006). En general, se han clasificado cuatro clases de actividades farmacológicas con potencial antimalárico (Frederich *et al.*, 2002): esquizonticidas hepáticos, esquizonticidas sanguíneos, gametocitocidas y esporontocidas.

La complejidad de la biología y la gran flexibilidad genética del parasito son también responsables del limitado número de fármacos antimaláricos disponibles. Los fármacos usados más frecuentemente son derivados de las quinolonas, derivados del antifolato, derivados de la artemisina y antibióticos. Debido a la aparición de resistencias se recomienda una terapia combinada con al menos dos fármacos que actúen en dianas diferentes.

Una de las drogas derivadas de las quinolonas, la 4-aminoquinolina sintética o cloroquina (CQ), se introdujo durante la segunda guerra mundial (Loeb *et al.*, 1946) y debido a su baja toxicidad y a su efectividad, ha sido el principal tratamiento en la lucha contra la malaria durante años (Tilley *et al.*, 2001).

Resistencia a fármacos antimaláricos

La resistencia frente a fármacos antimaláricos se ha definido como la capacidad de una cepa de parásito a sobrevivir y/o multiplicarse a pesar de la administración y absorción de un fármaco suministrado en dosis iguales o mayores que aquellas usualmente recomendadas, pero con tolerancia del paciente (Bloland, 2001).

Las resistencias se han desarrollado fundamentalmente en *P. falciparum,* que es la especie con mayor prevalencia en África tropical, Amazonía y Suroeste de Asia y, en general, ésta originada por mutaciones en una gran variedad de genes (Le Bras *et al.*, 2006).

La resistencia a la CQ se observó por primera vez en Tailandia en 1957 y en la frontera entre Colombia y Venezuela en 1959. Desde 1988 la resistencia se ha extendido fundamentalmente por toda África subsahariana y hoy en día, la CQ ha perdido su eficacia en casi todas las áreas del mundo (Hyde, 2007). Además, la expansión e incremento de la resistencia a otros fármacos existentes contribuye enormemente a las dificultades para el control de la enfermedad (Hyde, 2007).

Gracias al desarrollo de nuevos métodos de análisis molecular y a la manipulación genética de *Plasmodium* se ha ido progresado en la comprensión de los mecanismos moleculares que utiliza *P. falciparum* para revertir y adaptarse a estructuras químicas farmacológicas que se utilizan en la terapia antimalárica (Hyde, 2007).

Dianas potenciales para nuevos antimaláricos

La secuenciación del genoma de P. *falciparum*, la emergencia de resistencias a drogas y los avances en técnicas moleculares han redirigido los esfuerzos para el descubrimiento de fármacos. Hasta la fecha existen diversos estudios dedicados a identificar dianas metabólicas que puedan ser utilizadas con fines terapéuticos (Jana and Paliwal, 2007; Linares and Rodriguez, 2007). De esta manera, se han identificado moléculas implicadas en la biosíntesis de membranas (Vial, 1996; Ancelin *et al.*, 2003; Roggero *et al.*, 2004), transportadores (Joet *et al.*, 2003; Kirk *et al.*, 2005), proteasas (Bailly *et al.*, 1992; Rosenthal *et al.*, 1996; Coombs *et al.*, 2001; Singh and Rosenthal, 2001 ; Lee *et al.*, 2003; Pandey *et al.*, 2005), la detoxificación del grupo hemo (Padmanaban and Rangarajan, 2000; Kannan *et al.*, 2005), reguladores de las modificaciones post-transcripcionales (Jomaa *et al.*, 1999; Sullivan *et al.*, 2006; Fennell *et al.*, 2007), componentes centrales del sistema redox (Krauth-Siegel and Coombs, 1999; Wang *et al.*, 1999; Luersen *et al.*, 2000), del sistema mitocondrial (Krungkrai *et al.*, 1997; Srivastava *et al.*, 1997; Mi-Ichi *et al.*, 2005), del metabolismo de los ácidos nucleicos (Nduati *et al.*, 2005; Ting *et al.*, 2005) y del apicoplasto (McFadden and Roos, 1999; McLeod *et al.*, 2001; He *et al.*, 2004).

Proteasas

Desde hace dos décadas, las proteasas de los organismos del género *Plasmodium* se han considerado potenciales dianas para la quimioterapia antimalárica debido a la importancia que sus actividades tienen en el ciclo de vida del parásito y a la posibilidad de diseñar inhibidores específicos de las mismas (Wu et al., 2003). Muchas de las proteasas de *Plasmodium* sp. que han sido caracterizadas parecen tener papeles cruciales en el ciclo de vida intraeritrocítico; por ello son potenciales dianas quimioterapéuticas (Rosenthal, 2001). Así, el importante papel de las proteasas del *Plasmodium* en la ruptura del eritrocito, la invasión y la degradación de la hemoglobina, han sido el objeto de numerosos estudios (McKerrow *et al.*, 1993; Rosenthal, 1998; Eggleson *et al.*, 1999; Blackman, 2000; Klemba and Goldberg, 2002; Rosenthal, 2002; Rosenthal, 2004; Ersmark *et al.*, 2006).

Además, la secuenciación completa del genoma de *P. falciparum* proporciona las bases necesarias para identificar nuevas proteasas susceptibles de ser utilizadas como dianas para la quimioterapia antimalárica (Wu et al., 2003). El análisis genómico comparativo, realizado mediante búsquedas de secuencias por similitud, ha permitido revelar la presencia en este parásito de 92 secuencias putativas de genes que codifican para enzimas proteolíticos pertenecientes a 26 familias de las cinco clases catalíticas

descritas: aspártico, cistein, metalo, serin y treonin proteasas., De estas 92 secuencias, se ha demostrado que, al menos 88, codifican productos génicos tanto a nivel de mRNA como de proteína, según revelan los datos obtenidos con estudios de microarrays y proteómica (Wu *et al.*, 2003).

Modificación post-transcripcional de proteínas

La liberación de una cadena polipeptídica completa desde un ribosoma, no suele ser el último paso en la formación de una nueva molécula de proteína. Con frecuencia ocurren diversas modificaciones covalentes, que tienen lugar durante o después de la síntesis de la cadena polipeptídica, de hecho, la mayoría de las proteínas experimentan modificaciones co- y/o post-traduccionales Las modificaciones post-traduccionales cumplen con un elevado número de propósitos dentro de diversos procesos celulares como son la regulación enzimática, transducción de señales, localización de proteínas, interacciones entre éstas e incluso su estabilidad (Reinders and Sickmann, 2007).

Las proteínas pueden ser modificadas de forma post-traduccional mediante un proceso proteolítico de las cadenas polipeptídicas recién sintetizadas, por el acoplamiento de moléculas de distintas clases formando enlaces covalentes, mediante la formación de uniones intra- e intermoleculares, o, por una combinación de varios de estos eventos (Eichler and Adams, 2005).

Estrés oxidativo

Las especies reactivas del oxígeno (ROS) están presentes como contaminantes en la atmósfera, son generadas como subproductos en procesos metabólicos normales; y se forman ante exposiciones a radiación X-, λ-, o U.V. (Stadtman and Levine, 2003). En la célula, las ROS son generadas constantemente a baja concentración en condiciones fisiológicas y participan en la regulación redox de la misma (Dalle-Donne *et al.*, 2006). El daño oxidativo celular se desarrolla cuando el equilibrio entre los sistemas de formación y eliminación de ROS se inclina en favor del primero (Dalle-Donne *et al.*, 2006).

Las proteínas son las dianas principales de las ROS, ya que son los componentes mayoritarios de la mayoría de los sistemas biológicos (Davies *et al.*, 1999). Modificaciones irreversibles como la carbonilación y uniones proteína-proteína, son generalmente las responsables de que se pierda de forma permanente la función de las proteínas dañadas, las cuales son posteriormente degradadas o se acumulan progresivamente en inclusiones intracitoplasmáticas, como se observa en algunos procesos neurodegenerativos (Ghezzi and Bonetto, 2003).

Carbonilación de proteínas y detección del grupo carbonilo

La carbonilación de proteínas mediada por ROS es un marcador de la oxidación proteica y su detección sirve como sistema indicador del daño oxidativo que pueden sufrir las proteínas en determinadas condiciones de estrés oxidativo, envejecimiento y desórdenes fisiológicos (Butterfield and Kanski, 2001).

Un método altamente sensible para la detección de proteínas carboniladas implica la derivatización del grupo carbonilo con 2,4-Dinitrofenilhidracina (DNPH), que conduce a la formación de 2,4-Dinitrofenil (DNP) hidrazona estable que puede ser posteriormente detectada por diferentes métodos (Dalle-Donne *et al.*, 2003). Asi, la determinación de proteínas carboniladas es posible gracias a la disponibilidad comercial de anticuerpos específicos anti-DNP que permiten su identificación mediante inmunodetección (Dalle-Donne *et al.*, 2003). Es por ello que el contenido de grupos carbonilo en proteínas individuales puede ser también determinado mediante espectrofotometría de masas de las proteínas objeto de estudio separadas previamente mediante electroforesis bidimensional (2-DE) y que han sido identificados tras la transferencia a membrana e inmunodetección con anticuerpos anti-DNP (Dalle-Donne et al., 2003).

P. falciparum y estrés oxidativo

Todos los organismos aerobios están expuestos a las ROS generadas por su propio metabolismo. Los protozoos parásitos no sólo tienen que eliminar sus metabolitos tóxicos endógenos, sino que también deben soportar el ambiente oxidativo del sistema inmune del hospedador (Muller *et al.*, 2003).

Se ha demostrado que el parásito que produce la malaria es particularmente vulnerable al estrés oxidativo que se genera durante los estadios eritrocíticos de su ciclo biológico (Hunt and Stocker, 1990; Simoes *et al.*, 1992; Muller *et al.*, 2003; Becker *et al.*, 2004). Esto no es sorprendente, dado que estos parásitos viven en ambientes pro-oxidantes que contienen hierro y oxígeno, componentes fundamentales para la formación de ROS vía la reacción de Fenton (Muller, 2004). La causa principal del estrés oxidativo en el parásito es la degradación de la hemoglobina del hospedador ya que esta proteína por si misma representa la mayor fuente de energía metabólica y sintética en *Plasmodium* (Becker *et al.*, 2004). En el eritrocito, el hierro del grupo hemo está, casi por completo, en estado ferroso (+2); durante su degradación en las vacuolas digestivas del parásito, el hierro se oxida pasando a estado férrico (+3). Los electrones liberados en la reacción de oxidación se combinan con el oxígeno molecular, produciendo aniones superóxido (O_2^-). Esta combinación conduce inevitablemente a la generación de peróxido de hidrógeno y posteriormente radicales hidroxilo, ambos altamente reactivos e

intermediarios tóxicos del oxígeno (Francis *et al.*, 1997; Liochev and Fridovich, 1999). Además, el grupo hemo tóxico (ferri/ferroprotoporfirina IX; FP IX), es liberado en la digestión de la hemoglobina y por tanto debe ser detoxificado. La toxicidad del FP IX liberado es debida a que posee propiedades similares a los detergentes interfiriendo en la integridad de la membrana del parásito y además, porque tiene la capacidad de inducir reacciones redox, causando la generación de ROS por su unión con el hierro (Muller, 2004). La mayoría de FP XI liberado (hasta un 90%) se biomineraliza o biocristaliza (Egan *et al.*, 2002; Hempelmann, 2007) hasta formar hemozoína inerte, aunque se ha demostrado que una cantidad considerable de éste (incluso hasta el 50%) escapa a la biomineralización y debe ser degradado o secuestrado por otros medios (Loria *et al.*, 1999; Zhang *et al.*, 1999) para prevenir daños en la membrana y la muerte del parásito (Atamna and Ginsburg, 1993; Tilley, 2001; Becker *et al.*, 2004). Entre otras vías de detoxificación se incluyen la degradación del FP IX (Zhang *et al.*, 1999), la reacción con glutatión (Ginsburg *et al.*, 1998) y la unión a proteínas aceptoras de FP IX (Harwaldt *et al.*, 2002; Campanale *et al.*, 2003). Estas vías podrían contribuir también a la detoxificación del grupo hemo, ya que escapando sólo pequeña cantidad del FP IX de los procesos de detoxificación (Loria *et al.*, 1999), éste puede causar daño oxidativo a las proteínas y a la membrana del hospedador y del parásito (Campanale *et al.*, 2003; Famin and Ginsburg, 2003).

Fármacos antimaláricos que interfieren en el metabolismo redox: mecanismo de acción

Los antimaláricos quinolínicos como la CQ, representan una clase importante de fármacos antimaláricos que actúan de forma específica sobre vías de la degradación de la hemoglobina. La amplia distribución mundial de cepas *P. falciparum* resistentes a CQ han supuesto un problema y un desafío para el control de la malaria durante las últimas décadas, hasta el punto que la CQ se ha vuelto ineficaz en muchas regiones endémicas de esta enfermedad, ya que alrededor del 80% de la población parasitaria es resistente a este fármaco (Ehrhardt *et al.*, 2007) y, por lo tanto, ésta ha sido sustituida por otros fármacos o por terapias combinadas. A pesar de haberse utilizado durante más de 50 años, el mecanismo de acción de la CQ y los mecanismos de resistencia a la misma, son sólo parcialmente conocidos (Koncarevic *et al.*, 2007). Entre las diferentes teorías existentes, la más ampliamente aceptada es la hipótesis de interacción entre CQ y FP IX, que plantea que la CQ y otras 4-aminoquinolonas ejercen su acción antimalárica previniendo la detoxificación de hemo libre que se genera durante la degradación de la hemoglobina del hospedador en la vacuola digestiva del parásito (Koncarevic *et al.*,

2007). La CQ se acumula en la vacuola digestiva (Egan, 2001; Spiller *et al.*, 2002) donde interacciona con el dímero μ-oxo, formado por hemo oxidado (una de las formas químicas posibles de dímeros de FP IX que se encuentran en la vacuola digestiva, [Fe(III)FPIX]2O) (Chou *et al.*, 1980; Moreau *et al.*, 1982; Egan *et al.*, 1997; Dorn *et al.*, 1998; Leed *et al.*, 2002). Estudios realizados con RMN han sugerido que la interacción de la CQ con FP IX implica apilamiento π – π (Moreau *et al.*, 1982). La interacción de la CQ con el μ-oxo FP IX previene la formación del dímero de hemozoína, que presumiblemente es un intermediario en la formación de hemozoína cristalizada y, de ese modo se impide la detoxificación del FP IX (Becker *et al.*, 2004). Como se mencionaba anteriormente, la acumulación de FP IX libre daña membranas y enzimas debido a las propiedades detergentes del FP IX, así como su capacidad para participar en reacciones redox (Pandey *et al.*, 2001; Tilley, 2001; Sullivan, 2002).

El estrés oxidativo que se genera en el mecanismo de acción de la CQ se ha considerado siempre como una diana directa o indirecta de la acción de esta droga (Becker *et al.*, 2004). Se ha sugerido que la acumulación de FP IX libre y del complejo CQ-FP IX podría ser letal para el parásito, aumentando la toxicidad de las ROS producidas durante la degradación de la hemoglobina (de Almeida Ribeiro *et al.*, 1995; Loria *et al.*, 1999). La implicación de los eventos oxidativos en la cascada de efectos ocasionados por la CQ está apoyada por estudios de modulación de la acción de éste fármaco por distintos niveles de GSH (Becker *et al.*, 2004). Se cree que el hemo no polimerizado existe en la vacuola digestiva y es posteriormente degradado por glutatión (Ginsburg *et al.*, 1998). Se ha demostrado que la CQ inhibe de forma competitiva la degradación de hemo por glutation. En cierto modo, el incremento de los niveles celulares de glutation conlleva un aumento de la resistencia a la CQ, mientras que la disminución de los mismos resulta en un aumento de sensibilidad del parásito al fármaco (Ginsburg *et al.*, 1998).

II. OBJETIVOS

En este trabajo de tesis doctoral se han llevado a cabo estudios conducentes a profundizar en la biología de *P. falciparum* a nivel transcripcional, traduccional y post-traduccional, con el fin de encontrar potenciales dianas para el desarrollo de fármacos terapéuticos efectivos, así como clarificar el mecanismo de acción de la cloroquina como antimalárico de gran eficiencia en el pasado para conocer con detalle las modificaciones post-traduccionales que aparecen en el parásito con fenotipo resistente.

Para alcanzar estos propósitos, los objetivos específicos a nivel experimental han sido las siguientes:

1- Estudios de expresión de mRNA del gen PfNna1, en relación con el efecto inhibidor de carboxypeptidasas (PCI) en el ciclo biológico del parásito.

2- Análisis de polimorfismos del gen PfNna1 en las cepas Dd2 y 3D7 de *P. falciparum*.

3- Análisis de la enzima bifuncional G6PD-6PGL de *P. falciparum*, como modelo para el estudio del procesamiento de proteínas en este protozoo.

4- Caracterización del proteoma redox de *P. falciparum* a lo largo del ciclo intraeritrocítico del parásito y sus modificaciones en respuesta a la cloroquina.

III. CONCLUSIONES

Los resultados obtenidos en el presente trabajo de investigación representan nuevas perspectivas para un mejor conocimiento de la biología del parásito y la quimioterapia antimalárica. De dichos resultados se elevan las siguientes conclusiones:

1- En *Plasmodium falciparum*, los elevados niveles de expresión del gen PfNna1 y su sobreexpresión a nivel transcripcional por el PCI en estadios maduros del parásito, sugieren la implicación de esta proteína en actividades esenciales para el correcto desarrollo del mismo.

2- La proteína bifuncional única G6PD-6PGL de *Plasmodium* varía de tamaño dependiendo del estadio intraeritrocítico del parásito. De hecho, se puede sugerir la existencia de un patrón controlado de procesamiento de la PfG6PD-6PGL durante la maduración del mismo. La detección de dos bandas de diferente tamaño utilizando anticuerpos anti-G6PD y anti-6PGL implica que esta proteína bifuncional podría madurar rindiendo dos polipéptidos diferentes con distintas actividades enzimáticas.

3- El perfil proteómico de la oxidación es dependiente de los distintos estadios intraeritrocíticos de *P. falciparum,* tanto en parásitos tratados como en los no tratados con cloroquina.

4- El oxiproteoma de P. *falciparum* revela daño oxidativo en algunos componentes clave de las siguientes funciones celulares del parásito: plegamiento, recambio y procesamiento proteolítico de proteínas, metabolismo energético, transducción de señales y patogénesis.

5- La detección de aductos 4-hidroxi-2-nonenal específicos indica que la lipoperoxidación de proteínas se inhibe por la cloroquina, particularmente en estadios tempranos del desarrollo del parásito.

IV. APORTACIONES FUNDAMENTALES

El presente trabajo de tesis contribuye con cuatro beneficios fundamentales:

(I) Los estudios de expresión de ARN mensajero de una metalocarboxipeptidasa codificada por el gen PfNna1 en *P. falciparum* muestran su elevado nivel de expresión en los estadios maduros del parásito así como una sobreexpresión transcripcional de la misma inducida por el inhibidor de carboxipeptidasas PCI. Estos hallazgos sugieren la implicación de esta proteína en actividades esenciales para la supervivencia del parásito, por lo tanto, y de acuerdo con su potencial inhibición, esta proteasa resulta ser un candidato en la selección de nuevas dianas para la quimioterapia antimalárica.

(II) Los estudios post-traduccionales de la G6PD-6PGL muestran evidencias que sugieren la existencia de un potencial procesamiento de la proteína en dos unidades funcionales separadas.

(III) La obtención de cultivos de P. *falciparum* con más del 80% de parasitemia ha permitido llevar a cabo con rigurosidad, los estudios de proteómica a lo largo del ciclo intraeritrocítico del parásito. Hay muy pocos laboratorios que hayan desarrollado esta capacidad.

(IV) La información que se ha obtenido a partir de los estudios de proteómica redox puede ser de gran ayuda en la comprensión de los mecanismos implicados en el desarrollo y progreso de la malaria. Además, el análisis de proteómica redox de la resistencia de *P. falciparum* a la cloroquina proporciona nuevas potenciales dianas terapéuticas para el tratamiento de esta enfermedad, en la que proteínas particulares juegan un papel clave, tanto en el desarrollo del ciclo intraeritrocítico del parásito como para explicar la resistencia frente a este fármaco.

Summary

1. SUMMARY

The complex biology and life cycle of *Plasmodium falciparum* has hindered attempts to control malaria infections and prevent transmission. In addition, there is increasing resistance of the malaria parasites to currently available drugs. Therefore, there is a need for the development of new chemotherapeutic agents against malaria and understanding the basis of the chemoresistance. Recent strategies for antiparasitic drug discovery include seeking for specific inhibitors of critical biological activities within the complex parasite cycle. In this context, protease genes have already demonstrated that play key roles in the biology of malaria parasites and offer potential new chemotherapeutic targets.

The first part of the present thesis focus on the analysis of mRNA expression of the PfNna1 gene, a coding gene for a new metallocarboxypeptidase class member, in relation with the inhibition effect of potato carboxypeptidase inhibitor, PCI, on the parasite life cycle that allowed establishing a link between the inhibitor and the Nna-1 gene in *P. falciparum*. The Nna-1 like gene-mRNA expression pattern along the intraerythrocytic phase demonstrated that is maximally expressed in the mature developmental stages, phase at which PCI arrested parasite growth. Moreover, the transitional exposure of the parasite to PCI up-regulated PfNna1 gene expression. These observations suggest the involvement of this putative peptidase gene in essential activities for the parasite survival.

The genome from *P. falciparum*, the causative agent of human malaria, is characterized by an extreme high A+T content associated with abundant low complexity inserts within their proteins. The enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (G6PD-6PGL) found in all *Plasmodium* species has unique structural and bifunctional characteristics. Here, at the second part of thesis, we report the expression analysis of *P. falciparum* G6PD- 6PGL along the intraerythrocytic cycle by immunological analysis with antibodies raised against its N- and C- terminal domains. The pattern modification of band sizes at the different stages of parasite development suggests intracellular protein processing involving the cleavage of the native bifunctional form to produce two main fragments. *In vitro* RNA-mediated PfG6PD-6PGL gene silencing, studied along short-term parasite development also revealed the apparent intracellular protein modification dependent on the parasite stage. Fragment sizes were consistent with separating both catalytic functions of the enzyme. The proteolytic machinery underlying this specific PfG6PD-6PGL processing is still unknown in *P. falciparum* but suggests the existence of distinctive mechanisms in the parasite to deal with unique protein structures of essential function resulting from its genome evolution.

In the post-genomic era, when all proteins can be linked to the genes encoding them, comparative studies of the proteins expressed at any given life cycle stage or tissue

is a critical requirement for a full understanding of biological phenomena. The methods developed by Trager and Jensen in 1976 for the continuous culture of the erythrocytic stages of *P. falciparum* make this organism available to a large variety of scientists. As a result, much has been learned about *P. falciparum* during the past 30 years. Recent developments in the diverse aspects such as chemotherapy, drug resistance, vaccine development, pathogenesis, molecular biology and biochemistry emphasize the usefulness of the culture method in research. In fulfilling comparative proteomic studies in *P. falciparum*, highly parasitized cultures were needed. Here, at the third part of the research work, we report a modified method of the Trager and Jensen' culturing method in a way that synchronized cultures of *P. falciparum* with more than 80% parasitized cells are obtained.

Chloroquine resistance in *P. falciparum* has been worldwide spread in the last decade hindering malaria control in endemic areas. Current hypotheses on the chloroquine action mechanism involve ultimate interference with the parasite oxidative defence systems. In the fourth part of the thesis, carbonyl derivatization by 2,4 dinitrophenylhydrazine and subsequent immunoproteomics have allowed to identify and compare oxidatively modified proteins across the different intraerythrocytic stages of control and IC_{50} chloroquine-treated cultures of the drug-resistant *P. falciparum* strain Dd2. Functional groups of plasmodial proteins that were widely represented among those oxidatively damaged are central to parasite physiology, including protein folding, protein fate, energy metabolism, signal transduction and pathogenesis. Whether a nearly constant number of oxidized proteins across the *P. falciparum* life cycle were observed, chloroquine increased both, the amount of protein oxidation signal and the number of different oxidized proteins as the intraerythrocytic cycle proceeds to mature stages. Nevertheless, detection of specific 4-hydroxy-2-nonenal adducts indicated that protein lipoperoxidation was inhibited by chloroquine. Identification of those oxidatively modified proteins provides new insights into the molecular mechanisms triggering the parasite response to chloroquine, as well as its normal protein-oxidation modifications which could be involved in development and pathophysiology and whose targeting might provide ways of fighting against chloroquine-resistance and novel antimalarial targets.

Introduction

2. INTRODUCTION

2.1. Malaria

Malaria is caused by apicomplexan protozoa from the genus *Plasmodium* which are capable of parasitizing a wide range of hosts including mammals, birds, and reptiles (Klemba and Goldberg, 2002). The human infection is transmitted by the bite of an infective female *Anopheles* sp. mosquito and it is the major parasitic disease affecting the populations of tropical and subtropical areas of the world, as well as an increasing number of travellers to these destinations. Although malaria occurs in over 100 countries, the African continent carries the major burden of disease (Fig. 1), with over 90% of all deaths reported from this region (Williams *et al.*, 1999). Four species of *Plasmodium* cause malaria in humans, but *Plasmodium falciparum* is the causative agent of the most severe form of human malaria being annually responsible for the death of more than 1 million people every year mostly children under the age of five (Klemba and Goldberg, 2002).

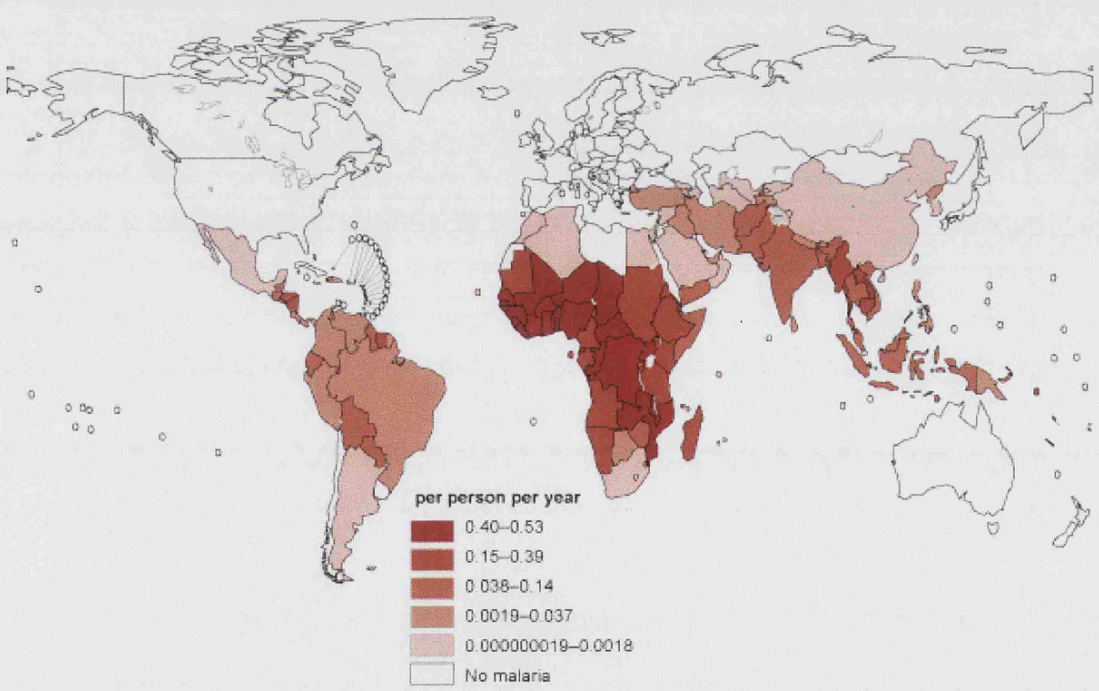


Fig. 1. Clinical incidence of malaria around the world (World Malaria Report 2005, OMS).

2.1.1. The Life Cycle of the Malaria Parasite

The life cycle of the malaria parasite is complex (Fig. 2) and involves two stages: a sexual reproductive stage with multiplication (sporogony) which occurs in the midgut of the mosquito, and an asexual reproduction phase with multiplication (schizogony), which takes place in the human host. In the human part of the cycle , two important phases: the

exoerythrocytic (hepatic) phase and the erythrocytic phase can be distinguished (Frederich *et al.*, 2002).

As depicted in Figure 2, the sporozoites are transmitted to humans by a bite of an infected female mosquito of the genus *Anopheles*. They circulate for a short time in the blood stream, and then invade liver cells, where they develop into exoerythrocytic schizonts during the next 5 to 15 days. *Plasmodium vivax* and *ovale* have a dormant stage, the hypnozoite (Krotoski *et al.*, 1982), that may remain in the liver for weeks or many years before the development of exoerythrocytic schizogony. This results in relapses of infection. *P. falciparum* and *P. malariae* have no persistent phase, but *P. malariae* could persist in the blood for many years if inadequately treated (Frederich *et al.*, 2002). A hepatic (exoerythrocytic) schizont contains 10 000 to 30 000 merozoites, which are released and invade the red blood cells (Frederich *et al.*, 2002). Invasion of erythrocytes by malarial parasites involves a complex series of events that depend on receptor interactions between the surface of both the erythrocyte and merozoite (Chitnis and Blackman, 2000). The entire invasion process takes about 30 seconds. Once the merozoites invade erythrocytes, they begin to undergo their asexual reproduction which leads to the formation of erythrocytic schizonts, through ring and trophozoite (erythrocytic schizogony) (Frederich *et al.*, 2002).

The erythrocyte membrane containing the mature schizont is disrupted and the merozoites are released, invading other red blood cells. In the course of these events, some merozoites which have invaded erythrocytes could differentiate into microgametocytes (male) and macrogametocytes (female). Gametocytes are transmitted to a female *Anopheles* mosquito during a blood meal to complete there their life cycle. Mature gametocytes taken into the midgut of the *Anopheles* mosquito are released from the human erythrocyte to form gametes. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Within18 to 24 hours, the zygote elongates into a slowly motile ookinete.

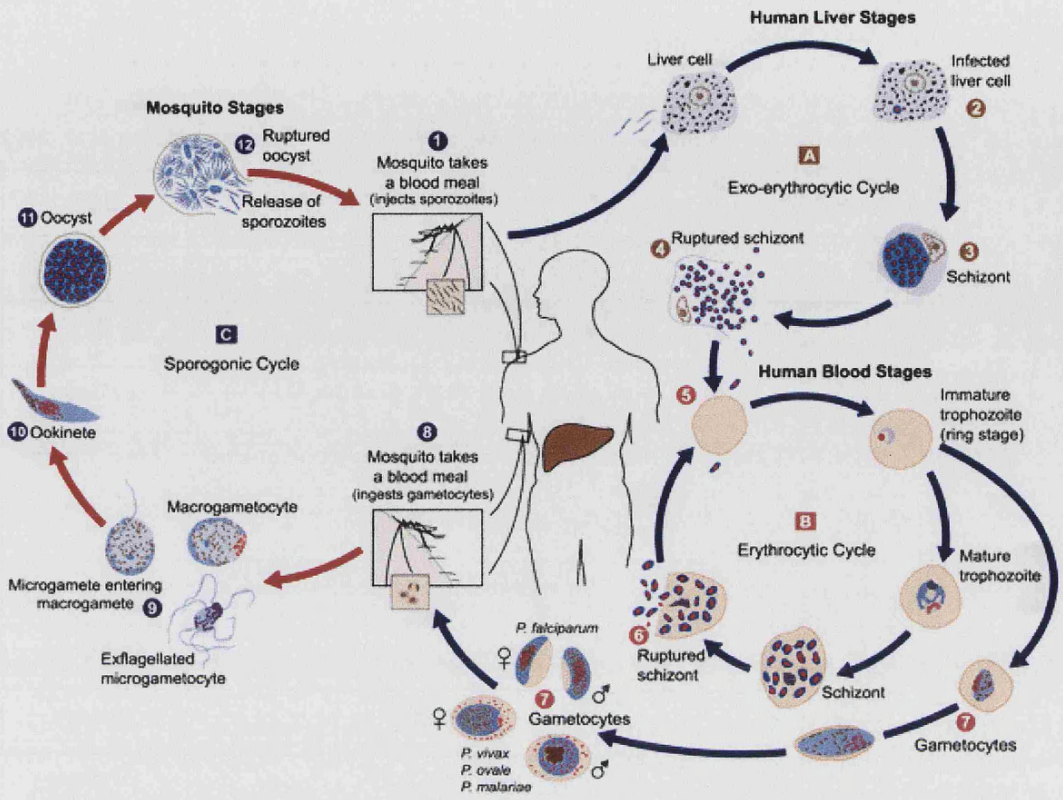


Fig. 2. Life cycle of *Plasmodium* species. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷ and mature into schizonts ❸, which rupture and release merozoites ❹. (In *P. vivax* and *P. ovale* a dormant stage, hypnozoites, can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells ❺. The ring stage develops into trophozoites which mature into schizonts, that disrupt the erythrocyte to release merozoites ❻ to maintain a continuous erythrocytic cycle B. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's digestive tract, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿ which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle ❶. Scheme adopted from www.dpd.cdc.gov/dpdx.

The ookinete cross the peritrophic membrane and the epithelial cell of the midgut, and then transforms into an oocyst beneath the basement membrane of the midgut epithelium. Between 7 and 15 days postinfection, depending on the *Plasmodium* species and ambient temperature, a single oocyst forms more than 10000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate in the acinar cells of the salivary glands. When an infected mosquito bites a susceptible vertebrate host, the

Plasmodium life-cycle begins again (Frederich *et al.*, 2002). The length of *P. falciparum* life cycle is mentioned in Table 1.

Table 1. Length of the different stages of life cycle of *P. falciparum* (Frederich *et al.*, 2002).

ORDER	STAGE NAME	TIME
1	Ookinetes formation	24 to 48 hours
2	Oocyts maturation	9 days
	Time for invasion of salivary glands (1 + 2)	**10 days**
3	Time of circulation of sporozoites in the blood stream	Max. 1 hour
4	Hepatic schizogony	6 days
5	Hypnozoites	Non existent
6	Erythrocytic schizogony	**48 hours**
7	Gametocytogony	10 days
	Complete cycle (1 to 7)	**27 days**

2.1.2. The *P. falciparum* genome

The malaria parasite is a complex multi-stage organism, which has co-evolved in mosquitoes and vertebrates for millions of years. Designing drugs or vaccines that substantially and persistently interrupt the life cycle of this complex parasite will require a comprehensive understanding of its biology (Florens *et al.*, 2002). To this respect, the *P. falciparum* genome sequence is considered fundamental and represents new starting points in the long search for solutions to the malaria problem (Gardner *et al.*, 2002).

An international effort was launched in 1996 to sequence the *P. falciparum* genome with the expectation that the genome sequence would open new avenues for research. The genome sequence of *P. falciparum,* clon 3D7, was fulfilled in 2002 (Gardner *et al.*, 2002).

The 22.8 Mb genome of *P. falciparum* is comprised of 14 linear chromosomes (Gardner *et al.*, 2002), a circular plastid-like genome, and a linear mitochondrial genome (Bozdech *et al.*, 2003). 14 chromosomes of the *P. falciparum* 3D7 rang in size from approximately 0.643 to 3.29 Mb (Gardner *et al.*, 2002). The structures of protein-encoding genes were predicted using several gene-finding programs and manually curated. Approximately 5,300 protein-encoding genes were identified (Gardner *et al.*, 2002). The

number of protein-coding genes is comparable to those in the free-living yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, although its genome is considerably larger than the genomes of these latter organisms (Carlton et al., 2002; Gardner et al., 2002; Mewes et al., 1997; Wood et al., 2002).

It should be emphasized that the *P. falciparum* genome is the most A + T rich genome sequenced to date. In fact, the overall A +T composition is 80.6% in coding regions (Gardner *et al.*, 2002). This bias pervades throughout all chromosomes; intergenic regions frequently show A+T percentages greater than 90%, while protein coding regions, generally, have a lower bias because of their codon use under the universal genetic code (Aravind *et al.*, 2003). However, high and unusual amount of adenine and thymine can be seen in the huge amount of AAT and AAA codons, translating as asparagine and lysine in many proteins of *Plasmodium*. On the other hand, a high amount (around 70%) of guanine and cytosine (G+C) can be seen in other species of *Plasmodium*, such as *P. vivax* or *P. cynomolgi* (McCutchan *et al.*, 1984).

Introns have been predicted in 54% of the *P. falciparum* genes. Excluding introns, the mean length of *P. falciparum* genes is 2.3 Kb, substantially larger than in other unicellular eukaryotic organisms in which the average gene lengths range from 1.3 to 1.6 kb. Thus, *P. falciparum* genes showed a markedly greater proportion of genes (15.5%) longer than 4 kb compared to *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, 3.0% and 3.6%, respectively (Gardner *et al.*, 2002). The explanation for the increased gene length in *P. falciparum* is not clear. Many of these large genes encode uncharacterised proteins that may be cytosolic proteins, as they do not possess recognizable signal peptides (Gardner *et al.*, 2002). In fact, comparison of the initial annotation of the *P. falciparum* nuclear genome with other genomes showed that 60% of the predicted genes cannot be functionally assigned (Kooij *et al.*, 2006). The products of at least 1.3% of the *P. falciparum* genes are known to be involved in cell-to-cell adhesion or invasion of host cells, and a further 3.9% are postulated to have a role in evasion of the host immune response; many of these 250–300 proteins possess host-like extracellular adhesion domains (Kooij *et al.*, 2006). Curiously, only 8% of the *P. falciparum* genes could have assigned functions in metabolism, in contrast to 17% of the genes of the yeast *S. cerevisiae* (Goffeau *et al.*, 1996; Kooij *et al.*, 2006). This suggests either that enzymes are more difficult to identify by sequence homology in *P. falciparum* owing to its great evolutionary distance from other well-studied organisms or that the *P. falciparum* genome contains fewer enzymes as a consequence of its parasitic lifestyle (Kooij *et al.*, 2006).

2.1.3. The *P. falciparum* proteome

Several detailed high-throughput mass-spectrometry studies of the *P. falciparum* proteome have been published. Reassuringly, the protein content of the different blood stages agrees well with the presence of transcripts of the genes encoding these proteins (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). In addition, the proteome of four stages of sporozoites, merozoites, trophozoites and gametocytes has also been profiled (Florens *et al.*, 2002), identifying up to 2415 proteins sorting them into 10 functional categories. It should be emphasized that 51% of them are classified as hypothetical proteins and therefore with unknown function. In addition, over half of the secreted proteins and integral membrane proteins (743 predicted) were annotated as hypothetical. This large class of proteins that has no homology to known proteins represents potential *Plasmodium*-specific proteins that may provide targets for new drug and vaccine development (Florens *et al.*, 2002). It has also been reported that the sporozoite proteome is markedly different from the other stages and about half of the sporozoite proteins are unique to this stage. In contrast, trophozoites, merozoites and gametocytes have fewer unique proteins, sharing a greater proportion of the total. Of the proteins found in multiple stages, the most common were mainly housekeeping proteins such as ribosomal proteins, transcription factors, histones and cytoskeletal proteins (Florens *et al.*, 2002). The analysis undertaken by Lasonder and coworkers was similar except that they studied strain NF54 of *P. falciparum* (Lasonder *et al.*, 2002) instead of the reference genomic and proteomic *P. falciparum* strain 3D7. However, this group adopted a complementary approach to sample classification, dividing the sexual stages into the gametocytes (formed in the human erythrocyte) and gametes (formed in the mosquito gut after the blood meal), while treating the asexual blood stages as just a single group. In this way, they identified 1289 proteins, 714 of which were present in asexual blood stages, 931 in gametocytes and 645 in gametes. Among the last two categories 575 appeared to be unique to the sexual stages and included a subset of proteins containing domains indicative of a role in cell-cell interactions.

Proteome studies have also shown that in many cases, the proteins from *P. falciparum* are consistently bigger than their homologous counterparts from other species, but the role of these parasite-specific inserts in the sequences of *P. falciparum* proteins is uncertain (Pizzi and Frontali, 2001).

Additional proteomes from the ring, schizont and gamete stages were also included later which increased number of identified proteins to 2904 (Le Roch *et al.*, 2004).

In addition to the reported proteomes of the whole life-cycle stages, proteome studies have focused on specific organelles and structures in malaria parasite. In fact, proteome analysis of subcellular components of *Plasmodium* is at the early days for the difficulties encountered in their purification (Di Girolamo *et al.*, 2005). Components of rhoptries, apical secretory organelles of merozoites, most probably involved in parasitophorous vacuole biogenesis, have been identified through proteomic combined with bioinformatic approaches (Sam-Yellowe *et al.*, 2004). Among proteins predicted to be located in this compartment are proteases possibly involved in the processing of merozoite proteins targeted to the rhoptries, enzymes of lipid metabolism that might be implicated in the establishment of the vacuolar membrane, as well as, proteins known to localize to the vacuolar membrane to support the involvement of rhoptry components in the genesis of parasitophorous vacuole (Di Girolamo *et al.*, 2005).

Other complementary studies have also been reported. One of them is devoted to Maurer's cleft proteomics (Vincensini *et al.*, 2005) where provided new insights on the important biological functions of this parasite-derived compartment. Maurer's clefts, flattened elongated vesicles close to the erythrocyte plasma membrane (Langreth *et al.*, 1978), are part of a parasite membrane network extending or budding from the parasitophorous vacuole membrane (Deitsch and Wellems, 1996). This study confirms that Maurer's cleft have characteristics of a secretory compartment addressing parasite proteins to the red cell surface. Moreover, it suggests that in addition to protein trafficking, these structures are also involved in signal transduction.

Another study based on high throughput proteomics have also identified antigens on the surface of infected erythrocyte (Florens *et al.*, 2004). Proteins on the surface of parasite-infected erythrocytes have always been one of the major focuses of malaria research due to their role in pathogenesis and their potential as targets for immunity and drug interference.

Moreover, quantitative proteomics of *P. falciparum* by isotope metabolic labeling presented an innovative method for identification and accurate comparative quantification of labelled peptides from expressed proteins synthesized by in vitro cultures subjected to different stimuli along the cell cycle (Nirmalan et al., 2004). This research has identified divergent patterns of protein and reported transcript levels suggesting modulation at translational level and provided evidence for significant levels of post-translational modification in the parasite. In addition, these studies demonstrate differential effects for a number of proteins unrelated to likely targets of tetracycline and pyrimethamine (Nirmalan et al., 2004).

Proteomic studies are at present very informative particularly for revealing several candidate proteins as antimalarial drug targets and blood-stage malaria vaccine (Kooij *et al.*, 2006).

2.2. Strategies to fight against malaria

The Roll Back Malaria (RBM) Programme, launched in 1988, represents a new approach to the war against malaria (http://www.who.int/malaria/). The RBM relies primarily on six main strategies: curative measures based on research and evidence, early diagnosis, prompt and appropriate treatment, multiple prevention methods, dynamic global movement against the problem, and broad-based coordinated actions backed by focused research.

Past and present malaria control programmes, as well as the most recent Malaria Control Programme Plan, have achieved limited successes in eradicating the scourge. In fact, the malaria situation has steadily deteriorated. Several factors account for limited successes in eradicating malaria such as lack of political will and commitment, poor awareness of the magnitude of the malaria burden, poor health practices by individuals and communities, and the increased resistance to drugs and insecticides.

In general, malaria control efforts include attempts to develop an effective vaccine, eradicate mosquito vectors, and develop new drugs (Oaks *et al.*, 1991; Olliaro *et al.*, 1996). Development of an effective malaria vaccine that could be deployed widely in endemic areas has proved to be more difficult than was anticipated, when the first clinical trials of malaria vaccines were conducted 30 years ago. In fact, a safe, effective and affordable vaccine, that provides sustained protection to the residents of malaria endemic areas, is still many years away (Greenwood, 2005). Efforts to control *Anopheles* mosquitoes have also had limited success, although the use of insecticide-impregnated bed nets does appear to reduce malaria-related death rates (Alonso *et al.*, 1991). In addition, methods to replace natural vector populations with mosquitoes unable to support parasite development are under study and may contribute to malaria control in the long term (Collins and Besansky, 1994). However, the current limitations of vaccine and vector control, as well as the increasing resistance of malaria parasites to existing drugs, highlight the continued need for new antimalarial agents.

2.2.1. Antimalarial drugs

Antimalarial drugs have been used for centuries. Early natural products, including the bark of the cinchona tree in South America and extracts of the wormwood plant in China, were among the first effective antimicrobial agents to be used. Cinchona bark was

used in Europe beginning in the 17th century, and upon its isolation from bark in 1820, quinine became widely used. In the last 50 years, extensive efforts, including the screening of hundreds of thousands of compounds, have led to the development of a number of effective synthetic antimalarial drugs (Rosenthal, 1998).

Drug therapy remains the mainstay of treatment and prevention of malaria. *Plasmodium* has a complex life cycle involving an arthropod vector and distinct stages within the human host. Each parasite stage plays a unique role in transmission, disease, and latency. These different stages may vary in their response to the various antimalarial compounds. Antimalarial compounds should be evaluated in the context of life cycle stage effect, molecular target, and half-life. Drug effect on each life cycle stage varies because of stage-specific biology (Daily, 2006).

Traditionally, four classes of potential antimalarial activities can be categorized according to their stage of action (Frederich *et al.*, 2002):

a) Tissue schizonticides: These drugs act on the primary tissue forms of the plasmodia which after growth within the liver, initiate the erythrocytic stage. By blocking this stage, further development of the infection can be theoretically prevented.

b) Blood schizonticides: These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in antimalarial chemotherapy.

c) Gametocytocides: These drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito.

d) Sporontocides: These drugs prevent the development of oocysts in the mosquito and thus ablate the transmission.

Therapy usually poses challenging problems, because of the complexity of parasite which has led to the limited number of available antimalarial drugs. The most commonly used antimalarial drugs are detailed below.

2.2.1.1. Quinolines

Quinoline-containing antimalarial drugs, such as chloroquine, quinine, and mefloquine, are vital compounds in our chemotherapeutic armory which have long been used to combat malaria (Tilley *et al.*, 2001). Quinine has been used for 300 years-ever since bark extracts from the *Cinchona* tree were first shown to have antimalarial activity.

Early synthetic work in Germany produced the 8-aminoquinolines, primaquine and pamaquine. Primaquine is still used to eradicate the liver-stage hypnozoites of *P. vivax* and *P. ovale* (Tilley *et al.*, 2001). During World War II, the synthetic 4-aminoquinoline, chloroquine (CQ) was introduced (Loeb *et al.*, 1946). Because of its low toxicity and, for many years, its effectiveness, CQ has been a mainstay in the fight against malaria ever since.

In general, the quinoline antimalarials function by targeting the parasite-specific pathway of haemoglobin breakdown (Becker *et al.*, 2004). CQ is a weakly basic amphipath that has been shown to accumulate in the food vacuole (Egan, 2001; Spiller *et al.*, 2002). In this location it is thought to interact with oxidised haem (FP-FeIII), the free toxic haem released by proteolysis of haemoglobin, and interfere with its detoxification process to make inert crystalline substance called haemozoin (Egan *et al.*, 2002; Becker *et al.*, 2004).

Resistance to CQ reported in many parts of the world by the end of the 1950s (White, 2006) and therefore lack of their therapeutical efficacy has contributed to a great deal of the difficulties in controlling malaria (Tilley *et al.*, 2001; Ehrhardt *et al.*, 2007).

2.2.1.2. Antifolates

The most important antifolate drug is pyrimethamine which targets the dihydrofolate reductase (DHFR) activity of the bifunctional DHFR–thymidylate synthetase protein. DHFR is present in both host and parasite being essential to maintain a constant supply of fully reduced forms of folate cofactors for essential one-carbon transfer reactions, including the provision of nucleotides for DNA synthesis and the metabolism of certain amino acids (Hyde, 2007). Pyrimethamine is normally used in combination with other drugs as initially uses alone after its introduction in the 1940 / 1950s arose resistance rapidly (Hyde, 2007). Unfortunately, falciparum parasites are able to quickly accumulate multiple genetic mutations producing enzymes resistant to such drugs (Shanks, 2006). Other antimalarials of this family are proguanil, and sulfonamides such as sulfadoxine and sulfalene (Hyde, 2007). Although pyrimethamine and proguanil were initially used alone after their introduction in the 1940 / 1950s, resistance to them also arose rapidly. To fight drug resistance, combinations such as pyrimethamine–sulfadoxine were then produced. However, resistance to this combination is increasing and therefore its therapeutical action is highly limited to the areas were not resistance reported (Warhurst, 2002).

2.2.1.3. Artemisinins

The artemisinins are sesquiterpene lactones that derive ultimately from the Chinese herb qinghao (*Artemisia annua*), used for centuries to treat malaria and other parasitic diseases (Hyde, 2007).

Artemisinins are highly active, decreasing the parasite biomass 10 000-fold in a single asexual cycle (White, 1997; Woodrow *et al.*, 2005). This makes artemisinins the most active and rapid-acting antimalarial drugs known today (Schlitzer, 2007).

A key structural feature of all artemisinins is the 1,2,4-trioxane substructure or, more precisely, the endoperoxide, which is mandatory for antimalarial activity. Despite the growing importance of artemisinins, their exact mechanism of action is still unresolved and remains a matter of intense debate (Schlitzer, 2007). It has been proposed that iron(II)-mediated cleavage of the endoperoxide leads to the formation of different carbon-centered radicals (Schlitzer, 2007). For a long time it was thought that the formation of carbon radicals from artemisinin takes place in the digestive vacuole and that FP IX (ferri/ferroprotoporphyrin IX) is the activating species. The reactive carbon radicals are thought to subsequently react more or less indiscriminately with different protein targets as well as with FP IX itself, thus preventing haeme detoxification and inhibiting important enzyme activities. However, this concept has been questioned owing to some contradictory findings: artemisinins act against all developmental parasite stages, including those which do not produce haemozoin (Schlitzer, 2007). Recently, has been suggested that endoperoxide cleavage should take place in the cytoplasm catalyzed by a cytoplasmic iron(II) source. The resulting reactive species then very specifically inhibits an ATP-dependent Ca2+ pump (PfATP6) located on the endoplasmic reticulum, but remains unknown how the inhibition of PfATP6 leads to the rapid killing of the parasite (Schlitzer, 2007; Hunt *et al.*, 2007).

Special properties of artemisinins make them the most important class of antimalarial agent, and a mainstay against multidrug resistant *P. falciparum.* In early 2006, due to global concerns about the need to safeguard the efficacy of this antimalarial, the WHO urged the malaria community to stop the production and use of artemisinin monotherapies and switch to artemisinin-based combination therapies. But, the major problem is that their use in many countries has been severely restricted by cost, and also artemisinins in combination are several-fold more expensive than the now almost useless CQ, or sulfadoxinepyrimethamine, whose efficacy is also declining (Woodrow *et al.*, 2005).

To document the existence of drug resistance it should be mentioned that although *P. falciparum* isolates may vary in their *in vitro* sensitivity to artemisinin, no mutations or

copy number changes were apparent in any of the genes that have been potentially implicated in the resistance of *P. falciparum* (Afonso *et al.*, 2006). However, the studies, which support PfATP6 as the major target of artemisinins, showed that parasites harbouring a single point mutation in PFATP6 are resistant to artemisinin (Uhlemann *et al.*, 2005). Recently, a critical mutation associated with resistance to artemisinin in CQ-resistant *P. chabaudi* has been found in a deubiquinating enzyme involved in the ATP-dependent protein degradation pathway. Such mutation might lead to an increase in degradation of proteins by the proteasome, eliminating artemisinin-induced damaged proteins (Hunt *et al.*, 2007).

2.2.1.4. Antibiotics

At first sight, it is surprising that several antibacterial agents display considerable activity against the eukaryotic malaria parasites, as antibiotics are known to specifically target prokaryotic structures. This apparent contradiction can be explained by the presence of two organelles, the mitochondrion and the apicoplast. Both organelles have their own DNA and bacteria like machinery for replication, transcription, and translation (Schlitzer, 2007). Apart from tetracyclines, which are thought to act mainly against the mitochondrion (Kiatfuengfoo *et al.*, 1989) all other antibiotics seem to act on the apicoplast (Vaidya, 2004). Tetracycline and derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis (Bloland, 2001). It has been shown that doxycycline blocks the expression of apicoplast genes (Dahl *et al.*, 2006). Characteristically, most antibiotics do not exert any visible effect in the first intracellular cycle, but during the second cycle the parasites are killed after the invasion of the new host cell. This phenomenon is known "delayed kill effect." As a result of the delayed kill effect, fever and parasite clearance times are significantly longer when antibiotics are administered as single agents in comparison with classical antimalarials (approximately 4 versus 2 days). Because this delay may be fatal in non-immune patients, antibiotics are used only in combination with a faster-acting drug for the therapy of acute malaria (Schlitzer, 2007). For example, in areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates (Bloland, 2001). However, antibiotics, especially doxycycline, can be used prophylactically as single agents, although they are not registered for this indication in most countries (Schlitzer, 2007).

2.2.1.5. Antimalarial drug combination therapy

Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination (WHO, 2001). The basis of combination chemotherapy in malaria is typically formed when a rapidly acting drug (quinine or artemisinin) is combined with a slower acting drug (tetracycline, mefloquine etc) given over enough time (at least four parasite generations which is about eight days) to kill any residual parasites (White, 2004).

When two effective drugs are used, the probability of selecting a mutant parasite with resistance to both drugs seems unlikely. Of currently available combinations which have been proven effective in field trials, quinine-tetracycline and chlorproguanil-dapsone have been reported (Shanks, 2006). As with any medication, each combination has its own advantages and disadvantages based on cost, tolerability and ease of administration (Shanks, 2006). It should also be emphasized that any benefits of combination therapy in preventing development or intensification of resistance may be lost due to unofficial and incorrect use of the component drugs outside of official health services (Bloland, 2001).

2.2.1.6. Protease inhibitors

Proteolytic enzymes play important roles in the life cycles of all medically important protozoan parasites, including the organisms that cause malaria (Rosenthal, 1999). Protease inhibitors have significantly been valuable reagents for studying the biological roles of proteases of malaria parasites and have demonstrated potent antimalarial effects (Rosenthal, 1999; Rosenthal, 2004). The range of protease inhibitors evaluated as potential antimalarial agents is small but growing in number (Go, 2003). Increasing evidence suggests that inhibitors of plasmodial proteases such as aspartic proteases (plasmepsin I and plasmepsin II) and cysteine protease (falcipain-2) have potent antimalarial activity and may be appropriate antimalarials (Rosenthal, 2001).

A collection of peptidomimetic compounds, designed as aspartic proteinase inhibitors, significantly blocked haemoglobin degradation and kill parasites in culture (Francis *et al.*, 1994; Moon *et al.*, 1997). Pepstatin A, a general inhibitor of aspartic proteases was also reported to inhibit haemoglobin degradation in *P. falciparum* (Gluzman *et al.*, 1994).

Incubation of *P. falciparum* culture in the presence of cysteine protease inhibitors, leupeptin and E64 results in accumulation of undegraded haemoglobin in the food vacuole (Rosenthal, 1995 ; Gamboa de Dominguez and Rosenthal, 1996). Production of hemozoin

was also blocked by E64 (Asawamahasakda *et al.*, 1994). Cysteine protease inhibitors also block rupture of the host cell membrane and release of merozoites which leads to clustered merozoite structures. Therefore, thiol proteinase inhibitors such as E64, leupeptin, and chymostatin appear to be a valuable template for the development of new inhibitors specific to individual plasmodial proteases (Bailly *et al.*, 1992).

Several aspartic and cysteine proteases have been evaluated as candidate drug targets (Singh and Rosenthal, 2001; Pandey *et al.*, 2005). Inhibition of hemoglobinases in *Plasmodium* remains as one of the most outstanding therapeutic strategies. In fact, use of inhibitors for both above mentioned classes of proteases in combination showed synergistic killing of the parasite (Semenov *et al.*, 1998).

The metal chelators such as EDTA, dipicolinic acid, and 1,10-phenanthroline inhibit falcilysin, a metallopeptidase involved in haemoglobin catabolism. Moreover, GM6001, a well-known inhibitor of both the mammalian matrix and disintegrin metalloprotease family showed participation of malaria parasite metalloproteases during host cell invasion (Grobelny *et al.*, 1992; Ito *et al.*, 2004). Reduction of invasion by above mentioned metalloprotease inhibitor, 1,10-phenanthroline, has also been reported (Kitjaroentham *et al.*, 2006), pointing out the importance of metal-requiring proteases in *P. falciparum* merozoite entry into host red blood cells.

Amino acid dipeptide analogs, such as bestatin and its derivatives, which are potent inhibitors of the aminopeptidases also blocked the growth of *P. falciparum* in culture (Stack *et al.*, 2007).

The parasite proteasome has also been inhibited by MLN-273, a small molecule dipeptidyl boronic acid proteasome inhibitor, which caused an overall reduction in protein degradation in *P. falciparum* and blocked erythrocytic development at an early ring stage (Lindenthal *et al.*, 2005).

In addition, recent advances in the characterization of other haemoglobinases and in proteases that are required for rupture or invasion suggest that inhibitors of many of these enzymes may also demonstrate antiparasitic activity and therefore can be considered as antimalarial candidates (Rosenthal, 2001).

2.2.2. Antimalarial drug resistance

Antimalarial drug resistance has been defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject (Bloland, 2001).

Drug resistant malaria is mostly due to *P. falciparum*, the highly prevalent species in tropical Africa, Amazon, and Southeast Asia and, in general, originates from chromosomal mutations. (Le Bras *et al.*, 2006).

In the continuing absence of clinically proven vaccines, preventing or treating malaria parasite infections in the human host depends upon chemoprophylaxis and chemotherapy. Since the advent of the first synthetic antimalarials in the 1930s, only a small number of compounds has proved suitable for licensing as drugs for human use, and several of these are now greatly compromised by the spread of drug-resistant parasite strains, including the cheapest formulations that are important to underdeveloped countries, namely CQ and pyrimethamine–sulfadoxine (Hyde, 2007).

Resistance of malaria parasites arises from several factors, including overuse of antimalarial drugs for prophylaxis, inadequate or incomplete therapeutic treatments of active infections, a high level of parasite adaptability at the genetic and metabolic levels, product instability in tropical climate, and a massive proliferation rate that permits selected populations to emerge relatively rapidly (Hyde, 2007).

CQ resistance was first observed in Thailand in 1957 and on the Colombian–Venezuelan border in 1959. By 1988 resistance had spread to essentially all of sub-Saharan Africa and today CQ has lost its efficacy in almost all areas of the world (Hyde, 2007). Molecular studies have shown multiple CQ resistance founder mutations and a large scale CQ resistance selective sweep from Southeast Asia to Africa as well as other sweeps across the Amazon in South America and in Papua New Guinea (Wootton *et al.*, 2002).

Despite multiple independent origins of CQ resistance worldwide, CQ resistance parasites share some common phenotypes such as increased IC_{50} (Ringwald *et al.*, 2000), reduced CQ accumulation in the acidic digestive vacuole (Sanchez *et al.*, 2003; Bennett *et al.*, 2004), reduced pH in digestive vacuole (Bennett *et al.*, 2004) and universally shared point mutations in a putative transporter gene termed *P. falciparum* chloroquine resistance transporter (pfcrt), which is the major determinant of CQ resistance (Jiang *et al.*, 2006).

Widespread and increasing resistance to other available drugs contributes enormously to the difficulties in controlling malaria (Hyde, 2007). Figure 3 represents the geographical distribution of resistance to the most common antimalarials in the endemic areas of *P. falciparum* malaria.

The principal antifolate drugs are pyrimethamine, proguanil, and the sulfonamides (such as sulfadoxine). *P. falciparum* can quickly create multiple genetic mutations which lead to resistance to these drugs (Shanks, 2006). In fact, resistance arose rapidly by

monotherapy of pyrimethamine and proguanil after their introduction in the 1940/1950s. Then, combination of pyrimethamine-sulfadoxine were produced (Hyde, 2007). However, in South East Asia from the mid-1960s onwards, pyrimethamine–sulfadoxine combination failed as primary therapy in a very short period of time once it was in general use (Wongsrichanalai *et al.*, 2002). These same resistance genes have rapidly spread across Asia and into Africa such that treatment with a single administration of sulfadoxine-pyrimethamine often fails to cure (Peterson *et al.*, 1988; Terlouw *et al.*, 2003; Abacassamo *et al.*, 2004). However, despite a rapid spread of pyrimethamine–sulfadoxine resistance and its diminishing efficacy, this reasonably priced combination has been extensively used to combat chloroquine-resistant parasites in Africa since the early 1990s (Hyde, 2007).

Mefloquine, a quinoline methanol derivative which introduced in the 1984 to fight against parasites that had become resistant to CQ and the antifolates, developed significant resistance within 6 years (Price *et al.*, 2004).

Sodium artesunate so far is the most effective artemisinin and can rapidly reduce parasite numbers ~ 10^4-fold in a single erythrocytic cycle (Hyde, 2007). However, the danger of using this type of drug in monotherapy was emphasized by a study where decreased sensitivity was observed in recrudescent parasites after 7 days of treatment with artesunate alone (Menard *et al.*, 2005). Therefore, artemisinin combination therapies have recently been adopted as first-line therapy for *P. falciparum* infections in most malaria-endemic countries (Alker *et al.*, 2007) and WHO has recommended the use of this combination in the treatment of uncomplicated *P. falciparum* infections (Faye *et al.*, 2007). Although new generation artemisinin-based combination therapies are highly effective and life-saving in all but most advanced cases (World Malaria Report 2005, OMS), there is doubt to the effectiveness of certain of these combinations where the partner drug is already resistant (Duffy and Mutabingwa, 2006). Moreover, the main obstacles to the success of combination treatment in preventing the emergence of resistance will be incomplete coverage, or inadequate treatment. In fact, irrespective of the epidemiological setting, ensuring that patients with high parasitaemias receive a full course of adequate doses of artemisinin combination treatment would be an effective method of slowing the emergence of antimalarial drug resistance (White, 2004). Artemisinin combination therapies offer hope, but it is not clear that which drug combinations are most effective, or most sustainable, in different populations. That knowledge requires many more studies and continued surveillance for resistance after use of such combinations (Duffy and Mutabingwa, 2006). To this respect, it should be stressed that resistance to subcurative doses of artemisinin in the CQ-resistant clones of the rodent malaria parasite *P. chabaudi*

have been experimentally obtained (Afonso *et al.*, 2006; Hunt *et al.*, 2007) and therefore, it is expected that resistance to artemisinins in human clinical practice would also be developed in a future.

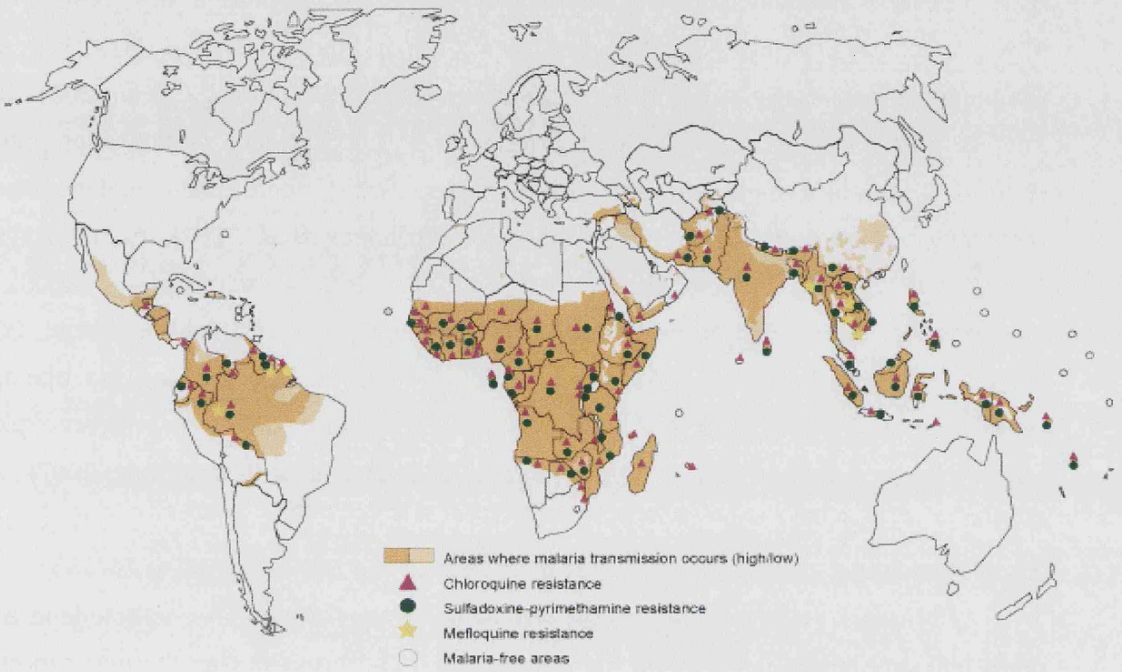


Fig. 3. Drug resistance to *P. falciparum* around the world (World Malaria Report 2005, OMS).

2.3. Potential targets for new antimalarials

While in the past century has been generated significant progress in anti-malarial drug development, many of the drugs are currently losing their efficacy due to the rise of drug resistant *Plasmodium* strains. This increasingly serious problem has led to an urgent need to develop new and effective antimalarial molecules. This goal can be achieved in two ways: either by focusing on validated targets in order to generate new drug candidates; or by identifying new potential targets for malaria chemotherapy (Jana and Paliwal, 2007). Sequencing of the *P. falciparum* genome, advances in molecular techniques, characterization of gene expression directly at the proteomic level, and the rapidly-expanding field of metabolomics have refocused antimalarial drug discovery efforts. However, despite substantial advances in our understanding of parasite biology and biochemistry, it appears that these organisms often use novel modes of metabolism, signaling, protein trafficking pathways, cell division and extracellular communication mechanisms. The proteins involved in these processes often possess significant sequence variation between them and show distinct biochemical or catalytic properties

(Jana and Paliwal, 2007). Structural studies on desirable proteins are also necessary to do a rational drug design using structural information. In addition, understanding of the mechanisms of action and resistance to traditional antimalarial drugs is important in order to design newer and more effective drugs.

Several parasite biochemical pathways such as membrane biosynthesis (Vial, 1996; Ancelin *et al.*, 2003; Roggero *et al.*, 2004), transporters (Joet *et al.*, 2003; Kirk *et al.*, 2005), proteases (Bailly *et al.*, 1992; Rosenthal *et al.*, 1996; Coombs *et al.*, 2001; Singh and Rosenthal, 2001; Lee *et al.*, 2003; Pandey *et al.*, 2005), haem detoxification pathway (Padmanaban and Rangarajan, 2000; Kannan *et al.*, 2005), post-translational modification pathways (Jomaa *et al.*, 1999; Sullivan *et al.*, 2006; Fennell *et al.*, 2007), the redox system (Krauth-Siegel and Coombs, 1999; Wang *et al.*, 1999; Luersen *et al.*, 2000), the mitochondrial system (Krungkrai *et al.*, 1997; Srivastava *et al.*, 1997; Mi-Ichi *et al.*, 2005), nucleic acid metabolism (Nduati *et al.*, 2005; Ting *et al.*, 2005), and the apicoplast (McFadden and Roos, 1999; McLeod *et al.*, 2001; He *et al.*, 2004), have been exploited for drug target identification (Jana and Paliwal, 2007; Linares and Rodriguez, 2007).

2.3.1. Proteases

Proteases from a variety of protozoan parasites have been characterized at the molecular and cellular levels, and the many roles that proteases play in these organisms are coming into focus. Central roles have been proposed for proteases in diverse processes such as host cell invasion, catabolism of host proteins and host cell rupture (Klemba and Goldberg, 2002).

Malarial proteases have long been considered potential targets for chemotherapy due to the above mentioned roles in the parasite life cycle, and the feasibility of designing specific inhibitors (McKerrow *et al.*, 1993; Rosenthal, 1998; Blackman, 2000; Rosenthal, 2002). Important advances over about the last decade have led to the biochemical and molecular characterization of a number of plasmodial proteases. Moreover, the completion of the *P. falciparum* genome provides a basis to identify new proteases and therefore subsequent targets for antimalaria chemotherapy (Wu *et al.*, 2003). The comparative genomic analysis, using an extensive sequence similarity search, has predicted in the *P. falciparum* genome up to 92 putative proteases belong to 26 families of five catalytic classes of aspartic, cysteine, metallo, serine, and threonine in this parasite. Of the 92, at least 88 have been demonstrated to code for gene products at the transcriptional levels, based upon the microarray and proteomics data (Wu *et al.*, 2003).

The biological processes where specific proteases have essential function in different intraerythrocytic stages *P. falciparum* has further been detailed below.

2.3.1.1. Host cell invasion

The *P. falciparum* invasion into the host erythrocytes requires the activity of parasite proteases (Blackman, 2004). Mature merozoites, the end product of the asexual replication cycle, burst out of the depleted host erythorcyte and rapidly enter new erythrocytes. The invasion process can be broken down into several steps: attachment of the merozoite to specific red blood cell receptors, reorientation to bring the apical end of the parasite in contact with the erythrocyte surface, release of the contents of specialized secretory organelles, and simultaneous formation of a parasite-red cell junction through wich the merozoites enteres (Klemba and Goldberg, 2002).

The invasion by *P. falciparum* merozoites has generally been shown to be inhibited by serine protease inhibitors such as chymostatin. Such observation suggested that some proteases play critical role in this process (Dluzewski *et al.*, 1986).

Subtilase-1 and Subtilase-2, two homologous serine proteases are expressed in schizonts and merozoites and demonstrated to be involved in merozoite invasion (Blackman *et al.*, 1998; Barale *et al.*, 1999; Hackett *et al.*, 1999). The gp76 GPI-anchored serine protease, which cleaves host erythrocyte surface proteins in *P. falciparum* (Braun-Breton *et al.*, 1988), is believed to facilitate parasitophorous vacuole formation and function in invasion (Roggwiller *et al.*, 1996). Two other *Plasmodium* serine proteases, serine repeat antigen 5 (SERA5) and acid basic repeat antigen (ABRA), have been described to be involved in erythrocyte invasion (Blackman, 2004). SERA5 is an abundant component of the parasitophorous vacuole that was present in a soluble form at merozoite release. Antibodies against it could interfere with merozoite release and erythrocyte invasion (Blackman, 2000). ABRA is a protein that accumulates during schizont maturation in the parasitophorous vacuole in a soluble form, but is also bound to the merozoite surface (Blackman, 2004). An slight reduction in invasion has been observed in transgenic parasites lacking surface form of ABRA (Mills *et al.*, 2002).

Besides serine proteases, cysteine protease may also play a role in invasion. It has been reported that falcipain-1, a cysteine protease, was active during the invasive merozoite stage (Greenbaum et al., 2002).

Finally, the malaria parasite may possibly employ metalloproteases during host cell invasion. In fact, mRNA expression analysis (Bozdech *et al.*, 2003), and also invasion inhibition of merozoites by 1,10-phenanthroline have revealed involvement of the metalloproteases in this process (Kitjaroentham *et al.*, 2006).

2.3.1.2. Haemoglobin degradation

The parasite ingests and degrades most of the host cell haemoglobin during the different intraerythrocytic stages of ring, trophozoite, and schizont (Goldberg, 1993). However, the metabolic activity varies between them and is most pronounced during the trophozoite stage (Francis *et al.*, 1997). Studies using protease inhibitors have proven haemoglobin degradation to be essential for parasite survival (Bailly *et al.*, 1992; Francis *et al.*, 1994; Rosenthal, 1995; Olson *et al.*, 1999).

Several enzymes have been shown to be involved in haemoglobin proteolysis. In *P. falciparum* these are aspartic proteases of plasmepsin I, II, IV, and the closely related histoaspartic protease (HAP) (Banerjee *et al.*, 2002), cysteine proteases of falcipain-1 (Salas *et al.*, 1995; Sijwali *et al.*, 2004), falcipain-2 (Shenai *et al.*, 2000), falcipain-2′ (Sijwali and Rosenthal, 2004), and falcipain-3 (Sijwali *et al.*, 2001), a metalloprotease (falcilysin) (Eggleson *et al.*, 1999), and dipeptidyl aminopeptidase 1 (DPAP1) (Klemba *et al.*, 2004). The degradation process appears to follow an ordered pathway (Gluzman *et al.*, 1994; Eggleson *et al.*, 1999). However, it has been difficult to determine the exact sequence of events, especially whether a plasmepsin or a falcipain catalyzes the initial cleavage (Gluzman *et al.*, 1994; Sijwali *et al.*, 2001; Coombs *et al.*, 2001; Ersmark *et al.*, 2006). The general pathway is outlined in Figure 4.

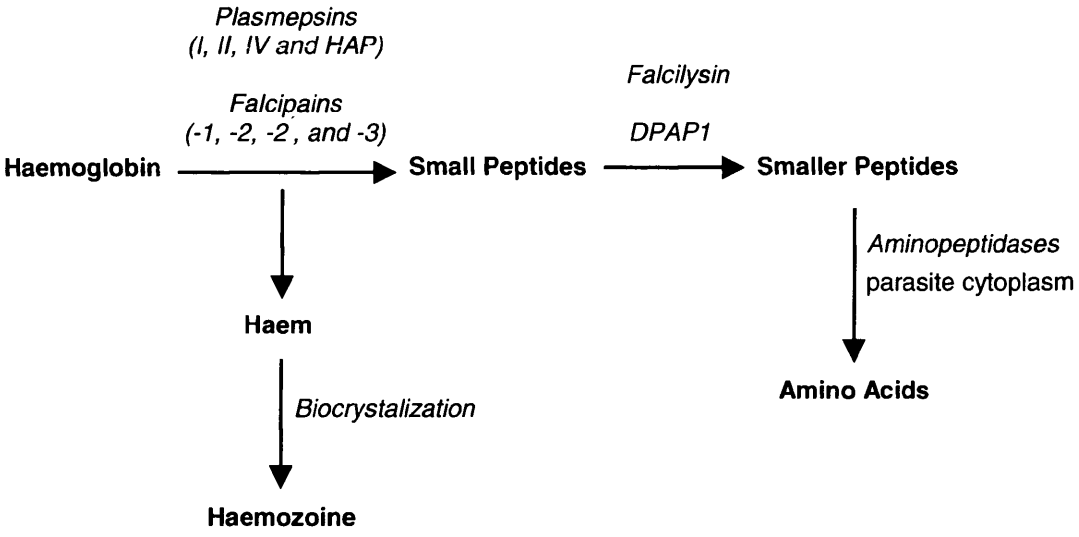


Fig. 4. The general pathway for haemoglobin metabolism in *P. falciparum* (Ersmark *et al.*, 2006). Degradation into small peptides can be accomplished by both plasmepsins and falcipains (Francis *et al.*, 1997). Falcilysin is only able to cleave small polypeptides, producing even shorter oligopeptides (Eggleson *et al.*, 1999). DPAP1 cleaved off dipeptides from haemoglobin-derived oligopeptides in the food vacuole (Klemba *et al.*, 2004). Last hydrolysis to free amino acids is considered to take place in the parasite cytoplasm by aminopeptidases (Gavigan *et al.*, 2001).

2.3.1.3. Host cell rupture

Once an intracellular parasite has completed its development in the host cell, it must release to infect new cells and accompany the completion of the erythrocytic cycle (Klemba and Goldberg, 2002). Studies with different protease inhibitors showed the involvement of proteases in this step (Debrabant and Delplace, 1989; Hadley et al., 1983; Lyon et al., 1986; Salmon et al., 2001; Wickham et al., 2003).

Proteases capable of disrupting the erythrocyte membrane skeleton have been considered as responsible factors (Klemba and Goldberg, 2002). There are evidences that in addition to haemoglobin degradation, plasmepsins II and IV may also be involved in cleavage of the red cell membrane skeleton as they could degrade the host cytoskeletal protein, spectrin, in schizonts (Le Bonniec *et al.*, 1999; Banerjee *et al.*, 2002; Wyatt and Berry, 2002).

Another erythrocyte skeletal protein, ankyrin, was cleaved by a cysteine protease activity in *P. falciparum* extracts (Raphael *et al.*, 2000). Older studies showed the accumulation of mature schizonts in cultures treated with leupeptin, a cysteine protease inhibitor (Hadley *et al.*, 1983; Lyon *et al.*, 1986; Debrabant and Delplace, 1989). Two other studies have further explored this event. Thus, E-64, another cysteine protease inhibitor, blocked the lysis of the parasitophorous vacuole membrane (Salmon *et al.*, 2001). This result suggested the implication of cysteine protease activity in the hydrolysis of parasitophorous vacuole membrane-associated proteins to mediate merozoite release. A second report showed that two cysteine protease inhibitors, leupeptin and antipain, blocked lysis of the erythrocyte membrane (Wickham *et al.*, 2003). Accordingly, it appears that the release of merozoites is a two-step process, requiring hydrolysis of proteins associated with the parasitophorous vacuole and erythrocyte membranes, and therefore cysteine protease activity appears to be required for both steps (Rosenthal, 2004).

2.3.2. Post-translational modification of proteins

With complete genome sequences appearing, attention is becoming increasingly directed towards the gene products of a given organism, in a specific situation. Proteomic studies have revealed that the repertoire of expressed proteins can expand beyond what is predicted by direct translation of the complement of open reading frames contained within a genome. For example, the proteome can acquire additional levels of complexity with differential expression of individual polypeptides or members of protein families as a function of developmental stage or in response to environmental cues. However, one of the most important and fundamental aspects of proteomic complexity comes from the various processing events that many proteins experience following their synthesis, i.e.,

post-translational modification (Eichler and Adams, 2005). While posttranslational modifications enhance the already high complexity of a cellular proteome, they have key functions with many different purposes in various cellular processes such as enzyme regulation, signal transduction, mediation of protein localization, interactions and stability (Reinders and Sickmann, 2007). Genomic data can only partly predict such modifications although specific software and databases are rapidly evolving (Blom *et al.*, 2004; Chen *et al.*, 2006; Lee *et al.*, 2006; Xue *et al.*, 2006). Therefore, proteomics is the method for the analysis of modified proteins and peptides. However, the analysis of posttranslational modifications is probably the most versatile and difficult, but also most frequently studied area of interest in proteomics research (Reinders and Sickmann, 2007).

Proteins can be modified post-translationally by proteolytic processing of the newly synthesized polypeptide chain, by covalent attachment of one or more of several classes of molecules, by the formation of intra- or intermolecular linkages, or by any combination of these events (Walsh *et al.*, 2005).

Proteolytic processing of newly synthesized polypeptide chains allows the cell to control the folding and function of a protein. By removing specific targeting sequences or other stretches of amino acid residues, the cell is able to control where, when, and how a protein will act. As such, post-translational modifications can significantly modulate the physicochemical and biological properties of a protein through effects on protein function, subcellular localization, oligomerization, folding, or turnover (Eichler and Adams, 2005).

By chemically linking of different modifying groups either permanently or temporarily and by allowing for changes in the molecular composition of the modifying moieties, covalent modifications can provide proteins with properties that are very different from those that are predicted by the encoding genes. Examples of such covalent modifications include glycosylation, lipid attachment, phosphorylation, methylation and protein oxidation (Eichler and Adams, 2005; Reinders and Sickmann, 2007).

In common with studies on many other organisms, mass spectrometry of *P. falciparum* extracts separated on 2-D gels, highlights the presence of many proteins that are represented by more than one spot in the gel. These isoforms of a particular protein assumed to be post-translationally modified variants of that protein (Nirmalan *et al.*, 2004; Sims and Hyde, 2006). Biochemical studies have indicated that glycosylation (Gowda and Davidson, 1999) and phosphorylation (Jones and Edmundson, 1990; Suetterlin *et al.*, 1991) occur quite widely on parasite proteins, while acetylation has been observed on the parasite histones (Freitas-Junior *et al.*, 2005) and actin (Schmitz *et al.*, 2005).

A comprehensive analysis of different post-translational modifications in parallel is usually not possible on a global scale. Therefore, the analysis should either be focussed on a single or very few distinct proteins or be directed towards a certain type of modification (Reinders and Sickmann, 2007).

2.3.2.1. Proteolytic processing

Protein processing is considered as a regulatory mechanism in function of many proteins such as receptors, kinases, transcription factors, and structural components. Such modification can remove regulatory proteins when they are not needed, alter an existing function or transform others from the inactive forms into the biologically active state. The latter mechanism often involves a subsequent change of cellular localization (Ehrmann and Clausen, 2004).

Proteolytic processing is a common posttranslational modification of a number of proteins involved in attachment and invasion of apicomplexan parasites such as *Plasmodium* spp. (Carruthers and Blackman, 2005). Processing of these proteins occurs either during transport through the secretory pathway or after secretion onto the parasites' surface. In many cases, proteolytic processing of these proteins has been shown to be crucial for invasion of host cells by these parasites (Carruthers and Blackman, 2005; Wanyiri *et al.*, 2007).

In *P. falciparum*, a comparison of orthologous proteins reveals that *Plasmodium* proteins can be up to 50% longer than the yeast proteins (Aravind *et al.*, 2003). This increased size is in striking contrast to the marked reduction in protein size that is seen in another parasitic eukaryote, *Encephalitozoon* (Katinka *et al.*, 2001). Analysis of this size difference brings up a remarkable feature of *Plasmodium* proteins; they are vastly more enriched in stretches that are predicted to form nonglobular structures than most other eukaryotes. These stretches show a characteristic composition in terms of being biased toward one or a few amino acids and are often characterized by homopolymeric runs of residues (most frequently asparagines) (Aravind *et al.*, 2003). These stretches possess low entropy or complexity (Wootton, 1994). In contrast, those regions of a protein that form globular structures show a greater diversity of amino acids in any given stretch of primary structure and are accordingly high entropy or high complexity regions (Wootton, 1994). Enrichment of low complexity regions in asparagines distinguishes the *Plasmodium* from most other eukaryotes (except *Dictyostelium discoideum*), whose low complexity regions are typically enriched in glutamate and glutamine (Aravind *et al.*, 2003). In most eukaryotes studied to date, low complexity regions are seen mainly in transcription factors and nuclear proteins, and some large cytoskeletal proteins. In contrast, *Plasmodium*

contains low complexity regions in several proteins that have never been observed to contain such segments in other eukaryotes. Furthermore, in *Plasmodium* the low complexity regions invade even ancient compact globular domains, and may vary in length, from small inserts of less than 10 amino acids to inserts of more than 100 amino acids (Aravind *et al.*, 2003).

Among proteins possessing such insertions, *P. falciparum* glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (G6PD-6PGL), a bifunctional enzyme exclusive to *Plasmodium* species, can be mentioned (Clarke *et al.*, 2001). This protein has a subunit molecular mass of 107 kDa (Kurdi-Haidar and Luzzatto, 1990). Its C-terminal half (residues 311–911) is clearly homologous to other described G6PDs (with glucose 6-phosphate dehydrogenase activity), though sequence similarity is interrupted by a 62 amino acid stretch with no similarity found to date (Clarke *et al.*, 2003). It has been nonetheless experimentally shown that this 62 amino acid insertion is necessary for the activity of the bifunctional enzyme (Clarke *et al.*, 2003). In contrast, the 310 amino acid protein sequence of the N-terminal region clearly differs from most eukaryotic and prokaryotic G6PDs, and shows 6-phosphogluconolactonase activity. The first two steps of the pentose phosphate pathway (PPP) is catalyzed by this bifunctional enzyme (Clarke *et al.*, 2001). In both the host and parasite, the PPP is essential for neutralizing reactive oxygen species during red blood cell infection with the malaria parasite. Therefore, PPP activity is greatly increased in infected erythrocytes compared to noninfected ones, and the parasite PPP is responsible for 82% of this activity (Atamna *et al.*, 1994 ; Clarke *et al.*, 2001).

In cultures of *P. falciparum*, the effects of PfG6PD-6PGL gene silencing on the mRNA expression of parasite antioxidant defense genes have been shown (Crooke *et al.*, 2006). This gene silencing caused arrest of the trophozoite stage and increased gametocyte formation. Moreover, an immediate transcriptional response was shown by thioredoxin reductase suggesting the physiological importance of this bifunctional enzyme in the specific response of the parasite to intracellullar oxidative stress (Crooke *et al.*, 2006).

Therefore, *P. falciparum* G6PD-6PGL could be considered as a candidate therapeutic target not only because of its structural characteristics which make it different from its human equivalent, but also because of the importance of this enzyme in the intraerythrocytic stage (Clarke *et al.*, 2003).

2.3.2.2. Oxidative stress

2.3.2.2.1. Reactive oxygen species

Reactive oxygen species (ROS): (a) are present as pollutants in the atmosphere; (b) are generated as by-products of normal metabolic processes; and (c) are formed during exposure to X-, λ-, or U.V.-irradiation (Stadtman and Levine, 2003). In the cell, ROS are constantly generated at low concentrations under physiological conditions, playing a part in the cellular redox regulation (Dalle-Donne *et al.*, 2006). ROS can also occur as the outcome of acute cell stresses or as a secondary effect of pharmacological treatment (Dalle-Donne *et al.*, 2006). Cellular oxidative damage develops when the balance between ROS-generating systems and ROS-scavenging ones tilts in favour of the former (Dalle-Donne *et al.*, 2006).

The primary cellular target of oxidative stress can vary depending on the cell type, the absolute level and duration of oxidant production, the species of ROS generated, its site of generation (intra- *vs.* extra-cellular), and the proximity of the oxidant to a specific cellular substrate. Therefore, the extent of damage to particular targets depends on different factors. Proteins are major targets for ROS as they are the major component of most biological systems (Davies *et al.*, 1999). Higher concentrations of the target protein considers as one of the crucial factors which cause more oxidation damage (Dalle-Donne *et al.*, 2006). There is increasing evidence that accumulation of oxidized dysfunctional molecules in the cell over a lifetime contributes to the reduction of its half-life (Berlett and Stadtman, 1997; Beckman and Ames, 1998).

Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events (Cabiscol and Ros, 2006). Irreversible modifications such as protein carbonylation, and protein-protein cross-linking are generally responsible for permanent loss of function of the damaged proteins which are subsequently degraded or may progressively accumulate in intra-cytoplasmic inclusions as observed in some neurodegenerative diseases (Ghezzi and Bonetto, 2003). In fact, such permanent modifications are implicated in the etiology or progression of a number of disorders and diseases (Stadtman and Levine, 2000).

2.3.2.2.2. Oxidation of the protein backbone by free radicals

Free amino acids and amino acid residues in proteins are highly susceptible to oxidation by one or more reactive species (ROS) which include radical species such as superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), and nonradical species such as hydrogen peroxide (H_2O_2). (Berlett and Stadtman, 1997; Dean *et al.*, 1997). The mechanisms of these ROS-mediated oxidations reactions were

elucidated by different groups (Garrison *et al.*, 1962; Schuessler and Schilling, 1984; Garrison, 1987) who exposed solutions of amino acids, peptides, and proteins to ionizing radiation under conditions where $^{\bullet}OH$ or a mixture of $^{\bullet}OH$ and $O2^{\bullet-}$ are formed. Results of these studies demonstrated that the $^{\bullet}OH$-dependent abstraction of a hydrogen atom from the α-carbon of amino acids and the protein polypeptide backbone and also from the aliphatic side chains of hydrophobic amino acid residues of proteins are initial sites of attack (Stadtman and Levine, 2003). As illustrated in Fig. 5, abstraction of the hydrogen atom leads to formation of a carbon-centred radical (reaction c), which in the presence of oxygen is rapidly converted to the peroxyl radical (reaction d). This peroxyl radical is readily converted to the alkyl peroxide by reaction with the protonated form of the superoxide radical (reaction e) or by abstraction of a hydrogen atom from another molecule (reaction f). Further reactions with $HO2^{\bullet}$ can lead to formation of the alkoxyl radical (reaction h) and its conversion to the hydroxy derivative (reaction j). Although the reaction sequence illustrated in Fig. 5 was established by using ionizing radiation for the generation of $^{\bullet}OH$ and $HO2^{\bullet}$, it is likely that cleavage of H2O2 by iron or copper (reaction b, Fig. 5) is a major source of $^{\bullet}OH$ under physiological conditions. Furthermore, as shown in Fig. 5, Fe(II) is also able to replace $HO2^{\bullet}$ in the reactions leading to formation of the alkyl peroxide, alkoxyl radical, and the hydroxy derivative (reactions g, i, and k). Significantly, all reactions depicted in Fig. 5, following the hydrogen abstraction by $^{\bullet}OH$, are dependent upon the addition of O2 to the carbon-centered radical (reaction d). In the absence of O2, two carboncentered radicals can react with one another to produce carbon–carbon cross-linked derivatives (reaction l, Fig. 5) (Stadtman and Levine, 2003).

In addition to the reactions illustrated in Fig. 5, the oxidation of proteins by ROS can lead also to the cleavage of peptide bonds (Stadtman and Levine, 2003). The alkoxyl radicals and alkylperoxide derivatives of proteins can undergo cleavage by either α-amidation or diamide pathways (Garrison, 1987). In the α-amidation pathway, the C-terminal amino acid of the fragment derived from the N-terminal region of the protein will exist as the amide derivative and the N-terminal amino acid of the fragment derived from the C-terminal portion of the protein will exist as an α-keto-acyl derivative. In contrast, the C-terminal amino acid of the fragment derived from the N-terminal portion of the protein via the diamide pathway will exist as the diamide derivative and the N-terminal amino acid residue of the peptide fragment derived from the C-terminal region of the protein will exist as the isocyanate derivative (Stadtman and Levine, 2003).

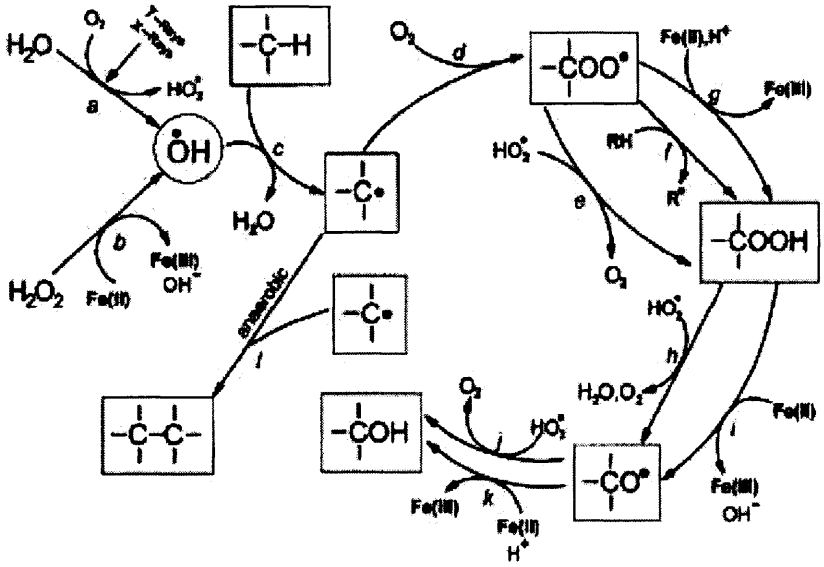


Fig. 5. Free radical-mediated oxidation of the protein polypeptide backbone (Stadtman and Levine, 2003).

2.3.2.2.3. Site-specific metal-catalyzed oxidation

In addition to the free radical-mediated oxidation of the protein polypeptide backbone (Fig. 5), the side-chains of amino acid residues of some proteins are readily oxidized by metal ion-catalyzed oxidation systems (Fucci *et al.*, 1983; Levine, 1983; Rivett *et al.*, 1985; Amici *et al.*, 1989; Stadtman *et al.*, 1990). Direct oxidation of the side-chains of lysine, arginine, proline, and threonine residues has been shown to yield carbonyl derivatives (Stadtman and Levine, 2003). In studies with *E. coli* glutamine synthetase, it was found that amino acid residues situated at metal binding sites on the enzyme are uniquely sensitive to metal-catalyzed oxidation by a site-specific mechanism (Farber and Levine, 1986; Stadtman and Levine, 2003). For example, lysine residues can be the target for Fe(II)-catalyzed oxidations where the chelate complex formed by the binding of Fe(II) to the ε-amino group reacts with hydrogen peroxide to generate a hydroxyl radical that will preferentially attack the lysine moiety leading to its conversion to a 2-amino-adipic-semialdehyde residue (Stadtman and Levine, 2003). Similar reactions of the Fe(II) with other amino acid targets lead to the generation of carbonyl derivatives (arginine, proline, and threonine residues) (Stadtman and Levine, 2003).

2.3.2.2.4. Protein carbonylation and carbonyl group detection

ROS-mediated protein carbonylation is an important marker of protein oxidation and its measurement is thought to be a good and the most widely used indicator for the

extent of oxidative damage of proteins associated with various conditions of oxidative stress, aging and physiological disorders (Butterfield and Kanski, 2001). Carbonyl groups may be introduced in the protein at different sites and by different mechanisms (Ghezzi and Bonetto, 2003). As it is explained, direct reaction of proteins with ROS can lead to formation of protein derivatives (Fig. 5) or peptide fragments possessing highly reactive carbonyl groups (ketones, aldehydes) (Stadtman and Levine, 2003). As illustrated in Fig. 6, proteins containing reactive carbonyl groups can also be generated by secondary reactions of primary amino groups of lysine residues of proteins with reducing sugars or their oxidation products (glycation/glycoxidation reactions) and also by Michael- addition reactions of lysine, cysteine, or histidine residues with α, β-unsaturated aldehydes formed during the peroxidation of poly-unsaturated fatty acids (Stadtman and Levine, 2003). A general scheme of origins of carbonylated proteins can be seen in Fig. 7.

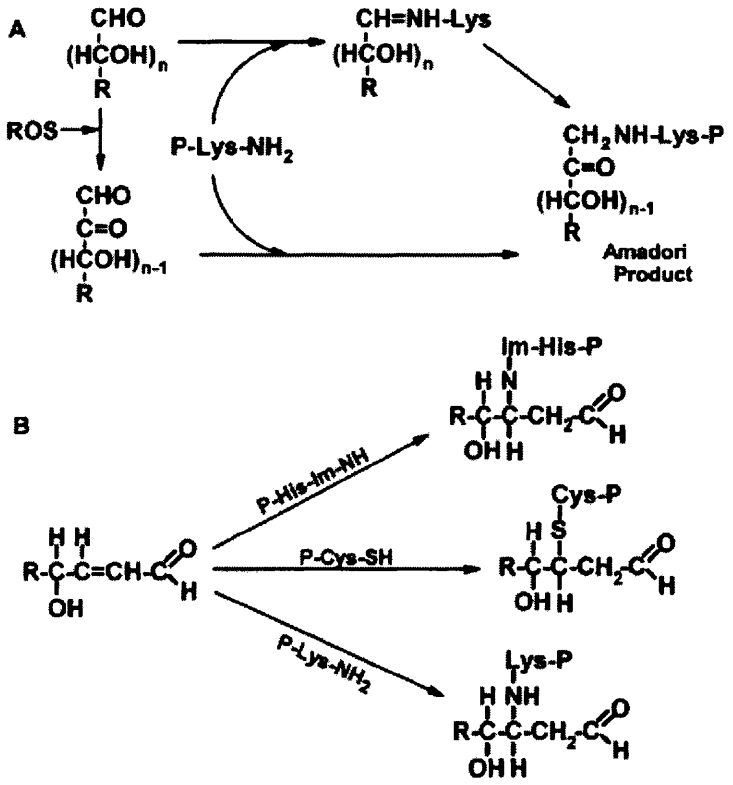


Fig. 6. Generation of carbonyl derivatives of proteins. A By glycation/glycoxidation of lysine amino groups. **B** By reactions of α-β-unsaturated aldehydes with lysine, cysteine, or histidine residues of proteins (Stadtman and Levine, 2003).

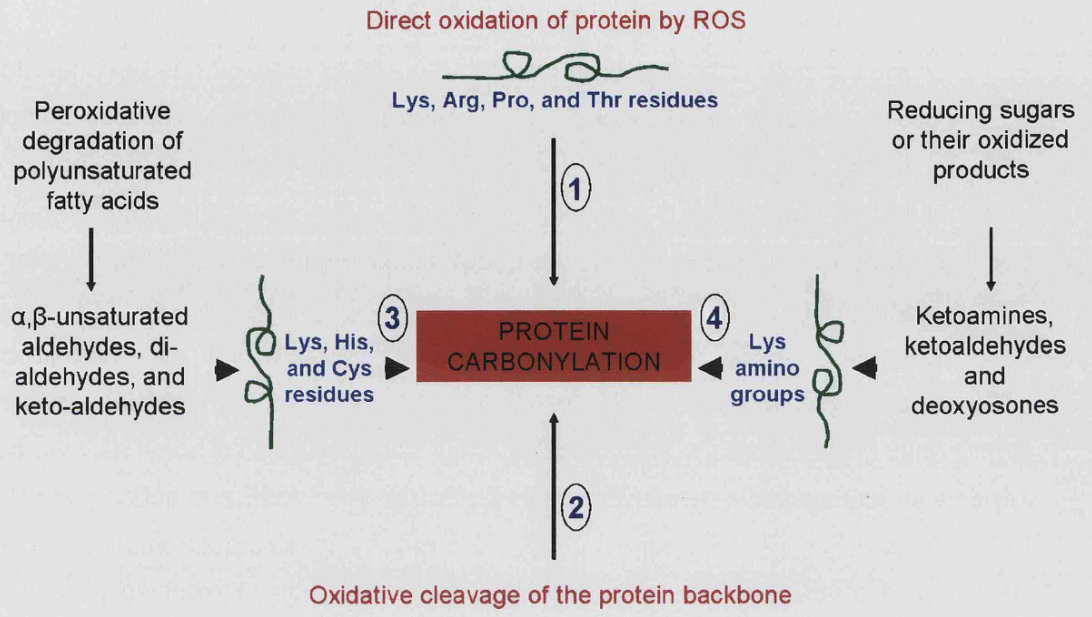


Fig. 7. Origins of carbonylated proteins. Protein carbonyl derivatives can be produced by different oxidative pathways. ROS can react directly with the Lys, Arg, Pro, and Thr side chains of proteins mainly through metal-catalysed oxidation (pathway 1). Direct oxidation of proteins by ROS can also yield highly reactive carbonyl derivatives resulting from the cleavage of peptide (pathway 2). Carbonyl groups may be introduced into proteins by adduction of reactive aldehydes derived from the metal-catalysed oxidation of polyunsaturated fatty acids (pathway 3). These lipoxidation products include α,β-unsaturated aldehydes which can undergo Michael-addition reactions and react with the sulfhydryl group of Cys, the ε-amino group of Lys or the imidazole group of His residues, di-aldehydes and γ-ketoaldehydes which react with Lys residues. Finally, carbonyl groups can also be generated by secondary reaction of the primary amino group of Lys residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidized products with lysine residues of proteins (pathway 4) (Dalle-Donne *et al.*, 2006).

Carbonyl (CO) groups (aldehydes and ketones), produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized, are chemically stable that is useful for both their detection and storage (Dalle-Donne *et al.*, 2003). Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation (Berlett and Stadtman, 1997; Chevion *et al.*, 2000; Shacter, 2000; Beal, 2002; Poon *et al.*, 2004; Reverter-Branchat *et al.*, 2004; Poon *et al.*, 2005; Chen *et al.*, 2006; Sultana *et al.*, 2006; Calabrese *et al.*, 2007; Vaishnav *et al.*, 2007; Yeh *et al.*, 2008). Highly sensitive assays for detection of protein carbonyls involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product. Then, the stable DNP adduct can be detected by various means (Dalle-Donne *et al.*, 2003). The DNP group itself absorbs ultraviolet light so that the total carbonyl content of a protein or mixture of proteins can be quantified by a spectrophotometric assay (Levine *et al.*, 1990), which can be coupled to protein fractionation by high-performance liquid chromatography (HPLC) to give greater

sensitivity and specificity than measuring total carbonyls in a protein mixture (Levine *et al.*, 1994).

In the last years, the identification of carbonylated proteins has been facilitated by the availability of commercial specific antibodies to anti-DNP that allow their detection by immunoblotting analysis (Dalle-Donne *et al.*, 2003). Immunoblotting assays based on the use of anti-DNP antibodies have been developed in an attempt to identify oxidatively damaged proteins in human tissues and body fluids (Keller *et al.*, 1993; Shacter *et al.*, 1994; Robinson *et al.*, 1999).

Moreover, carbonyl content in individual proteins can also be assessed by two-dimensional electrophoresis (2-DE) followed by Western blot immunoassay, which have significantly more sensitivity and specificity than all other total carbonyl assays, but they are only semiquantitative. (Dalle-Donne *et al.*, 2003). 2-D PAGE and subsequent Western blot immunoassay (2-D immunoblotting), followed by mass spectrometry, are actually widely used methods of redox proteomics for identification of oxidized proteins under oxidative stress (Choi *et al.*, 2002; Butterfield, 2004; Reverter-Branchat *et al.*, 2004; Poon *et al.*, 2004; Poon *et al.*, 2005; Tezel *et al.*, 2005; Sultana *et al.*, 2006; Vaishnav *et al.*, 2007).

2.3.2.2.5. Oxidative stress in Plasmodium

All aerobic organisms are exposed to ROS generated by their own metabolic activity. Parasitic protozoa not only have to eliminate their endogenous toxic metabolites but they must also cope with the oxidative burst of the host immune system (Muller *et al.*, 2003).

Although oxidative stress has been suggested to play a key role in the pathogenesis of malaria (Becker *et al.*, 2004), it has been shown that malaria parasites are particularly vulnerable to oxidative stress during their erythrocytic life stages (Hunt and Stocker, 1990; Simoes *et al.*, 1992; Muller *et al.*, 2003; Becker *et al.*, 2004). This is not surprising as the parasites live in a pro-oxidant environment that contains oxygen and iron, the key prerequisite for the formation of ROS via the Fenton reaction (Fig. 8) (Muller, 2004). Interestingly, its susceptibility to oxidative stress (Muller, 2004) has advocated this metabolism as a promising target for antimalarial chemotherapy (Becker *et al.*, 2004). Apart from the large amounts of reactive oxygen species (ROS) generated by the active metabolism of the growing and multiplying malaria parasite, a main source of oxidative stress is the degradation of host haemoglobin (Becker *et al.*, 2004). Haemoglobin represents the major source of amino acids for for parasite development, but its degradation in an acidic food vacuole results in the production of toxic free haem

(ferri/ferroprotoporphyrin IX; FP IX) and ROS (Muller, 2004). In the erythrocyte, haem iron is almost entirely in the ferrous (+2) state. Upon degradation in the parasite digestive vacuole, the iron is oxidized to the ferric (+3) state. Electrons liberated by oxidation combine with molecular oxygen to produce superoxide anions (O_2^-). This combination inevitably leads to the generation of hydrogen peroxide and subsequently hydroxyl radicals, both highly reactive and toxic oxygen intermediates (Fig. 8) (Francis *et al.*, 1997; Liochev and Fridovich, 1999). Furthermore, free FP IX is toxic because it has detergent-like properties interfering with membrane integrity and has the ability to undergo redox reactions causing the generation of ROS because of the bound iron (Muller, 2004). In *P. falciparum*, most of the released FP IX is biomineralized (Egan *et al.*, 2002; Hempelmann, 2007) to form inert haemozoin (Fig. 8). Alternative detoxification pathways, including FP IX degradation (Zhang *et al.*, 1999), reaction with glutathione (Ginsburg *et al.*, 1998), and the binding to FP IX-binding proteins (Harwaldt *et al.*, 2002; Campanale *et al.*, 2003), may also contribute to FP IX detoxification since escaping small amount of the FP IX from the detoxification processes (Loria *et al.*, 1999) could cause redox damage to host and parasite proteins and membranes (Campanale *et al.*, 2003; Famin and Ginsburg, 2003).

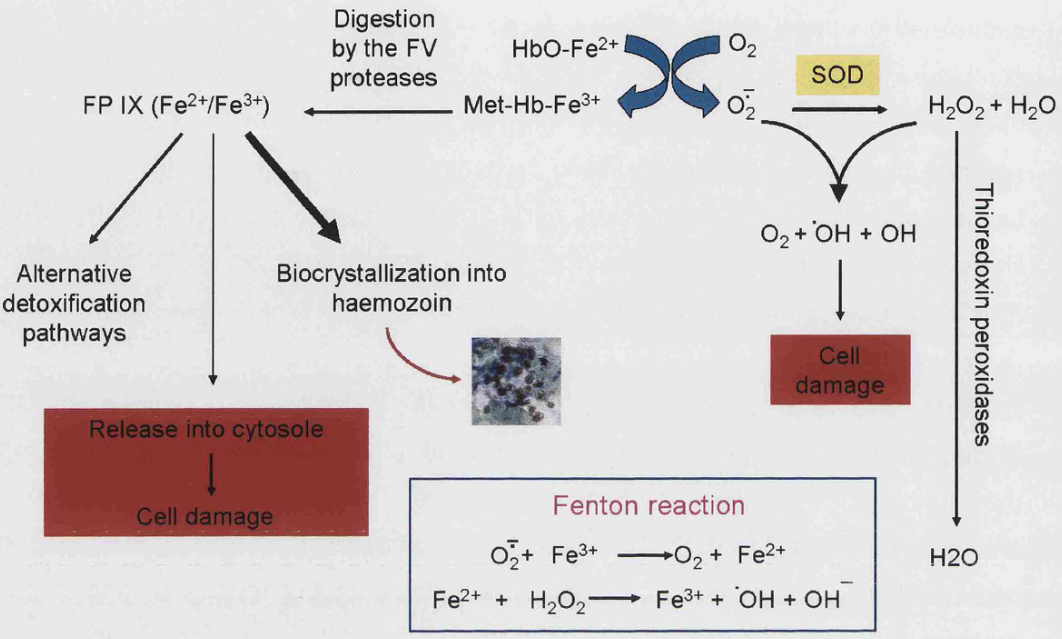


Fig. 8. Sources of ROS in *P. falciparum*. The major source for ROS in *P. falciparum* during its intraerythrocytic life is the digestion of host cell haemoglobin in the parasite's FV. FP IX is released from the digested haemoglobin and the majority of that is biocrystallized to haemozoin (Egan *et al.*, 2002). Alternative detoxification pathways (Ginsburg *et al.*, 1998; Zhang *et al.*, 1999; Harwaldt *et al.*, 2002; Campanale *et al.*, 2003) may also contribute to FP IX detoxification. However, some free FP IX (Loria *et al.*, 1999) releases from the FV into the parasite cytosol where it causes membrane damage and can undergo redox reactions which lead to the generation of superoxide anions in this parasite compartment. Superoxide anions, resulting from the oxidation of haem-iron in haemoglobin, are either detoxified by SOD, to yield H_2O_2, or can react in a spontaneous reaction with H_2O_2 leading to the formation of hydroxyl radicals. In addition, hydroxyl radicals are generated in the presence of free iron via the Fenton reaction. These radicals are highly reactive and cause cell damage. H_2O_2 generated by the SOD reaction has to be detoxified by reduction to H_2O. In *P. falciparum*, this is exclusively achieved by thioredoxin peroxidases because the parasites lack catalase and glutathione peroxidases. FP IX, ferri/ferroprotoporphyrin IX; FV, food vacuole; HbO-Fe^{2+}, oxy-haemoglobin containing ferriprotoporphyrin IX; MetHb-Fe^{3+}, methaemoglobin containing ferroprotoporhpyrin IX; SOD, superoxide dismutase. (Muller, 2004).

2.3.2.2.6. Antimalarial drugs interfering with redox metabolism

In spite of being used more than 50 years, the mode of CQ action and the mechanisms of resistance are only partly understood (Koncarevic *et al.*, 2007). Among different theories, the most widely spread hypothesis is the CQ-FP IX interaction hypothesis, which states that CQ and other 4-aminoquinolines exert their antimalarial action by preventing the detoxification of free haem which is generated during the degradation of host haemoglobin in the parasites' digestive vacuole (Koncarevic *et al.*, 2007), where CQ accumulates (Egan, 2001; Spiller *et al.*, 2002). In this location it is thought to interact with the μ-oxo dimer form of oxidized haem (one of the various possible chemical forms of dimeric FP IX, [Fe(III)FP IX]2O) (Chou *et al.*, 1980; Moreau *et al.*, 1982; Egan *et al.*, 1997; Dorn *et al.*, 1998; Leed *et al.*, 2002). Studies using NMR have

been taken to suggest that the interaction of CQ with FP IX involves π– π stacking (Moreau *et al.*, 1982). The interaction of CQ with the FP IX μ-oxo dimmer prevents the formation of the haemozoin dimer which is presumably an intermediate in the formation of the haemozoin crystal and CQ binding thereby prevents FP IX detoxification (Becker *et al.*, 2004). It has been suggested that the consequent build-up of free FP IX and CQ-FP IX complexes is lethal for the parasite, as free FP IX has been proposed to damage membranes and enzymes due to its detergent-like properties as well as its ability to participate in damaging redox reactions (de Almeida Ribeiro, 1995; Loria *et al.*, 1999; Pandey *et al.*, 2001; Tilley, 2001; Sullivan, 2002). Therefore, oxidative stress in the mechanism of action of CQ has always been thought as one of the indirect or direct targets of CQ action (Becker *et al.*, 2004).

The involvement of oxidative events in the downstream effects of CQ is also supported by studies of the modulation of CQ action by GSH levels (Becker *et al.*, 2004). Moreover, nonbiomineralized haem seems to be degraded by glutathione (Ginsburg *et al.*, 1998) and given that CQ competitively inhibits the degradation of haem by glutathione, the increasing cellular levels of glutathione leads to increased resistance to CQ, whereas decreasing them results in enhanced sensitivity to the drug (Ginsburg *et al.*, 1998).

Thus, the enhancement of intracellular ROS is considered the final consequence of the CQ action, but it remains unknown how this toxic environment modifies the essential functions of the parasite.

Objectives

3. OBJECTIVES

The complex biology and life cycle of *P. falciparum* has hindered attempts to control infections. In addition, there is increasing resistance of the malaria parasites to currently available drugs. In general, the efforts to control malaria have been focused on the chemotherapy, mosquito control and in the development of vaccines. Nowadays, the lack of an effective vaccine and the increasing parasite resistance to available drugs suggest the necessity to identify new targets that allow the development of new drugs and vaccines. Completion of *Plasmodium* genome sequences has provided a vast amount of molecular information. This, together with the transciptome and proteome analysis along the parasitic developmental stages pursue a deep understanding of the peculiar parasite biology in the context of exploring new therapeutic and immunization strategies.

The first part of this project explored a new protease, PfNna1 gene, encoded in *P. falciparum* genome with interest as a potential target for inhibitors. Transcription analysis of this new metalocarboxypeptidase and the effect of potato carboxypeptidase inhibitor (PCI) on the erythrocytic cycle of the parasite have been studied.

The particular bifunctional *P. falciparum* G6PD- 6PGL protein, which is exclusive to *Plasmodium* species and catalyses the first two stages of the pentose phosphate pathway, was explored in the second part of this thesis. The interest of this gene relies on its potential protein processing along the different intraerythrocytic stages. Such enzyme could be a potential therapeutic target not only because of its unique structural characteristics that make it different from its host, but also because of the importance of this enzyme in the parasite's intraerythrocyte cycle.

To achieve high yields of isolated proteins, which are necessary to work on protein level and to do a comparative study of protein profiling by proteomic analysis, highly parasitized cultures are needed. In the third part of the work, to fulfil requirements for proteome studies of intraerythrocytic stages of *P. falciparum*, we have therefore worked in large-scale and produced a high percentage parasitaemia.

In the fourth part of the present work, a proteomic approach has been applied to identify and compare oxidatively modified proteins in the different intraerythrocytic stages of non-treated and chloroquine-treated *P. falciparum* from a chloroquine-resistant strain. Such studies are informative with regard to the importance of such proteins in the life cycle of the malaria parasite and as potential new therapeutical targets to fight chloroquine and other quinolines resistant *P. falciparum* strains.

Overall, in this context the general objective of the work relies on studying the biology of *P. falciparum* in transcriptional, translational and post-translational levels to find potential targets for the development of effective therapeutic drugs. Particular emphasis was given to clarify the mechanism of action of chloroquine, as efficient antimalarial in the past, in order to know in detail the post-translational modifications shown by the resistant phenotype parasite. For this purpose, the specific experimental objectives were the following:

1- Studying mRNA expression of PfNna1 target gene in relation with the carboxypeptidase inhibitor (PCI) effect on the parasite life cycle.

2- Analyzing PfNna1 gene polymorphysm in the strains Dd2 and 3D7 of *P. falciparum*.

3- Analyzing the *P. falciparum* bifunctional enzyme of G6PD-6PGL as a model to study protein processing.

4- Characterizing the *P. falciparum* redox proteome across the intraerythrocytic cycle, and the modifications caused in response to chloroquine.

Research work

4. RESEARCH WORK

4.1. Expression analysis of a putative carboxypeptidase gene, PfNna1, in *Plasmodium falciparum*

Expression analysis of a putative carboxypeptidase gene, PfNna1, in *Plasmodium falciparum*

Contents

1. Abstract

The complex biology and life cycle of *Plasmodium falciparum* has hindered attempts to control infections and prevent transmission. In addition, there is increasing resistance of the malaria parasites to currently available drugs. Therefore, there is an urgent need for the development of new chemotherapeutic agents that can control the parasite. Recent strategies for antiparasitic drug discovery include seeking for specific inhibitors of critical biological activities within the complex parasite cycle. In this context, protease genes have already demonstrated that play key roles in the biology of malaria parasites and offer potential new chemotherapeutic targets.

The present study, focus on transcription analysis of the PfNna1 gene in relation with the potato carboxypeptidase inhibitor (PCI) effect on the *P. falciparum* erythrocytic cycle. Identification and characterization of the PfNna1 gene has been previously reported. Experimental confirmation that PCI inhibited parasite growth has been *in vitro* and *in vivo* assessed in a different project with participation of our research group. The reported specificity of the inhibition suggested the presence of a putative functional protease, which is essential for the parasite life cycle, in the *P. falciparum* genome. In fact, analysis of mRNA expression allowed establishing a link between the inhibitor and the PfNna1 gene in *P. falciparum*. The PfNna1 mRNA expression pattern along the intraerythrocytic phase demonstrated that is maximally expressed in the mature developmental stages, phase at which PCI arrested parasite growth. Moreover, the transitional exposure of the parasite to PCI up-regulated the expression of PfNna1 gene. These observations suggest the involvement of this putative peptidase gene in essential activities for the parasite survival.

Keywords:

Carboxypeptidase inhibitor / PfNna1 / *Plasmodium falciparum* / Proteases / Transcription

2. Introduction

Malaria caused by *Plasmodium* parasites kills approximately 1-3 million people and causes disease in 300-500 million people annually throughout the world (Tripathi *et al.*, 2005). The current limitations of malaria vaccine development, and the emergence and spread of insecticide-resistant vectors and drug-resistant parasites are major causes behind the re-emergence and severity of a worldwide malaria problem (Sharma, 2007).

In the last 50 years, extensive efforts, including the screening of hundreds of thousands of compounds, have led to the development of a number of effective synthetic antimalarial drugs. The most important of these, CQ, has been the mainstay of antimalarial chemotherapy for the last 50 years. However, resistance to CQ has been steadily increasing since the drug's initial use in South America and Southeast Asia in the late 1950s (Rosenthal, 1998). CQ resistance is now widespread in most *P. falciparum*-endemic areas of the world (Olliaro *et al.*, 1996). Thus, the use of CQ for treatment of falciparum malaria is usually no longer appropriate as about 80% of the parasite population is resistant to this drug (Ehrhardt *et al.*, 2007). Moreover, resistance to CQ of *P. vivax*, the second most lethal human malaria parasite, is increasing in South Asia (Murphy *et al.*, 1993).

In fact, the antimalarial drug resistance progressively acquired by many parasite strains all over the world do not allow the disease control and favour its transmission to other previously controlled areas (Bloland, 2001). Consequently, there is a need for new drugs and the identification of new chemotherapeutic targets. To this respect, publication of the complete genome sequence from *P. falciparum* (Gardner *et al.*, 1998; Bowman *et al.*, 1999; Gardner *et al.*, 2002) opens new possibilities for the development of new therapeutic drugs and vaccines.

All parasitic protozoa contain multiple proteases that some of which are attracting attention as drug targets (Coombs *et al.*, 2001). Aspartic proteases are already the targets of some clinically useful drugs, like chemotherapy of HIV infection (Wlodawer and Vondrasek, 1998; Tomasselli and Heinrikson, 2000), and a variety of factors make these enzymes potential targets for novel antimalarial therapies (McKerrow *et al.*, 1993).

Extensive evidence demonstrates that proteases play substantial roles during the malaria parasite life cycle (Rosenthal, 2001; Rosenthal, 2002). They appeared to be involved in multiple processes essential for metabolism and development of the parasite, including haemoglobin hydrolysis, the processing of parasite proteins, merozoite release, and invasion of red blood cells (Schrevel *et al.*, 1990).

The erythrocytic life cycle, which is responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes (Rosenthal, 1998). The intraerythrocytic parasites develop from small ring-stage organisms to larger, more

metabolically active trophozoites and then to multinucleated schizonts. The cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites. Erythrocyte entry and host cell rupture require the activity of parasite proteases, and these enzymes are therefore attractive targets for rational approaches to new drug development (Blackman, 2000). Malarial proteases play a role in at least two distinct aspects of host cell invasion; modification of parasite proteins involved in host cell recognition and entry; and restructuring of the host cell itself during and following invasion, and in order to allow parasite release from the host cell (Blackman, 2000). Moreover, proteases appear to be required for the degradation of haemoglobin (Fig. 1) that is necessary for the growth of erythrocytic malaria parasites (Rosenthal, 1998). These results strongly suggest that plasmodial proteases could be appropriate targets for the development of chemotherapeutic drugs (Rosenthal, 1998).

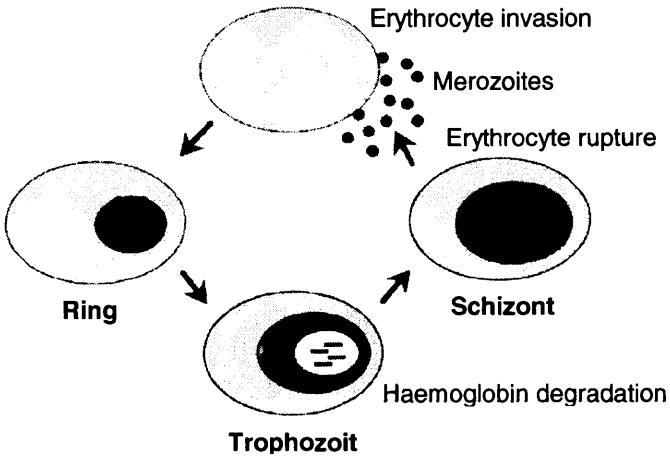


Fig. 1. Protease targets in erythrocytic malaria parasites. The *P. falciparum* erythrocytic life cycle is shown schematically.

The first indications that proteolytic activity is important during the malaria life cycle came from experiments investigating the effects of broad-spectrum protease inhibitors on invasion process and haemoglobin degradation (Blackman, 2000). Considering parasite proteases, that are required for its survival, as a potential new target for chemotherapy, protease inhibitors are in the spotlights as antimalarial drugs, however, protease inhibitors must be highly potent and specific for parasite proteases to be recognized as biological tools (Sharma, 2007). Although studies evaluating the antimalarial effects of protease inhibitors are limited to date, experience with other systems, such as HIV aspartic protease (Deeks *et al.*, 1997), suggests that inhibitors of essential proteases can be developed as antimalarial drugs (Rosenthal, 2002). Some of the main *P. falciparum* protease inhibitors identified up to date include cysteine protease inhibitor leupeptin

(Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Vander Jagt *et al.*, 1989) and E-64 (Rosenthal *et al.*, 1988; Vander Jagt *et al.*, 1989; Bailly *et al.*, 1992; Asawamahasakda *et al.*, 1994; Rosenthal, 1995); aspartic protease inhibitor pepstatin (Bailly *et al.*, 1992; Rosenthal, 1995; Banerjee *et al.*, 2002); serine protease inhibitor GlcA-valine-leucine-glycine-lysine-NHC_2H_5 (Mayer *et al.*, 1991); aminopeptidase inhibitor bestatin (Vander Jagt *et al.*, 1989; Nankya-Kitaka *et al.*, 1998) and proteasome inhibitor lactacystin (Gantt *et al.*, 1998). Protease inhibitor combinations also seems appropriate to inhibit parasite growth as it has been shown with cysteine and aspartic proteases which appear to act cooperatively in haemoglobin degradation (Francis *et al.*, 1994). Pepstatin used in combination with E64 or N-Me pipu-Leu-Hph-VSPh, gave promising results and are examples of such synergistic effects (Semenov *et al.*, 1998).

The potato carboxypeptidase inhibitor (PCI) (Aviles *et al.*, 2006), was used by our group to inhibit the growth of *P. falciparum*. PCI is a 39-amino acid protein naturally occurring in potatoes that can inhibit several carboxypeptidases (Vendrell *et al.*, 2000). Its inhibitory effect on tumor cell growth was reported before (Blanco-Aparicio *et al.*, 1998). Inhibition of *P. falciparum* cultures by PCI proves that a kind of carboxypeptidase activity should function in the malaria life cycle. Searching for the target gene of PCI in the molecular databases showed the existence of a new metallocarboxypeptidase, *P. falciparum* Nna1-like (PfNna1) gene of M14 family (Rodriguez de la Vega *et al.*, 2007). This gene has been reported to be highly up-regulated in spinal cord of mice subjected to sciatic nerve injury (Harris *et al.*, 2000) and has therefore been named Nna1 (Nervous system Nuclear protein induced by Axonomy). A detailed genomic scanning found about 100 Nna1 homologues in bacteria, Protista, and Animalia (Rodriguez de la Vega *et al.*, 2007). This study shows that Nna1-like proteins ranged from 400 to 2000 residues in length and comprise a common metallocarboxypeptidase domain of ~300 residues and a N-terminal conserved domain that is ~150 residues in length and contains two other conserved motifs such as FESG and PYTY. These peptidases conserve the Zn^{++} ligands and the catalytic residues of metallocarboxypeptidases, but where the motifs take place differ from each other and make different subfamilies (Rodriguez de la Vega *et al.*, 2007). As methalocarboxypeptidases, these enzymes catalyze the hydrolysis of C-terminal amino acids from peptides. The lack of the N-terminal signal peptide in the Nna1-like proteins of eukaryotes (Rodriguez de la Vega *et al.*, 2007) means that they function in the cleavage of cytosolic proteins. It is believed that these proteins are involved in the proteolytic processing of α-tubulin, the only cytosolic protein known to undergo C-terminal processing. α-tubulin is initially produced with a C-terminal tyrosine, which is removed (Hallak *et al.*, 1977) by possible function of Nna1-like proteins as tubulinyl-Tyr carboxypeptidase (Rodriguez de la Vega *et al.*, 2007; Kalinina *et al.*, 2007).

PfNna1 is a large gene (more than 1000 residues) with long N- and C-terminal extensions and extensive non-conserved insertions of up to 300 residues long (Rodriguez de la Vega *et al.*, 2007). *P. falciparum* growth inhibiton by PCI, alongside with the possible function of PfNna1 as tubulinyl-Tyr carboxypeptidase, considering tubulin as a fundamental protein for cytoskeleton, suggest PfNna1 as an important therapeutic target. Therefore, the gene expression analysis of this candidate protein for antimalarial chemotherapy has been done in this work.

3. Material and methods

Parasite and cultures

P. falciparum strains Dd2 (CQ resistant) and 3D7 (CQ sensitive) were cultured *in vitro* in group A^+ human erythrocytes using previously described conditions (Trager and Jensen, 1976), with some modification. Briefly, parasites were cultured in RPMI 1640 medium (Sigma), supplemented with 25 mM HEPES, 0.5% AlbuMAX I (Invitrogen), 1.77 mM sodium bicarbonate, 100 µM hypoxanthine, and 12.5 µg/ml gentamicin sulphate, at a pH of 7.2. Where needed, the cultures were synchronized by sequential treatments with 5% (w/v) sorbitol (Lambros and Vanderberg, 1979). Experiments were performed with a 5% haematocrit and parasitaemia between 1–2%.

Nucleic acid extraction

Genomic DNA was isolated from parasite extracts by treatment with lysis buffer (40 mM Tris-HCl, pH 8.0; containing 80 mM EDTA, 2% SDS and 2 mg/ml Proteinase K) and incubated for several hours at 37°C. The DNA was extracted by phenol/chloroform and recovered by ethanol precipitation (Singh *et al.*, 1996).

Total RNA was isolated from 12 µl of infected red blood cell cultures with 2-3% of parasitaemia using "RNA Blood-DNA Method" from Applied Biosystems on the ABI PRISM® 6100 Nucleic Acid PrepStation.

Reverse transcription/PCR

Isolated RNA was retrotranscribed to cDNA using the High-Capacity™ cDNA Archive Kit (Applied Biosystems) as described by the manufacturer. Both random primers and PfNna1 specific primers (reverse primers of Table 1) were used for generating different cDNA first strands in two separate reverse transcription (RT) reactions. All RT reactions were done at 37°C for 2 h.

Two different Reverse Transcription PCR (RT-PCR) were used, standard and quantitative. The standard RT-PCR reaction was performed to amplify the PfNna1 cDNA

to determine the length of the sequence involved in gene in transcription. Also, these fragments were used to sequence the whole transcription unit from two strains Dd2 and 3D7. For this cDNA amplification, the AmpliTaq Gold kit (Applied Biosystems) was used throughoutly. RT-PCR was performed using the following cycle profile: 94℃, 1 min; 58.1℃, 30 sec; 62℃, 3 min for 40 cycles with an i nitial 7-min denaturation at 95℃ and a final 7-min elongation at 62℃. The sequences of th e specific oligonucleotides used are given in Table 1. On the other hand, quantitative real time RT-PCR (qRT-PCR) was done to assess the relative abundance of PfNna1 mRNA along the parasite cycle. The cDNA was amplified in the ABI PRISM® 7000 Sequence Detector (Applied Biosystems). The TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) was used for ABI PRISM® 7000 amplification and detection. Molecular beacon probes and primers sequences are given in Table 2. According to our preliminary assays all primers and probe were used in a concentration of 300 nM. Real-time PCR involved 1 cycle each of 50℃ for 2 min and 95℃ for 10 min, followed by 45 cycles of 57℃ for 1 min, 95℃ for 30 sec, and 45℃ for 29 sec for fluorescence detect ion.

Primers and molecular beacons design

Primers for qRT-PCR, and the standard RT-PCR (Table 1) were designed (Primer Express 2.0 software, Applied Biosystems) in a way that they could cover the whole PfNna1 gene (GenBank Accession No. NC_004325) amplification with overlapping amplicons.

Molecular beacon and primers used for cDNA expression analysis (qRT-PCR) were designed according to previously published procedures (Bustamante *et al.*, 2004) and are shown in Table 2. The quantification of mRNA from the Nna-1 like gene was done using the constitutive gene 18S rRNA as endogenous control.

Probes were labelled at the 5′ end with 6-carboxyfluorescein (FAM). TAMRA was used as the 3′ quencher. Probes were purchased from Isogen Life Science (Maarssen, The Netherlands), and primers were purchased from Genotek (Barcelona, Spain).

Table 1. Primers of PfNna1 gene for qRT-PCR and the standard RT-PCR.

Primer name (F: Forward, R: Reverse)	Primer sequence	Fragment size (bp)
1F	ATGGTGTTTACTGTTTTACTTATAATAATTTTAG	201
1R	CAAATGGTTTGTCTCCTTGTCTTTTATTCTCTT	
2F	TGATAATATAAAAATGCAACAAGCC	165
2R	ATTTTTGTTCCTATTTCTACCATATACTATAGA	
3F	ATCCATATGATAATGTGAACGCA	150
3R	ATTTTTTCATCATTCATTTTGCTC	
4F	AATAAAGGAAGAAAATGACAAAGAAGT	102
4R	ATAACTAGCACTGAAATAGAACCACTG	
5F	AAAGGTGTATGAATCAATATTTGAATG	99
5R	AATTTATCACATGCGTCTAACGA	
6F	AAATAGAGAATATACAATGGGAAAGAAAT	151
6R	TGCATACGTAAAATCATAACTAAATTCTA	
7F	TCATGTGGGTTTGATTGCC	150
7R	CCATCCCATCAACCAATTTT	
8F	GTGGTAAATAATTTATTTGTTGATATGAAA	174
8R	AACATTCTTTACACATATTATCATTTGAAT	
9F	TCGAGTTATGCAATACATGGATT	180
9R	TCGGATTAGACCATTGCCTAT	
10F	GATTATATTTTTTTGTGATTTTCATAGTCA	180
10R	CTCATTATCTGCTTTATATACAGTGTCG	
11F	GGTAAAAGAGGTATATGAGTTAGGAAGAA	154
11R	CAGGACCTTTATACTTACCATTAGGAT	

Table 2. Primers and molecular beacons designed for cDNA expression analysis of PfNna1.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Molecular beacon 5'-3'
PfNna1 PFA0170c	ATGTGTGAAGTTTTCCCAGAAATAT	TTGCGCTCCCCTTATTCTC	CGCGGATTACCTTGGTTTGCTCTTGTCGACACC GCG
18S rRNA M19172	TGACTACGTCCCTGCCCTT	ACAATTCATCATATCTTTCAATCGG	GGGGGACACCGCCCGTCGCTCCCCC

The underlined sequence in the molecular beacon corresponds to the stem.

mRNA expression analysis using the $2^{-\Delta\Delta CT}$ method

The analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method described in detail in (Schmittgen and Zakrajsek, 2000). This involves comparing the target C_T values of the treated samples with a non-treated sample. The target C_T values of both treated and non treated samples must be normalized to the endogenous gene 18S rRNA. For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and internal control must be approximately equal. In order to validate our method, sample amplifications were performed on serial 10-fold dilutions using primers (Table 2) for the internal control (18S rRNA) and the target genes. The average C_T was calculated for both internal control and target genes and the ΔC_T (C_T,Target– C_T, 18S rRNA) was determined. Plots of the log DNA dilution versus ΔC_T were made. The absolute value of the slope (m) was below 0.1, so, the efficiencies of the target and internal control genes were considered as similar.

$\Delta\Delta C_T$ calculation for the relative quantification of targets were performed according to
Equation A: $\Delta\Delta C_T=(C_T,\text{Target}-C_T,\ 18S\ rRNA)_\chi-(C_T,\text{Target}-C_T,\ 18S\ rRNA)_\gamma$,
where χ = variable under investigation (treated samples) and γ=calibrator sample (non-treated samples). Results for each treated sample were expressed in N-fold changes in target gene expression relative to the same gene target in the calibrator sample, both normalized to 18S rRNA:
Equation B: N-fold $=2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

DNA sequencing

Final RT-PCR products were purified by QIAquick® PCR Purification Kit and were automated sequenced (Applied Biosystems 3730 DNA Analyzer) in both strands using separately the primers employed in PCR (Table 1). All the Data from sequencing was analysed using ABI PRISM® SeqScape® software version 2.0 (Applied Biosystems).

4. Results

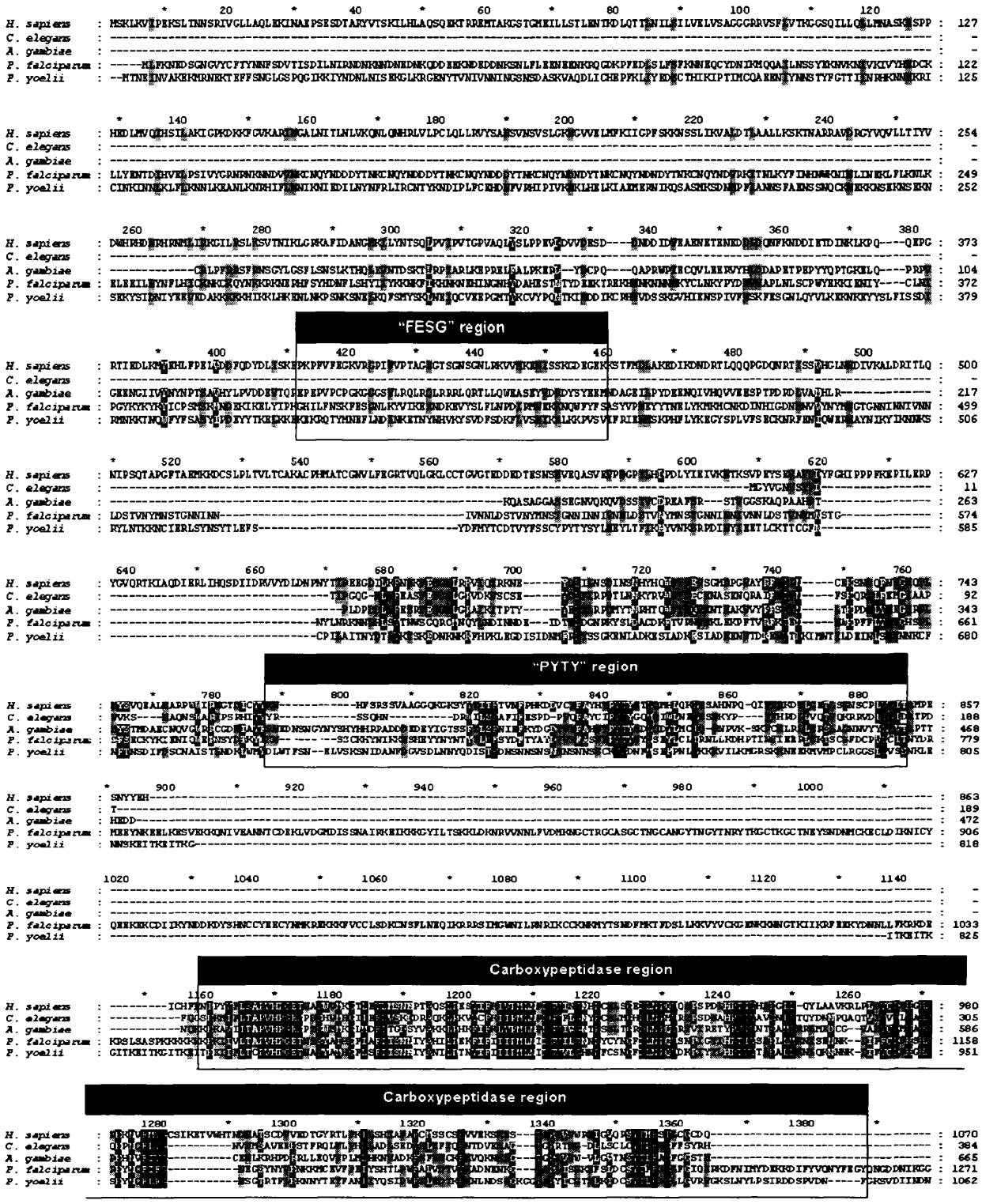
Expression pattern of PfNna1 across the *P. falciparum* intraerythrocytic cycle

Identification of PCI target gene, PfNna1, has been reported previously (Rodriguez de la Vega *et al.*, 2007). This gene was identified in PlasmoDB (www.plasmodb.org) as PFA0170c, protein-coding gene of the PfNna1 protein, an atypical putative zinc carboxypeptidase from *P. falciparum* strain 3D7. Clustal alignment of PFA0170c with homologous atypical carboxypeptidases (NP_056054.1 *Homo sapiens*, Q09296 *Caenorhabditis elegans*, XP_311036.1 *Anopheles gambiae* and EAA15579.1 *Plasmodium*

yoelii) showed homology not only with the carboxypeptidase domain, but also with two other regions near to N-terminus (Fig. 2).

Moreover, Clustal alignment of PFA0170c with metalocarboxypeptidases of the other species of *Plasmodium* can be seen in Figure 3.

In order to know whether the target gene (PfNna1) is expressed along the intraerythrocytic parasite stages that are inhibited in the presence of PCI, quantitative RT-PCR with the target gene-specific primers was assayed using total RNA extracted from synchronized parasites. Quantification of the gene was assessed using the molecular beacon probe and primers (defined in Table 2) designed from the CCP zone (a common motif in carboxypeptidases) of the PfNna1 gene. As shown in Figure 4, the expression analysis in different asexual erythrocytic stages demonstrated that this gene was expressed at highest level in the mature forms of the parasite life cycle in both strains assayed. In the strain Dd2, the maximum PfNna1 mRNA level was observed in schizonts which resulted 3-fold higher than in rings. In the 3D7, PfNna1 mRNA level was 3.3-fold higher in trophozoites, with the highest amount of the mRNA, than rings. Comparison of the gene expression between both strains showed that PfNna1 was expressed at higher level in 3D7 than in Dd2. Particularly, there was around 2-fold increase in trophozoites of 3D7 in comparison with maximum mRNA expression obtained in Dd2 (Figure 4).


"FESG" region
"PYTY" region
Carboxypeptidase region
Carboxypeptidase region

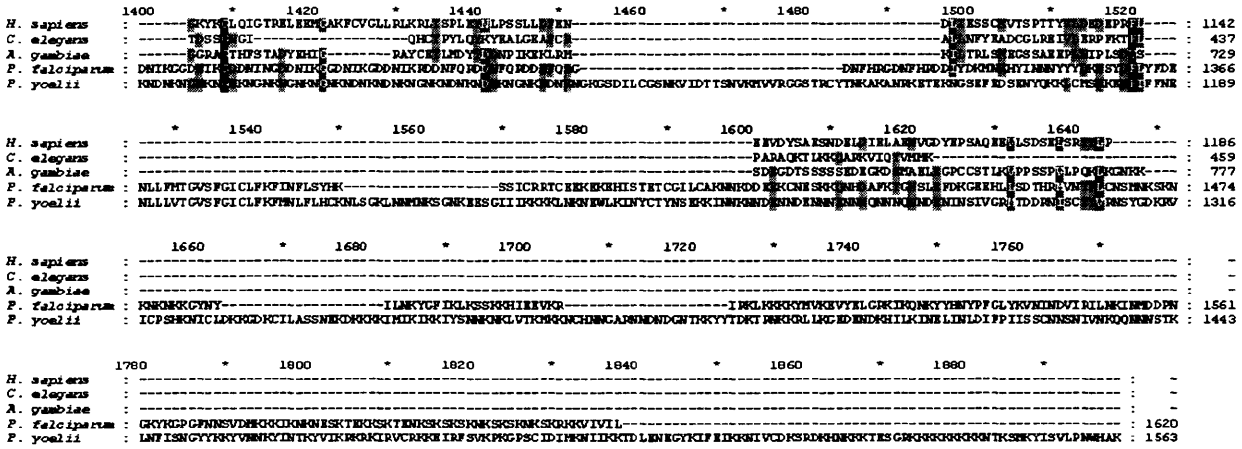


Fig. 2. ClustalX alignment of PfNna1gene of *P. falciparum* (PFA0170c) with atypical carboxypeptidases from other representative organisms (*H. sapiens, C. elegans, A. gambiae, and P. yoelii*). Boxes show homologies between motifs of "PYTY" and "FESG", and carboxypeptidase domain.


"FESG" region
"PYTY" egion
"PYTY" region
Carboxypeptidase region
Carboxypeptidase region
Carboxypeptidase region

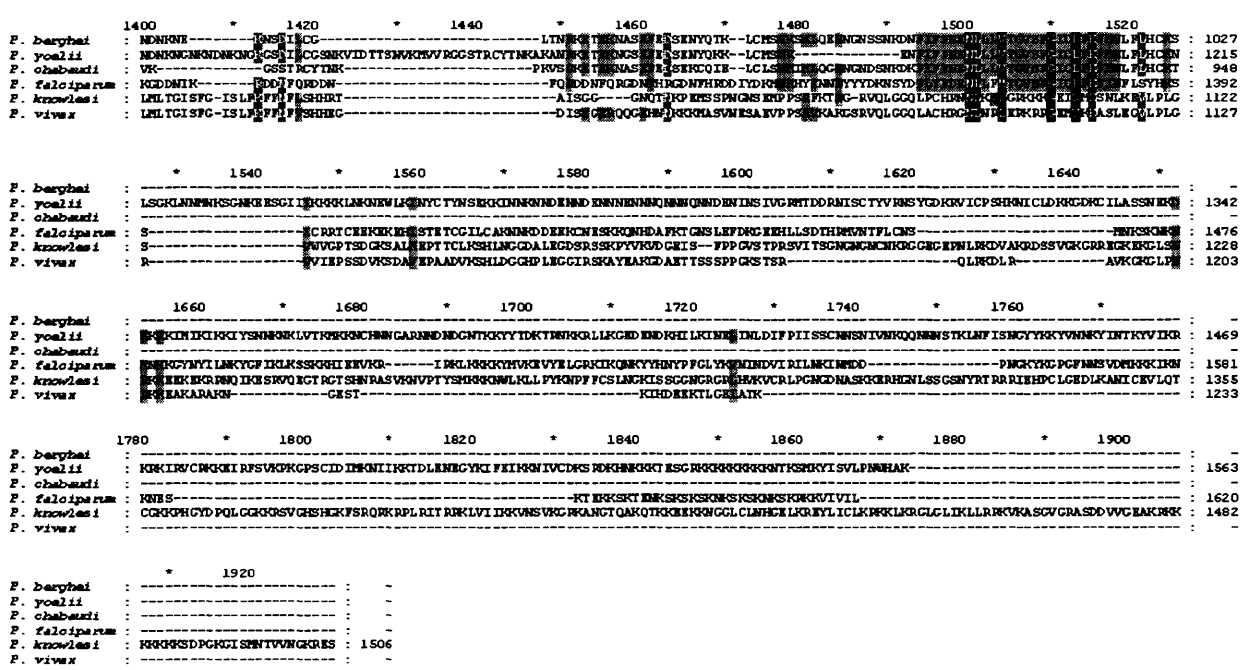


Fig. 3. ClustalX alignment of PfNna1 gene of *P. falciparum* (PFA0170c) with its homologues in *P. berghei*, *P. yoelii*, *P. chabaudi*, *P. falciparum*, *P. knowlesi*, and *P. vivax*. Boxes show homologies between motifs of "PYTY" and "FESG", and carboxypeptidase domain.

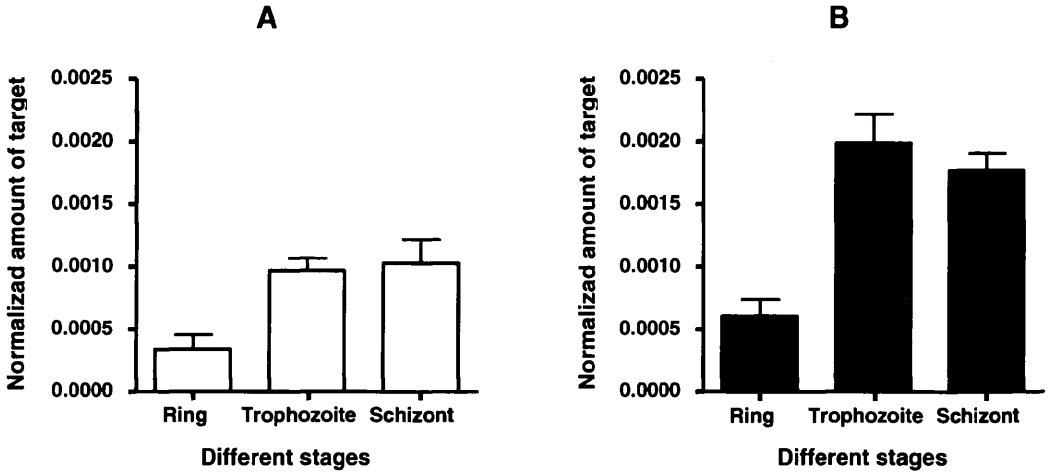


Fig. 4. Asexual stage-specific gene expression of PfNna1 determined in RNA isolated from *P. falciparum* cultures. Transcripts were detected in rings (10 h), trophozoites (27 h), and schizonts (39 h). Normalized expression levels against 18S rRNA expression are shown in both strains. A: Dd2 and B: 3D7.

To know whether the PCI effect on the parasite cycle could modify the mRNA expression pattern of the PfNna1 gene, and thus indicating its regulation by inhibiting the protein product, transient inhibitory assays were performed. To optimise the incubation time at which PCI modify PfNna1 gene expression, asynchronized *P. falciparum* (strain Dd2) microcultures were incubated with 40 µM of inhibitor over 8 h and aliquots were taken at 1, 2, 3, 4, 6 and 8 h. Then, the relative PfNna1 gene expression was quantified using the corresponding isolated RNA. Target gene expression was mostly inhibited after 3 h, and therefore, 3 h of incubation with PCI was selected as the treatment time-span.

PfNna1 mRNA expression was studied in synchronized cultures incubated with PCI during 3 h across the intraerythrocytic cycle. The experiment started with parasites (1.5% parasitaemia) in very young rings and lasted during 57 h. Six time intervals corresponding to main parasite stages were analysed during this complete intraerythrocytic cycle. Figure 5 shows the amount of PfNna1 mRNA expression in cultures incubated with PCI during 3 h in comparison with the control non-treated culture at the same time point. PfNna1 was highly expressed in PCI-treated cultures, and more particularly in the mature schizont forms at 45 h where the highest amounts of PfNna-1 mRNA were detected, reaching up to 4.7-fold the amount in the absence of PCI.

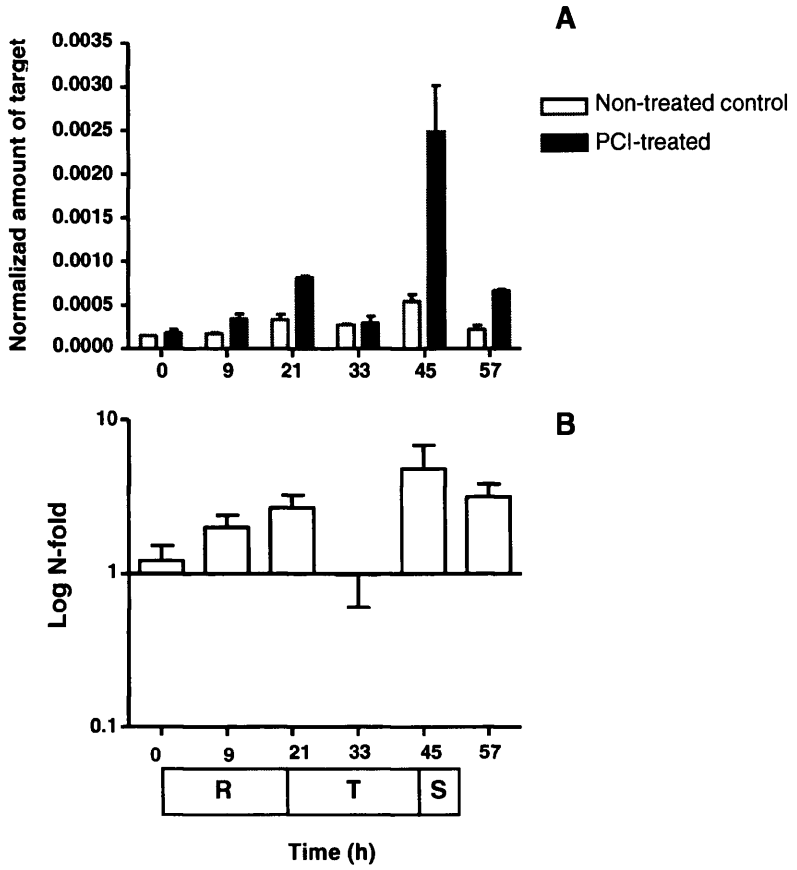


Fig. 5. Expression pattern of the PfNna1 gene along a single intraerythrocytic cycle of *P. falciparum*. Cultures were transiently incubated with PCI during 3 h *vs* control culture in the absence of PCI. The given time indicates the time points of harvesting after 3 h PCI incubation. Standard deviation of triplicate cultures is shown. (A), Time-course PfNna1 mRNA expression in PCI-treated cultures vs. non-treated control. (B) N-fold changes (in log scale) of PfNna1 expression in PCI-treated cultures vs. non-treated control, normalized to the 18S rRNA reference gene. R: Ring, ~ 0-20 h; T: Trophozoite, ~ 20-44 h; S: Schizont, ~ 44-50 h.

Transcript and sequence analysis of the Nna-1 like gene

In order to analyse possible post-transcriptional modifications or cryptic splicing, mRNA from the Nna-1 like gene was studied by RT-PCR. Eleven Pairs of primers were designed (Table 1) to amplify the full-coding sequence of the gene by overlapping PCR fragments in six combinations of primers pairs (Fig. 6A). All amplified fragments by RT-PCR (using both random and gene specific primers for reverse-transcription reaction), with expected and unexpected sizes, were analysed by DNA sequencing and cloning.

Some smaller fragments appeared when RT-PCR was done using specific primers. Cloning and sequencing of these fragments proved just mismatched priming effect in A+T rich regions in the retrotranscription at 37°C and consequent smaller size amplification (results not shown). Nevertheless, random primers in the reverse

transcription allowed the generation of the expected RT-PCR fragment sizes without producing any small unspecific fragment.

DNA sequencing of the expected size RT-PCR products (Fig. 6B & 6C) showed that these gene sequences did not show differences between cDNA and genomic DNA. Moreover, this was confirmed by the qRT-PCR analysis of mRNA expression along the gene with the same set of overlapping primers. As shown in Table 3, C_T values obtained with RNA correlate with the values obtained with genomic DNA, indicating that all fragments are in a relative proportion of expression level. These data suggest that not lack of expression of any fragment in the PfNna1 gene could reveal any cryptic of alternative splicing.

Table 3. Average C_T values for the PfNna1 gene amplification. qPCR and qRT-PCR were done using the eleven primer pairs shown at left. SYBRGreen I was used to detect and quantify double-stranded DNA.

Pair of Primers	C_T in DNA	C_T in cDNA
1F+1R	20,950 ± 0,100	31,450 ± 0,171
2F+2R	22,230 ± 0,141	31,663 ± 0,247
3F+3R	21,440 ± 0,06	29,597 ± 0,080
4F+4R	21,355 ± 0,019	27,197 ± 0,199
5F+ 5R	21,895 ± 0,054	28,410 ± 0,116
6F+6R	26,340 ± 0,171	37,987 ± 0,698
7F+7R	19,715 ± 0,133	26,147 ± 0,253
8F+8R	25,070 ± 0,084	33,120 ± 0,167
9F+9R	23,525 ± 0,038	28,450 ± 0,010
10F+10R	20,800 ± 0,098	32,970 ± 0,124
11F+11R	20,085 ± 0,038	28,677 ± 0,227

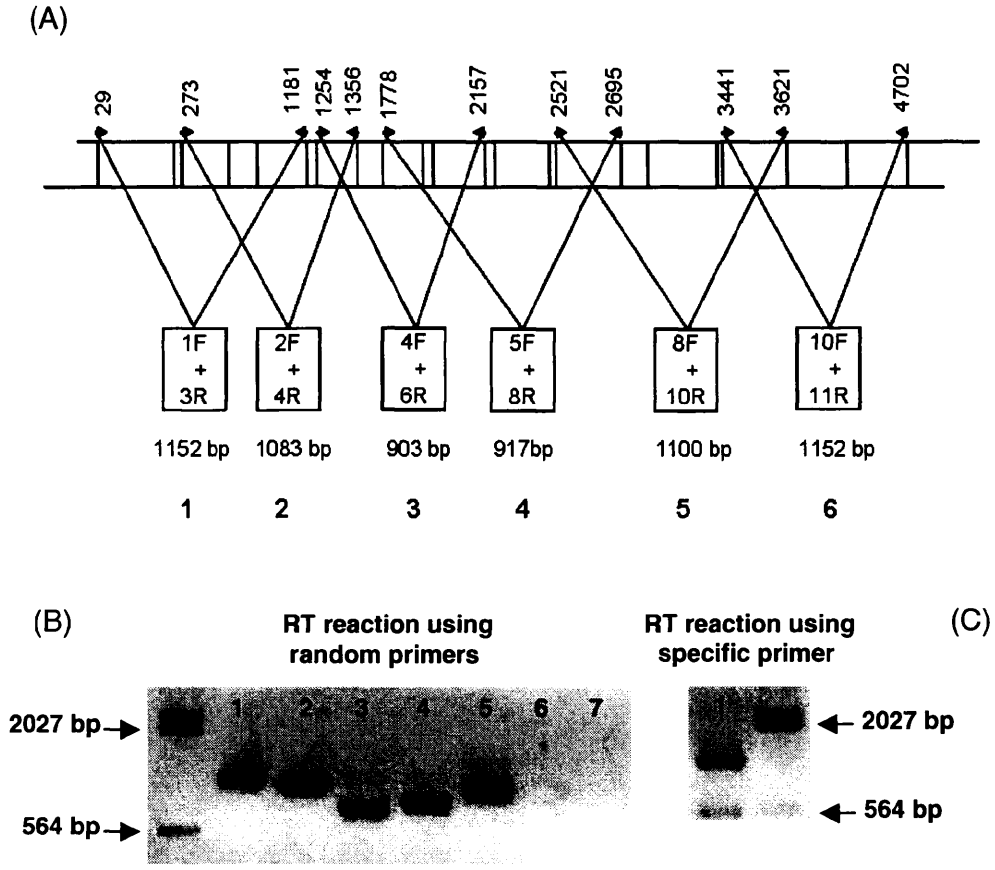


Fig. 6. Expression detection of the PfNna1 gene by RT-PCR using *P. falciparum* RNA from strain Dd2. (A): six combinations of primers (Table 1) over the full-coding sequence of the PfNna1 gene. (B): RT-PCR products. The first lane on the left: λ/HindIII molecular size marker. Nos. 1 to 5 show the amplicons using primer pairs: 1F+3R, 2F+4R, 4F+6R, 5F+8R and 8F+10R respectively. There was not any amplification using primer pair of 10F+11R (No. 6). No. 7 used RNA as a negative control. (C): RT-PCR product. The first line on the right: λ/HindIII molecular size marker. No. 1 is the amplicon using primer pair 10F+11R. Here, gene specific primer was used for the reverse-transcription reaction as there was not any amplification using random one (B, No. 6). RT, reverse transcription.

The sequence analysis of the overlapping fragments was performed in cDNA and DNA from strains 3D7 and Dd2. The complete genome sequence of *P. falciparum* (Gardner *et al.*, 1998; Bowman *et al.*, 1999; Gardner *et al.*, 2002) is available in strain 3D7. The comparison of the genomic DNA sequences from strains 3D7 and Dd2, detected two main mutation events. First, in the overlapping segment of fragments 1 and 2 (Fig. 6A), it was detected an inframe insertion of 36 bp at position 582-583 in the Nna-1 like gene from strain Dd2 with regard to the reference sequence in GeneBank from strain 3D7 (Fig. 7A). This mutation deduces the insertion of 12 new amino acids in the protein without the loss of the open reading frame. Second, in fragment 3 (Fig. 6A), it was revealed an inframe deletion of 63 bp in the Nna-1 like gene from strain Dd2, which with regard to the reference sequence in GeneBank from strain 3D7 is located at position

1609-1671 (Fig. 7B). This mutation deduces the lack of the 21 amino acids in the protein without the loss of the open reading frame.

Multiple alignment of metallocarboxypeptidases of *Plasmodium* genus (Fig. 3) showed that despite sharing large conserved parts between them, there are some sequences which are species specific in *P. falciparum.*

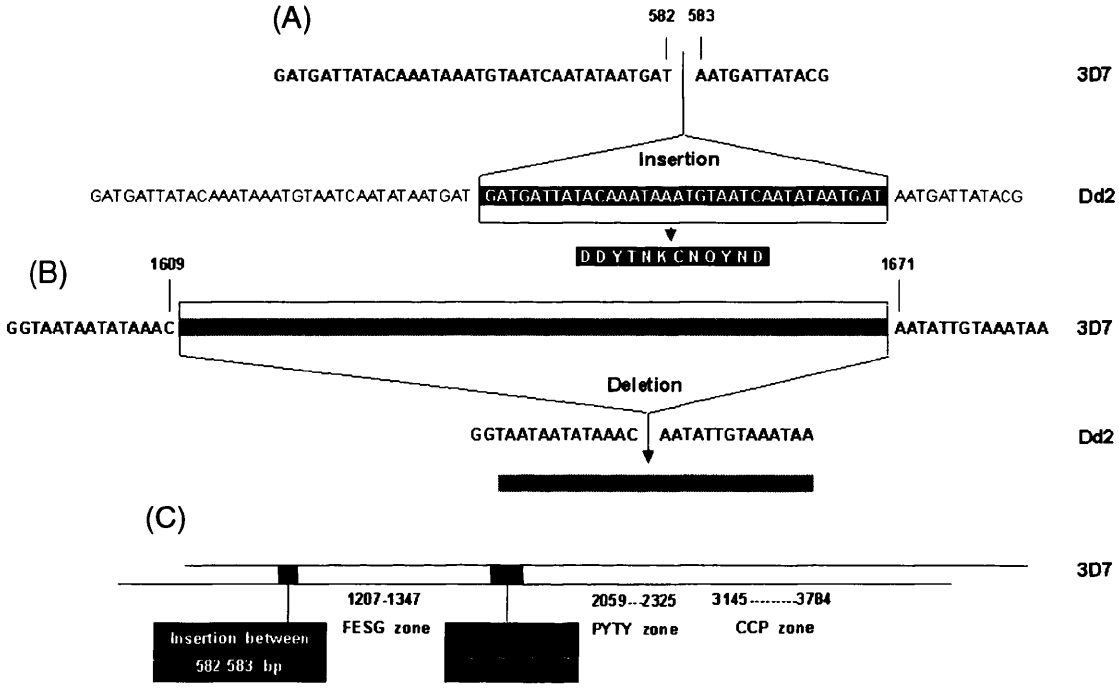


Fig. 7. Mutation event in the PfNna1 gene of *P. falciparum*, strain Dd2. (A) shows an insertion (duplication of the flanking sequence on the left) which exists in the Dd2 strain, 12 amino acids are repeated in PfNna1 protein in *P. falciparum*, Dd2 strain. (B) shows a deletion which occurred in the Dd2 strain, 21 amino acids deleted in PfNna1 protein in *P. falciparum*, Dd2 strain. (C) shows the situation of mutations over the PfNna1gene of 3D7 strain.

5. Discussion

The limitations of antimalarial chemotherapy underline the need for new drugs, ideally directed against new targets. Potential targets for chemotherapy include malarial proteases which play important roles during the malaria parasite life cycle (McKerrow *et al.*, 1993). Initial studies of partially purified protease activities and of the biological effects of protease inhibitors have now been complemented by molecular and biochemical analyses of important proteases of all four major catalytic classes (cysteine, aspartic, serine and metallo) due to the knowledge of the *P. falciparum* genome (Gardner *et al.*, 2002; Wu *et al.*, 2003). However, in the *P. falciparum* genome has been identified

sequences that likely encode additional proteases, therefore, more protease activities can still be expected (Rosenthal, 2001; Wu *et al.*, 2003).

The present study focus on the expression of a recently annotated carboxypeptidase gene (Rodriguez de la Vega *et al.*, 2007) in the malaria parasite *P. falciparum*. The evidence that PCI, as a carboxypeptidase inhibitor (Aviles *et al.*, 2006), had inhibited parasite growth indicated the important role of this new carboxypeptidase as a functional protease in parasite life cycle. Searching for the target gene of PCI in the molecular databases showed the presence of PfNna1 gene, a metallocarboxypeptidase, belonging to the M14 family (Rodriguez de la Vega *et al.*, 2007). These proteins hydrolyze single C-terminal amino acids from polypeptide chains (Grobelny *et al.*, 1992; Rodriguez de la Vega *et al.*, 2007). Neither its mechanism of action nor its protein or peptide target is actually known in *P. falciparum* or in other organisms (Harris *et al.*, 2000). Therefore, considering PfNna1 protein as the target for PCI, further molecular studies on that, as a target for chemotherapy in malaria, have been done by our group.

Both, mRNA expression pattern across intraerythrocytic stages and transcriptional up-regulation of the PfNna1 gene by PCI demonstrated high expression levels of this gene in mature stages. Time course analysis of PfNna1 mRNA expression showed that it is maximally expressed at 45 h post invasion, in schizonts, and being up-regulated in PCI treated cultures. Therefore, higher abundance of PfNna1 mRNA in mature erythrocytic stages and in PCI-treated schizonts suggested the critical role of PfNna1 in essential activities for the parasite survival.

The involvement of proteases is considered part of essential functions in *P. falciparum* life cycle. Cysteine and aspartic protease inhibitors are now under study as potential antimalarials (Rosenthal, 1998; Rosenthal, 2004; Ersmark *et al.*, 2006) for interfering haemoglobin hydrolysis, erythrocyte rupture, and erythrocyte invasion. GM6001, an inhibitor of both matrix and disintegrin metalloprotease family (Grobelny *et al.*, 1992; Ito *et al.*, 2004), was used to address the function of metalloproteases during host cell invasion. The IC_{50} of GM6001 for schizont-derived merozoite invasion was 112 µM, which implicates the role of metalloprotease. A *P. falciparum* metalloaminopeptidase belonging to M1 family has been also identified (Florent *et al.*, 1998). In addition to haemoglobin degradation (Gavigan *et al.*, 2001), this metalloaminopeptidase has been proposed to have a role in growth and invasion as bestatin and nitrobestatin inhibitors are able to block *P. falciparum* development (Nankya-Kitaka *et al.*, 1998; Allary *et al.*, 2002).

The participation of PfNna1, as tubulinyl-Tyr carboxypeptidase (tubCP), in the proteolytic processing of α-tubulin have been hypothesized (Kalinina *et al.*, 2007; Rodriguez de la Vega *et al.*, 2007). α-tubulin is initially produced with a C-terminal tyrosine, which is removed by the tubCP (Hallak *et al.*, 1977). Tubulin, a heterodimer of α-

and β-tubulin, is the major component of microtubules which are subcellular components present in all eukaryotes and have function in a wide range of cellular processes including chromosome separation during mitosis, intracellular transport of organelles and cell motility. Moreover, they maintain the structural integrity and cytoplasmic architecture of the cells (Hyams and Lloyd, 1994). Posttranslational modifications such as C- terminal detyrosination of α-tubulin makes more stabilized microtubules with slower rates of protein turnover in comparison with the tyrosinated α-tubulins associated with dynamically unstable microtubules with short half-lives (Webster *et al.*, 1987; Fennell *et al.*, 2007).

It has been reported that tubCP activity increased in the nervous cells and also in proliferating cells when cells undergo differentiation (Wehland and Weber, 1987; Contin and Arce, 2000). In addition, *in situ* analysis of developing embryonic nervous tissue showed Nna1 was highly expressed in differentiating neurons suggesting its contribution in differentiating and regenerating neurons (Harris *et al.*, 2000). Higher abundance of PfNna1 mRNA in mature stages (i. e. schizonts of the Dd2 strain) and PCI-induced transcriptional up-regulation of this gene in shizonts of *P. falciparum* could confirm tubCP activity of PfNna1 as schizonts are involved in cell division, differentiation, and biogenesis of motile merozoites. All these events need participation of tubulins (Bell, 1998), specifically detyrosinated α-tubulins which are associated with more stabilized microtubules (Webster *et al.*, 1987; Fennell *et al.*, 2007), and therefore imply the active presence of PfNna1 enzyme as tubCP in the schizont stage. The possible function could make PfNna1 a candidate target for chemotherapy of malaria.

In general, genes of malaria parasites and other unicellular organisms have larger exons with fewer and smaller introns than metazoans (Singh *et al.*, 2004). It is believed that such differences in gene structure cause simpler mechanisms for transcriptional control and mRNA processing (Singh *et al.*, 2004). The *P. falciparum* genome is highly A+T rich, reaching 80% in most genes (Gardner *et al.*, 2002; Szafranski *et al.*, 2005). This particular biased composition had led to suggest an elevated thermodynamic capacity for quick genetic change allowing faster adaptations, even at the level of gene rearrangements and intro-exon boundaries. According to the large and unique species-specific insertions found in the alignments of the PfNna1 gene we investigated whether cryptic splicing sequences could favour a post-transcriptional processing (Singh *et al.*, 2004) resulting in a different mature protein than that expected for the Nna-1 like gene. Interestingly, transcript and sequence analysis of PfNna1 gene showed that all parts of this large gene were involved in transcription, thus no splicing was detected. This proved that all sequence should code for mature polypeptide. Nevertheless during this study, we found two large inframe mutations between two strains, suggesting the easiness in *P. falciparum* for change in protein structures, since the two mutation events account for a

total of 33 amino acid differences. It should be noted that these two large changes were found in not conserved areas, and therefore allowing to maintain the main protein function of the Nna-1 like gene.

Large insertions have also been identified in several other plasmodial proteins, such as, protein kinase, heat shock protein-90, RNA polymerase, α-glutamyl cysteine synthetase, and both bifunctional enzymes of glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase and ornithine decarboxylase/S-adenosylmethionine decarboxylase (Giesecke *et al.*, 1991; Kappes *et al.*, 1995 ; Bonnefoy *et al.*, 1997; Luersen *et al.*, 1999 ; Muller *et al.*, 2000; Clarke *et al.*, 2001; Birkholtz *et al.*, 2004). All of these insertions are characterised by a high abundance of charged amino acids, such as Asp, Asn, Glu, Gln, Ser and Lys (Muller *et al.*, 2005). Our results show that the inserted part in PfNna1 gene contains such charged amino acids in abundance as well. The consequences of these inframe insertions/deletions could be similar to the ones caused by missense mutations. On the other hand, the effects of these kinds of mutations on the structure and functionality of a protein can be quite different. Occasionally, such mutations increase the efficiency of a protein, conferring some selective advantage for the organism itself. *P. falciparum* genome were found polymorphic, containing amplificatons and deletions (Kidgell *et al.*, 2006) in the genes that introduce drug resistance (Foote *et al.*, 1989 ; Price *et al.*, 2004) and those that change the mechanisms for invasion of erythrocytes (Triglia *et al.*, 2005). This suggests that such mutations could be important determinants for the survival and spread of the parasite and possibly for the outcome of the disease (Ribacke *et al.*, 2007).

Although in our case we have not the above-mentioned evidences, the existence of the gene duplication and deletion in PfNna1 gene of the CQ-resistant strain (Dd2) express new and interesting clues for further future studies of this gene and its any possible participation in pathogenesis. However, in general, the extent of these mutations, their function in plasmodial proteins, and their possible contribution to the virulence of the malaria parasite is still unknown (Aravind *et al.*, 2003; Muller *et al.*, 2005; Ribacke *et al.*, 2007).

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4.2. Protein processing in *Plasmodium falciparum?*

Protein processing in *Plasmodium falciparum?*

Contents

1. Abstract

The genomes from the organisms of the *Plasmodium* genus, the causative agents of human and animal malaria, are characterized by an extreme high A+T content and an associated abundant low complexity inserts within their proteins. The enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (G6PD-6PGL) found in *Plasmodium* species has unique structural and bifunctional characteristics. Here, we report the expression analysis of *P. falciparum* G6PD- 6PGL along the intra-erythrocytic cycle by immunological analysis with antibodies raised against its N- and C- terminal domains. The pattern modification of band sizes at the different stages of parasite development suggests intracellular protein processing involving the cleavage of the native bifunctional form to produce two main fragments. *In vitro* RNA-mediated PfG6PD-6PGL gene silencing, studied along short-term parasite development also revealed the apparent intracellular protein modification dependent on the parasite stage. Fragment sizes were consistent with separating both catalytic functions of the enzyme. The proteolytic machinery underlying this specific PfG6PD-6PGL processing is still unknown in *P. falciparum* but suggests the existence of distinctive mechanisms in the parasite to deal with unique protein structures of essential function resulting from its genome evolution.

Keywords:

Glucose-6-phosphate dehydrogenase / dsRNA / Gene silencing / Malaria / Protein processing

2. Introduction

Plasmodium falciparum is the causative agent of the most severe form of human malaria being responsible for the death of more than 1 million people a year (Snow *et al.*, 2005). The efforts to control the illness have been focused on the chemotherapy, mosquito control and in the development of vaccines. Nowadays, the lack of an effective vaccine and the increasing parasite resistance to available drugs suggest the necessity to identify new targets that allow the development of new drugs and vaccines. Completion of *Plasmodium* genome sequences (Gardner *et al.*, 1998; Bowman *et al.*, 1999; Gardner *et al.*, 2002) has provided a vast amount of molecular information. This, together with the transcriptome (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003) and proteomic analysis along the parasitic developmental stages (Lasonder *et al.*, 2002) pursue a deep understanding of the peculiar parasite biology in the context of exploring new therapeutic and

immunization strategies. Since 1993, different technologies of *P. falciparum* genetic manipulation have been developed as gene disruption by homologous recombination (Wu *et al.*, 1995; Crabb and Cowman, 1996; Crabb *et al.*, 1997; Lobo *et al.*, 1999; Omara-Opyene *et al.*, 2004; Sijwali and Rosenthal, 2004), antisense RNA (Gardiner *et al.*, 2000; Noonpakdee *et al.*, 2003) and more recently, RNA interference that has been successfully applied to understand the *in vivo* functions of *P. falciparum* genes (Kumar *et al.*, 2002; McRobert and McConkey, 2002; Crooke *et al.*, 2006).

Genome comparison from lower eukaryotes have shown that *Plasmodium* proteins are notably longer in size than their respective orthologous genes. This is due to the extraordinary attribute of *Plasmodium* proteins that are hugely enriched in stretches biased toward 1-3 residues (mainly Asn, Lys, and Ile) due to the high A+T content (about 80% average). Due to this particular composition these stretches acquire low entropy or complexity (Wootton, 1994). These regions are embedded into highly conserved domains that form globular structures with larger variety of residue composition with high entropy and complexity (Wootton, 1994). Although many eukaryotes have also low complexity areas in nuclear proteins, transcription factors, and some cytoskeletal proteins, *Plasmodium* species display low complexity stretches in unique genes, not observed before in genomes from other organisms. In addition, these unusual regions even penetrate in independent functional domains with unpredictably length between 10 and 100 residues (Aravind *et al.*, 2003).

P. falciparum G6PD-6PGL is a bifunctional enzyme exclusive to *Plasmodium* species (Clarke *et al.*, 2001) that probably arose from the fusion of two genes in a common ancestor (Scopes *et al.*, 1997). The deduced protein has a subunit molecular mass of 107 kDa, in agreement with the tetramer molecular weight calculated by size exclusion chromatography (Kurdi-haidar and Luzzatto, 1990). Its C-terminal half (residues 311-911) is clearly homologous to other described G6PDs (with glucose 6-phosphate dehydrogenase activity) though sequence similarity is interrupted by a 62 amino-acid stretch with no similarity found to date. It has been nevertheless experimentally shown that this 62 amino acid insertion is essential for the activity of the bifunctional enzyme (Clarke *et al.*, 2003). In contrast, the 310 amino-acid protein sequence of the amino terminal region clearly differs from most eukaryotic and prokaryotic G6PDs, and shows 6-phosphogluconolactonase activity; thus G6PD-6PGL catalyses the first two stages of the pentose phosphate pathway (Clarke *et al.*, 2001). The occurrence of large insertion sequences that differ with respect to their homologous proteins in other species has been often observed in many gene products of *P. falciparum* and other *Plasmodium* species, but their structural functions and origins are unknown (Pizzi and Frontali, 2001; Clarke *et al.*, 2003). We have been interested to know whether the low complexity invasions of

globular domains could have a role in regulating protein turnover along the parasitic cycle, and therefore, in the present paper, the unique bifunctional *P. falciparum* G6PD- 6PGL protein was explored as a model gene to study dynamics of its protein processing along the intraerythrocytic cycle and under gene silencing conditions.

3. Materials and methods

Parasite cultures and electroporation

P. falciparum strain 3D7 was grown and double synchronised using standard procedures (Trager and Jensen, 1976; Lambros and Vanderberg, 1979). Parasites (ring stage 8-10% parasitaemia) were transfected by electroporation with 40 µg of dsRNA as described (Wu *et al.*, 1995). The parasites transfected with dsRNA-G6PD or dsRNA-Rab5a were kept for 24 h in 75 cm2 flasks. The growth and development of each transfection was monitored by Giemsa staining blood films.

dsRNA design

A 21 basepair dsRNA (sense: UACAUCAUGCACCAACGAAdTdT; antisense: UUCGUUGGUGCAUGAUGUAdTdT) was designed for the target sequence (UACAUCAUGCACCAACGAA) of the G6PD-6PGL gene, following Dharmacon siDESIGN Center criteria (http://design.dharmacon.com/). In addition, a dsRNA corresponding to the PfRab5a gene (GenBank™ accession number AE001399) (target sequence: UAUGCAAGUAUUGUCCCAC; sense: UAUGCAAGUAUUGUCCCACdTdT; antisense: GUGGGACAAUACUUGCAUAdTdT) was also designed to use as control. All dsRNAs were obtained from Dharmacon Research (Lafayette, CO, USA) in annealed and lyophilised form and were suspended in RNase-Dnase-free water before use.

Immunodetection

Antibodies were raised against two different recombinant *P. falciparum* G6PD-6PDL polypeptides expressed in the vector pGEX (Amersham Biosciences), which contains the glutathione-S-transferase sequence upstream from the polylinker to produce a fusion protein with the insert. Sequences from ntG6PD-6PGL (AAYYICKEIYDKQQINKDGYVVIGLSGGRTPIDVYKNMCLIKDIKIDKSKL) and ctG6PD-6PGL (KILKSIPSIKLEDTIIGQYEKAENFKEDENNDDESKKNHS) (see Fig. 1 for their location within the G6PD protein) were amplified and cloned into pGEX. Expression in *E. coli* was achieved following the manufacturer's instructions and the two glutathione-S-transferase/G6PD-6PDL fusion proteins were separately purified using a glutathione sepharose affinity column (Amersham Biosciences). Cleavage of the fusion protein by

factor X and subsequent separation in SDS-PAGE provided pure protein for antibody production. Antibodies against ntG6PD-6PGL and ctG6PD-6PGL where raised separately in rabbits but used as a mixture in the Western blot analyses to increase the signal.

anti- 6PGL-domain **anti-G6PD-domain**

anti-6PGL domain (AAYYICKEIYDKQQINKDGYVVIGLSGGRTPIDVYKNMCLIKDIKIDKSKL)
anti-G6PD domain (KILKSIPSIKLEDTIIGQYEKAENFKEDENNDDESKKNHS)

Fig. 1. G6PD-6PGL protein and location of antibody reactivity. Scheme showing primary structure of the G6PD-6PGL gene with two main functional domains, the low complexity stretch (red) and the sequences of the peptides corresponding to the two antibodies raised (see text).

For Western blot analyses, infected red blood cells (IRBCs) from a 10 ml-culture were suspended in 2 volumes of 0.2% saponin in PBS and incubated for 15 min at 37°C, to lyse the RBC membranes. The released parasites were pelleted at 10000 x g for 10 min and washed three times in cold PBS. The parasite pellet was solubilized in 100 µl of TTP (PBS containing 1% Triton X-100 and a protease inhibitor cocktail, Complete Mini, Roche Diagnostics) and incubated for 30 min at 4°C with frequent vortexing. Next, the soluble parasite fraction was frozen (at -70°C for 5 min) and thawed (at 37°C for 5 min) only once and centrifuged for 10 min at 10000 x g. Approximately 10 µg of total protein supernatant was boiled in 5x loading buffer and separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes and probed with the above-mentioned antibodies at 1/200 dilutions. Antibody binding was detected by incubation with secondary 1/5000 HRP-conjugated sheep anti-rabbit Ig Abs, followed by chemoluminescence detection using SuperSignal West Pico (Pierce, Biotechnology Inc, Rockford, IL). Bands were quantified using Quantity One® software (BioRad, Hercules, CA).

4. Results

PfG6PD-6PGL protein expression patterns across the intraeryhrocytic cycle

Expression patterns at the protein level were examined through immunodetection of parasite G6PD-6PGL using antibodies against N- and C- terminal separately and a mixture of both of them. Bands with different molecular weight were consistently observed in western blots. As shown in Figure 2, a 107 kDa band corresponding to the theoretical molecular mass of the deduced protein sequence was abundant mainly in mature stages and detected with both antibodies. Also two other abundant bands of 73 and 69 KDa were observed, predominantly in the mature parasite. Thus, the 73 kDa band, detected by the anti-6PGL domain antibody, has a size that includes the N-terminal domain with the expected 6PGL activity and the main low-complexity stretch between both domains. The 69 kDa band, detected by the anti-G6PD domain antibody, match with the expected size of the G6PD domain. In addition, in the mature stages two other smaller bands were also detected: a 44 kDa band detected with the anti-6PGL domain and a 39 kDa band detected with the anti-G6PD domain, which could result from processing of their corresponding 73 kDa and 69 kDa band, respectively.

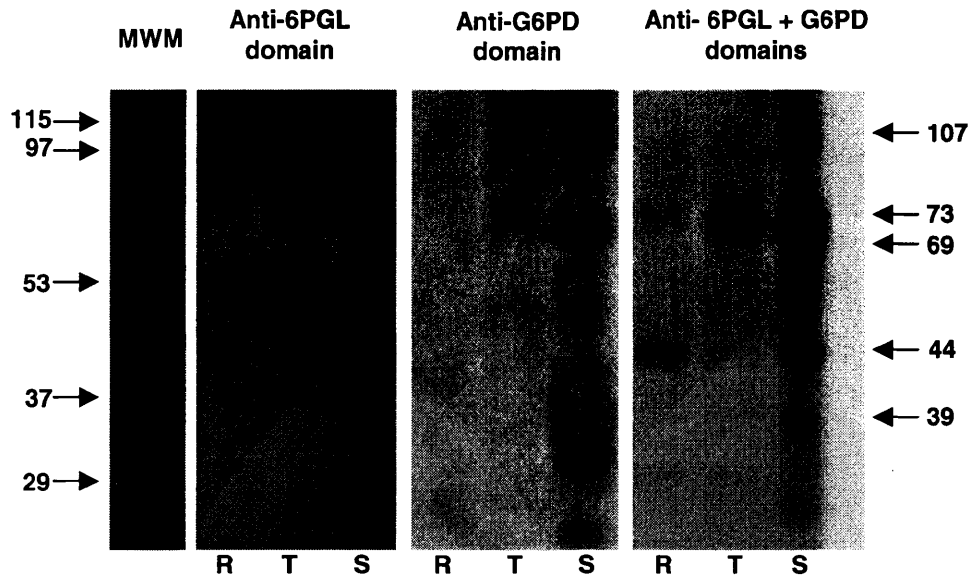

Fig. 2. G6PD-6PGL protein patterns across the intraerythrocytic cycle of *P. falciparum*. Expression patterns at protein level were examined through immunodetection of the parasite G6PD-6PGL. Parasites harvested at the three main stages, rings (R), trophozoites (T) and Schizonts (S) were transferred and immunodeveloped with three different conditions using anti-6PGL domain, anti-G6PD domain, and a mixture of both of them.

dsRNA silencing of the PfG6PD-6PGL and expression pattern

In an attempt at silencing the G6PD-6PGL gene, erythrocytes infected with ring-stage *P. falciparum* 3D7 (pyrimethamine-sensitive clone) were electroporated with a dsRNA-G6PD duplex, RNase-free water and dsRNA-Rab5a (the last two as controls) as previously reported (Crooke *et al.*, 2006).

The G6PD-6PGL protein band pattern during the *P. falciparum* intra-erythrocyte cycle, as revealed by immunodetection with both antibodies is shown in Figure 3A. In all stages, two main bands of different molecular weight were observed, a 107 kDa band corresponding to the theoretical molecular mass of the deduced protein sequence and the 73 kDa band with 2 other subforms in the mature parasites.

Compared to the control, gene silencing by dsiRNA reduced G6PD-6PGL immunoreactivity as observed in cultures at 3 and a 24 h (Fig. 3B). Thus, as earlier as 3 h, a 54% and 69% reductions in the 73 kDa and 107 kDa bands, respectively, were observed (Fig. 3C). After 24 h, this effect started to diminish, and only 14% and 19% decreases in the two bands, respectively, were observed (Fig. 3C). These data confirm previous findings that silencing through dsRNAG6PD instantly took place after 3 h of electroporation at the ring stage, with normal levels gradually restored at the trophozoite stage, 24 h post-transfection (Crooke *et al.*, 2006).

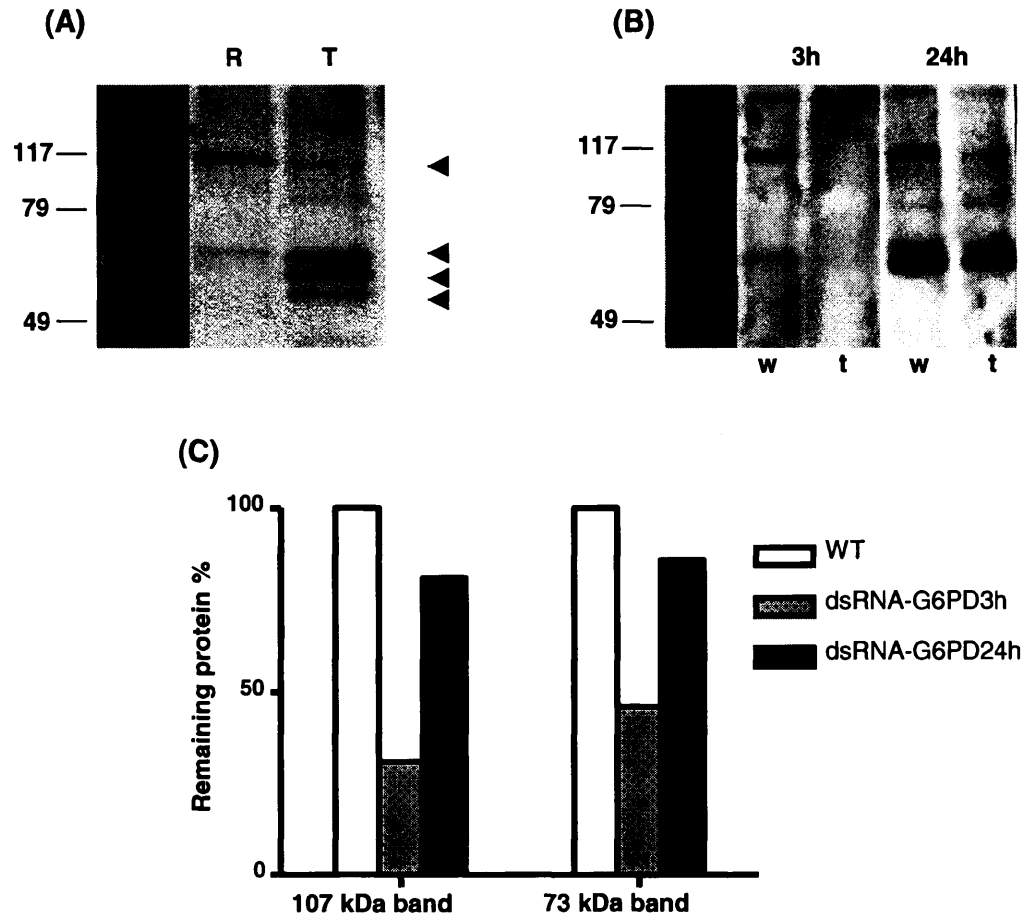

Fig. 3. Effect of dsRNA on parasite protein levels. (A) Immunoblot analysis of wild-type synchronized 3D7 rings and trophozoites. The G6PD-6PGL protein band pattern immunodetected during the intra-erythrocyte cycle of *P. falciparum* includes the 107, 73, 69 and 53 kDa bands labelled with arrows. (B) Immunoblot analysis of synchronized wild-type and dsRNA-G6PD parasites (10 µg of total protein per lane) at 3 and 24 h after electroporation. (C) Immunoblot quantification of synchronized wild-type and dsRNA-G6PD parasites. The 107 and 73 kDa bands signal detected were quantified by densitometry using a Fluor-S Multilmager and Quantity One quantitation software (Bio-Rad). Value for PfG6PD-6PGL in dsRNA-G6PD parasites is expressed as a percentage of control levels in wild-type parasites transfected with water. The positions of molecular mass standards are indicated (kD).

5. Discussion

The data presented above indicate that this unique bifunctional *Plasmodium* G6PD-6PGL protein varies in size depending on the parasite developmental stage. Thus, the 69 KDa band detected by the anti-G6PD domain antibody coincides with the expected size of the G6PD domain at the C-terminal, and the 73 kDa band identified by the anti-6PGL domain antibody could have a size that including the essential insertion (Clarke *et al.*, 2003) corresponds to the N-terminal of the protein with the 6PGL activity. Thus, it seems that the bifunctional protein could mature to render two different polypeptides with separate enzyme activities but sharing the essential insertion.

To assess specific biological gene function in the parasite, several systems for the functional analysis of *P. falciparum* genes have been developed, including gene silencing by antisense RNA (Gardiner *et al.*, 2000; Crooke *et al.*, 2006) or more recently, by RNA interference (Kumar *et al.*, 2002; McRobert and McConkey, 2002; Crooke *et al.*, 2006). Antisense RNA has been found in humans, mice, plants and protozoan parasites such as *P. falciparum*. The fact that endogenous antisense RNAs are widespread in *P. falciparum*, suggests that they could be a natural gene expression regulatory mechanism (Gunasekera *et al.*, 2004; Militello *et al.*, 2005). In our model, *P. falciparum* G6PD-6PGL was silenced *in vivo* through a dsRNA. Although mechanisms of RNAi silencing in many organisms are not well known, this technique has been used to study gene function in a great variety of organisms including other parasites (Ullu *et al.*, 2004). Despite the fact that, so far, the genes encoding the required RNAi machinery have not been detected in any of the currently available *Plasmodium* databases, RNAi silencing has been achieved in *Plasmodium* (Kumar *et al.*, 2002; McRobert and McConkey, 2002; Crooke *et al.*, 2006). Thus, it could be that the data reported for *Plasmodium*, as well as our results using dsRNA-G6PD, are the consequence of an antisense RNA rather than a direct RNAi effect. However, it is also true that, to date, 60% of the genes predicted for *P. falciparum* have no known homologues, and we have no clues as to their function (Gardner *et al.*, 2002).

The protein expression patterns examined by gene silencing showed that after 3 h of transfection, when most of the parasites are at the ring stage, the complete 107 kDa band predominates in both transfected and not transfected parasites. However, after 24 h of electroporation when the parasites were mainly at the trophozoite stage, the main band was the 73 kDa band. Again, this data suggest protein size change across parasite life cycle by protein processing or by alternative splicing. To this respect, different sizes of mRNA G6PD-6PGL have been observed in parasites in the ring and trophozoite stages (Cappadoro *et al.*, 1998). Thus, there seems to be specific *P. falciparum* mechanisms for processing this mRNA, controlled by the parasite's development cycle, which could be unique or shared with other genes (Singh *et al.*, 2004; Muller *et al.*, 2005). The 69 kDa band could coincide with the C-terminal end of the protein corresponding to G6PD activity in such a way that it would not show the N-terminal end that corresponds to the 6PGL activity. Based on the data shown, we can hypothesize a controlled pattern of PfG6PD-6PGL processing during parasite maturation as depicted in Figure 4.

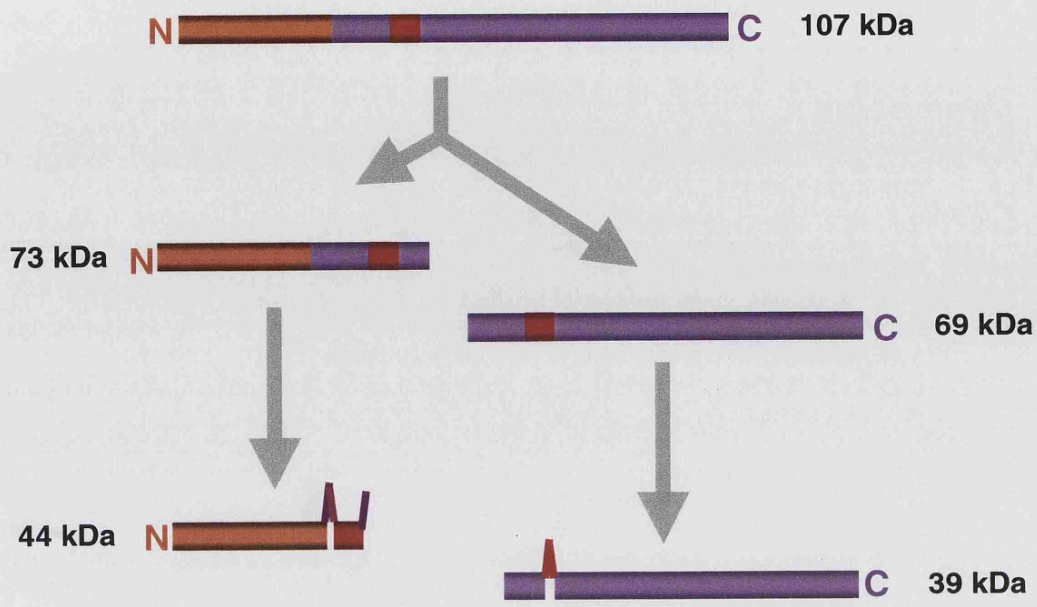

Fig. 4. Hypothetical processing of G6PD-6PGL. The protein product sizes observed by immunodetection analysis across the intraerytrocytic *P. falciparum* life cycle could be explained by two steps of controlled maturation of the protein to take apart, optimize and recycle, the two functional enzymatic activities in the parasite cell.

An independent role of the 6PGL function has been addressed, and although its 6-phosphogluconolactone substrate is highly unstable *in vitro*, some increase in the efficiency of the pathway may be evident (Clarke *et al.*, 2001; Miclet *et al.*, 2001). To this respect, the lactonase activity shown by the bifunctional enzyme is ten times lower than that of the monofunctional domain 6PGL (Clarke *et al.*, 2001), demonstrating its low efficiency for 6-phosphogluconolactone hydrolysis. Moreover, from a structural standpoint, it has also been questioned whether the bifunctional protein is more efficient in producing NADPH than two separate enzymes (Clarke *et al.*, 2001). NADPH is also the co-substrate of *Plasmodium* glutathione reductase and thioredoxin reductase enzymes that protects against oxidative stress caused mainly by digestion of host cell haemoglobin at the late ring-early trophozoite stage (Bozdech and Ginsburg, 2004). Thus, different NADPH efficiencies could be required at different developmental stages with particular specialization by the bifunctional protein (Clarke *et al.*, 2001). Another explanation of this apparent processing, is the genome economy shown by the small *Plasmodium* genome containing a proportionally high number of genes compared to similar genome sizes (Enright and Ouzounis, 2001; Veitia, 2002). Several other unique bifunctional enzymes have been described in *Plasmodium* species (Bzik *et al.*, 1987; Pashley *et al.*, 1997;

Muller *et al.*, 2000) reflecting the parasite's rapid evolution in its constant fight to overcome host defence mechanisms.

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4.3. High parasitaemia culture of *Plasmodium falciparum*

High parasitaemia culture of *Plasmodium falciparum*

Contents

1. Abstract

The methods developed by Trager and Jensen in 1976 for the continuous culture of the erythrocytic stages of *Plasmodium falciparum* make this organism available to a large variety of scientists. As a result, much has been learned about *P. falciparum* during the past 30 years. Recent developments in the diverse aspects such as chemotherapy, drug resistance, vaccine development, pathogenesis, molecular biology and biochemistry emphasize the usefulness of the culture method in research. In the post-genomic era, when all proteins can be linked to the genes encoding them, comparative studies of the proteins expressed at any given life cycle stage or tissue is a critical requirement for a full understanding of biological phenomena. In fulfilling proteomic studies, highly parasitized cultures were needed. Here we report a modified method of the Trager and Jensen's culturing method in a way that synchronized cultures of *P. falciparum* with more than 80% parasitized cells are obtained.

Keywords:

Culture / Parasitaemia / *Plasmodium falciparum*

2. Report

A large effort and time have been invested for *in vitro* growth of the erythrocytic stages of the *Plasmodium* life cycle which is the stage most often associated with the pathogenesis of malaria and a major target for vaccine and drug development (Trager and Jensen, 1997; Schuster, 2002). A significant accomplishment in this area was defining *in vitro* conditions for continuous cultivation of *P. falciparum*. This was accomplished by Trager and Jensen (Trager and Jensen, 1976; Trager and Jensen, 1977) using HEPES-buffered RPMI 1640, a tissue culture medium developed for *in vitro* cultivation of leukocytes, supplemented with human serum, erythrocytes, and sodium bicarbonate.

The methods for cultivation of the erythrocytic stages of *P. falciparum* have been usefully applied in nearly every aspect of research on malaria: chemotherapy, drug resistance, immunology and vaccine development, pathogenesis, gametocytogenesis and mosquito transmission, genetics, the basis for resistance of certain mutant red cells, cellular and molecular biology and biochemistry of the parasites and of their relationship with their host erythrocytes (Trager and Jensen, 1997).

To study the dynamic nature of the *P. falciparum* parasite, efforts to characterize gene expression directly at the proteomic level have strikingly come under the spotlight. To achieve high yields of isolated proteins which are necessary to study on protein level, highly parasitized cultures are needed. In this work, we have focused on reaching a high percentage parasitism to fulfil protein and proteomics studies on different intraerythrocytic stages of *P. falciparum.*

The chloroquine-sensitive 3D7 and chloroquine-resistant Dd2 *P. falciparum* isolates were used for cultivation. Parasites were grown *in vitro* in groups A^+ or O^+ human erythrocytes following previously described conditions (Trager and Jensen, 1976), with some critical modifications specially in applied methods. Parasites were cultured in RPMI1640 medium (Sigma), supplemented with 25 mM HEPES, 0.5% AlbuMAX I (Invitrogen), 1.77 mM sodium bicarbonate, 100 µM hypoxanthine, and 12.5 µg/ml gentamicin sulphate, at a pH of 7.2 in a gas mixture of 96% nitrogen, 3% carbon dioxide and 1% oxygen. The culture medium without sodium bicarbonate (incomplete medium) was prepared weekly and stored at 4°C. To avoid increasing pH, sodium bicarbonate was added freshly after heating the medium at 37°C and just before use.

The cultures were contained in 150 cm^2 culture flasks (Iwaki). Cultures were prepared using low red blood cell suspensions (0.8 % for first invasions, then up to 1% haematocrit for the last invasion) and up to four times daily replacement of culture medium after last invasion. The volume of the culture medium was usually 140 ml and the parasites were normally sub-cultured at intervals of 2 days.

Fresh CDP (citrate phosphate dextrose) whole blood obtained from the donor was stored at 4°C for at least one day. Afterward, the upper serum layer was removed and erythrocytes were washed to remove any left serum, CDP, and white blood cells. Thereafter, washing medium (RPMI1640 medium, 25 mM HEPES, and 12.5 µg/ml gentamicin sulphate) and Lymphoprep (Axis-Shield) was used to obtain isolated and purified red blood cells, that were stored at 4°C until use. While fresh whole CDP blood was stored up to 18 days after blood extraction at 4°C, the washed erythrocytes never were used after 5 store days.

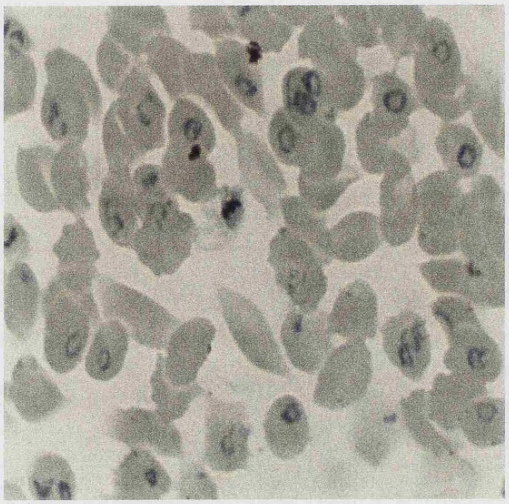
To study stage-specific differential proteins oxidation pattern, cultures were sorbitol synchronized as described previously (Lambros and Vanderberg, 1979). A detailed laboratory protocol is given in Annex I, where all experimental procedures to culture the parasite are shown.

Synchronized cultures of *P. falciparum* with more than 80% parasitized cells were obtained by our method (Fig. 1). It should be emphasized that parasite were

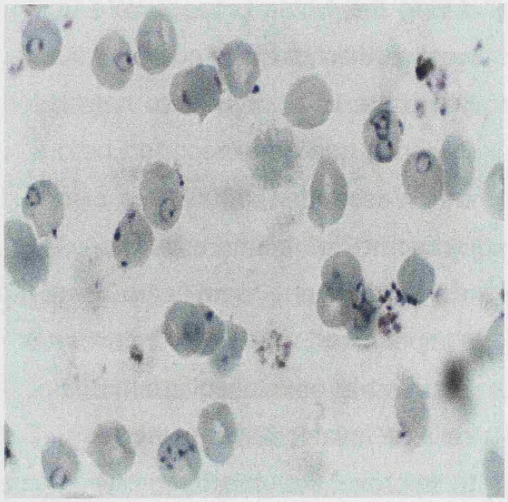
synchronized, where needed in appropriate time, up to two (mostly) to three (rarely) times in two weeks. Actually, the sequential treatment with sorbitol was preferably avoided during the whole process. We do believe that sequential treatment with sorbitol would weaken the red blood cells and subsequently decrease parasitaemia. Moreover, we found that there was an inverse correlation between parasite density and haematocrit. Such evidence has been reported before (Saute *et al.*, 2002; Ljungström *et al.*, 2004; Vernot and Wasserman, 1990). We also had more yields with sub-culturing every 48 hour and just some hours before invasion. This was done by dividing the contents of each flask into two to make one more flask. Then, the haematocrit and medium volume of the two flasks compensated with fresh erythrocytes and medium to keep the haematocrit between 0.8-1% in ~140 ml total culture medium. This helps parasites to get fresh and intact red blood cells for invasion every other day. Taking into account all above-mentioned critical points to improve the *in vitro* growth conditions, we could increase the parasitaemia of *P. falciparum* (Dd2 and 3D7 strains) cultures from 20% to higher than 80% (more than 4-fold yield increase).

In general, the procedure described here allows us to obtain very high level of invasion and therefore high parasitaemia, which is a necessity to study the proteins of *P. falciparum* that can be exploited in the search for vaccines and new drugs.

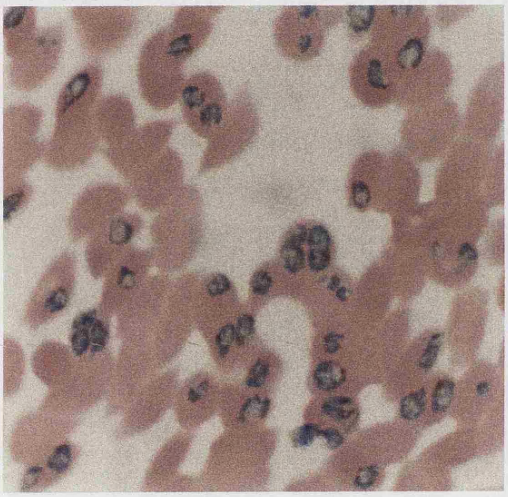
(A)



(B)



(C)



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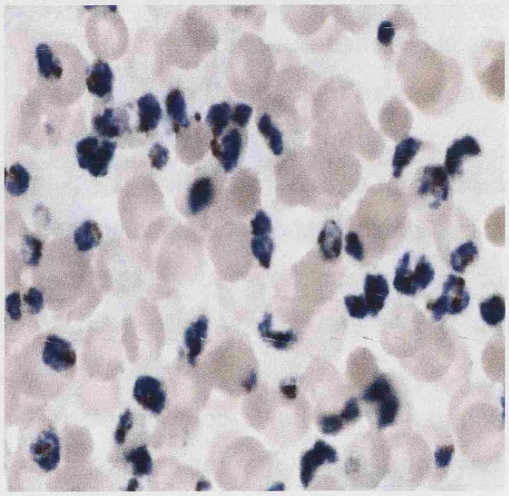

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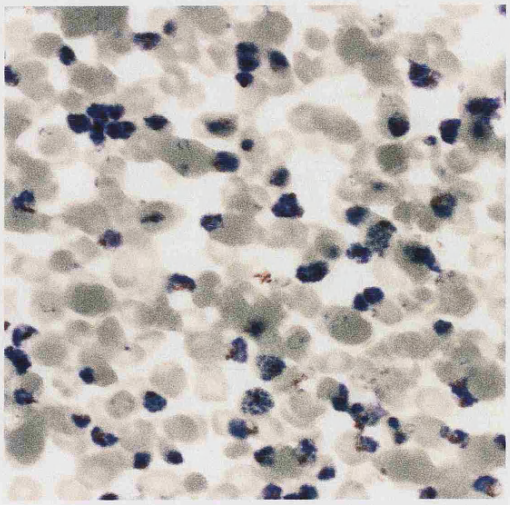

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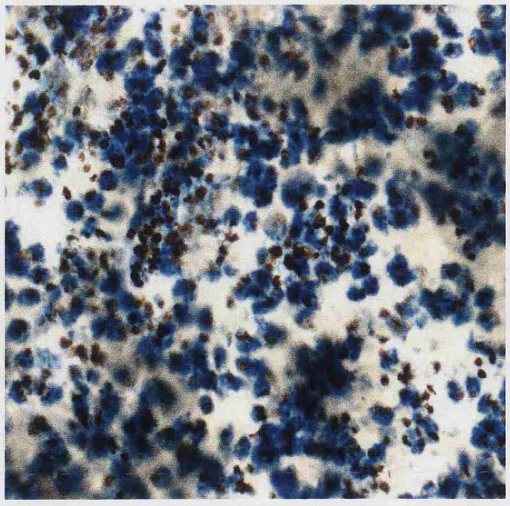

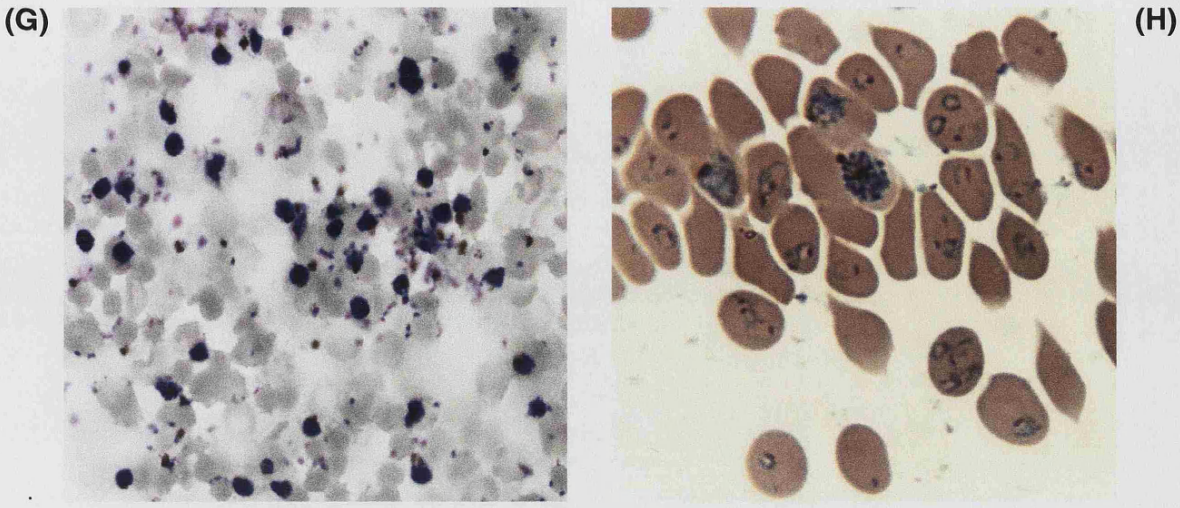

Fig. 1. Intraerythrocytic stages of highly parasitized cultures of *P. falciparum, strain* Dd2. Different stages were obtained from sorbitol synchronized cultures. (A) & (B) The ring stage: a thick rim of cytoplasm with nucleus in a dark blue condensed form can be seen in the ring stage. (C) & (D) The trophozoite stage: the surface area of the parasite has enlarged in this stage. Moreover, pigment vacuole, which contains haemozoin crystals, can be seen as yellow-brown part. (E) & (F) The schizont stage: parasite has grown greatly and covered the most parts of the erythrocyte in this stage. Pigment vacuole has also become compacted into a single, dense, rounded mass. In the more developed ones, budding merozoites from the surface of the schizont can be seen. (F) Specifically shows isolated schizonts of a highly parasitized culture after 5 min centrifugation at 2500 rpm. In such condition, schizonts situate on the top of the infected erythrocytes as a dark brown layer. (G), Schizonts, released merozoites and new rings (H).

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4.4. Chloroquine mediates specific proteome oxidative damage across the erythrocytic cycle of resistant *Plasmodium falciparum*

Chloroquine mediates specific proteome oxidative damage across the erythrocytic cycle of resistant *Plasmodium falciparum*

Contents

1. Abstract

Resistance of *Plasmodium falciparum* to chloroquine hinders malaria control in endemic areas. Current hypotheses on the action mechanism of chloroquine evoke its ultimate interference with the parasite's oxidative defence systems. Through carbonyl derivatization by 2,4-dinitrophenylhydrazine and immunoproteomics, we compared oxidatively-modified proteins across the parasite's intraerythrocytic stages in untreated and transiently IC_{50} chloroquine-treated cultures of the chloroquine-resistant *P. falciparum* strain Dd2. Functional plasmodial protein groups found to be most oxidatively damaged were among those central to the parasite's physiological processes, including protein folding, protein fate, energy metabolism, signal transduction and pathogenesis. While an almost constant number of oxidized proteins were detected across the *P. falciparum* life cycle, chloroquine treatment led to increases both in the extent of protein oxidation and number of proteins oxidized as the intraerythrocytic cycle progressed to mature stages. In contrast, by detecting specific 4-hydroxy-2-nonenal adducts, it was revealed that protein lipoperoxidation is inhibited by chloroquine. Our data provide new insight into the molecular mechanisms triggering the parasite response to chloroquine and the normal protein-oxidation modifications that could be involved in the development and pathophysiology of the parasite and strongly suggest that chloroquine causes specific oxidative stress sharing common features with eukaryotic cells. Targeting these processes might provide ways of combating chloroquine-resistance and developing new antimalarial drugs.

Keywords:

Chloroquine / *Plasmodium falciparum* / oxidized proteome

2. Introduction

Plasmodium falciparum is the causative agent of the most virulent form of human malaria with up to 2.7 million deaths per year attributed to this pathogen (Breman, 2001). Entry of the malaria parasite into its host red blood cell initiates the intraerythrocytic asexual cycle, which is responsible for the clinical manifestations of malaria. It is at this stage, that 75% of the parasite's genes are transcribed (Bozdech et al., 2003). Although oxidative stress has been suggested to play a key role in the pathogenesis of malaria (Becker et al., 2004), the fact that the malaria parasite is also vulnerable to oxidative stress (Muller, 2004) has advocated this metabolism as a promising target for antimalarial

chemotherapy (Becker et al., 2004). Apart from the large amounts of ROS generated by the active metabolism of the growing and multiplying malaria parasite, a main source of oxidative stress is the degradation of host haemoglobin (Becker et al., 2004). Haemoglobin digestion in the food vacuole is the main source of amino acids for parasite development, and the release of free haem (FP IX) upon degradation promotes ROS production (Muller, 2004). Free FP IX is toxic owing both to its detergent-like properties, whereby it disrupts membrane integrity through its incorporation in the lipid bilayer, and to its ferric-state (3+) bound iron, which catalyses and undergoes redox reactions resulting in ROS production (Muller, 2004). In *P. falciparum*, most haem aggregates into the insoluble crystalline FP IX dimer, known as malaria pigment or haemozoin (Egan et al., 2002). Alternative detoxification pathways such as FP IX degradation (Zhang et al., 1999), reaction with glutathione (Ginsburg et al., 1998), and binding to FP IX-binding proteins (Campanale et al., 2003; Harwaldt et al., 2002) also contribute to avoiding intracellular oxidative damage. However, small amounts of FP IX escaping from detoxification processes (Loria et al., 1999) could cause redox damage to host and parasite proteins and membranes (Campanale et al., 2003; Famin and Ginsburg, 2003).

Although CQ has been the standard antimalarial drug for over 50 years, its mechanism of action is not completely understood (Koncarevic et al., 2007). A widely accepted hypothesis is that CQ binding to free FP IX prevents its biocrystalisation to haemozoin, allowing the partial release of FP IX (Koncarevic et al., 2007), which ultimately leads to lethal oxidative stress in the parasite (Becker et al., 2004). Thus, increased intracellular ROS is considered the final consequence of the mechanism of CQ action, but it remains unknown how this toxic environment modifies the essential functions of the parasite. As a by-product of normal metabolism and also as a secondary effect of pharmacological treatment, constantly generated ROS are known to cause oxidative damage to cell components such as proteins, DNA, and lipids. Such damage leads to structural modifications and loss of biological function (Berlett and Stadtman, 1997). Superoxide anions cause the release of iron-sulphur clusters of several enzymes leading to their inactivation (Gardner and Fridovich, 1991). The hydroxyl radical generated by H_2O_2 and transition metals, such as iron, is involved in protein backbone fragmentation or modification of amino acid side chains (Stadtman and Levine, 2003). Oxidatively-modified proteins accumulate as a function of age in human erythrocytes and other cells (Oliver et al., 1987; Stadtman, 1992) and there is increasing evidence that the build-up of these dysfunctional molecules over a lifetime contributes to reducing their half-life (Beckman and Ames, 1998; Berlett and Stadtman, 1997).

The spread over the past decade of CQ-resistant *P. falciparum* strains has been a challenging problem in malaria control, reaching the point that CQ is virtually useless in

many of the world's regions, in which the high prevalence of clinical CQ-resistant isolates determines the need for substitute drugs or combined therapy (Ehrhardt et al., 2007). It should be, nevertheless, emphasised that CQ resistance in the parasite is a trait acquired by increasing the CQ-IC_{50} of a given strain by serial passage with subcurative doses of the drug. As such, this resistance should be considered as the adaptation of the parasite to higher concentrations of the drug through natural selection, since CQ levels above this newly tolerated IC_{50} value are still lethal for the parasite (Rosario, 1976). Accordingly, a *P. falciparum* strain is considered CQ-resistant when its CQ-IC_{50} value increases above the normal 6-17 nM (Quashie et al., 2006), as is the case for the strains Dd2 (IC50=89 nM), and K1 (IC_{50}=143 nM) (Akoachere et al., 2005).

Oxidative damage to proteins can be assessed by determining the carbonyl groups generated in some amino acid side chains under stress conditions (Levine et al., 1994; Shacter et al., 1994). Protein oxidation results in the formation of carbonyl groups (aldehydes and ketones) in susceptible amino acids, and these can be derivatized using DNPH. The derivatized proteins can be separated by two-dimensional electrophoresis (2-DE) and then analysed for their carbonyl contents by immunoassay using anti-DNP antibodies (Levine et al., 1994; Shacter et al., 1994). Moreover, lipid hydroperoxides can also be generated in membranes by lipid peroxidation during oxidative stress (Uchida, 2005). These oxidative modifications yield several breakdown products including HNE. This oxidative stress by-product can react with proteins through a Michael-type addition as an additional source of protein carbonylation (Dalle-Donne et al., 2006), which can also be assessed using specific anti-HNE antibodies.

In the present study, a proteomic approach was used to identify and compare oxidatively-modified proteins in the different intraerythrocytic stages of a CQ-resistant *P. falciparum* strain in the presence or absence of CQ. The results are discussed in terms of the role of such proteins in the life cycle of the malaria parasite and as potential functional targets for combating CQ-resistance.

3. Materials and methods

Highly parasitized cultures and in vitro CQ assay

The CQ-resistant strain of *P. falciparum* Dd2, was used in all experiments. Parasites were grown *in vitro* in group A^+ human erythrocytes under conditions described previously (Trager and Jensen, 1976) with some modification. Briefly, parasites were cultured in 150 cm^2 flasks in RPMI1640 medium (Sigma), supplemented with 25 mM HEPES, 0.5% AlbuMAX I (Invitrogen), 1.77 mM sodium bicarbonate, 100 µM hypoxanthine, and 12.5 µg ml^{-1} gentamicin sulphate, at pH 7.2 in an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen. Synchronised cultures of *P. falciparum*

showing more than 80% parasitized cells were obtained using low haematocrit (0.8-1%) red blood cell suspensions and replacing the culture medium three to four times daily after the last invasion. The volume of the culture medium was usually 140 ml and parasites were normally sub-cultured at intervals of 2 days. To examine stage-specific differential protein oxidation patterns, cultures were sorbitol synchronised as described previously (Lambros and Vanderberg, 1979) to obtain the stages ring (10-16 h), trophozoite (28-34 h) and schizont (39-45 h).

To test the effects of CQ on protein oxidation, the drug was included at IC_{50} (160 nM) in synchronised parasite cultures only 6 h before harvesting.

Two-dimensional gel separation of parasite proteins

Harvested parasites were mixed with 0.05% saponin/PBS (w/v) for 5 min at room temperature to lyse the erythrocyte membrane. The lysate was collected by centrifugation at 25000 g for 10 min at 4°C and washed three times with ice-cold PBS, followed by one wash in 10 mM Tris-HCl, pH 7.4, to reduce contamination with erythrocyte-derived β-haematin, which causes dark staining of the gels. Parasite pellets were resuspended in 3 volumes (w/v) of lysis buffer (PBS, 0.1% Triton X-100, one Roche Complete-Mini™ protease inhibitor cocktail tablet per 10 ml buffer). Parasite cells were lysed by subjecting them to 5 cycles of quick-freezing at -20°C and sub sequent thawing. Supernatants were clarified by centrifugation at 25000 g for 10 min at 4°C and protein concentrations determined. Protein carbonyl groups were derivatized with DNPH as previously described (Levine *et al.*, 1994).

Proteins from cell extracts were precipitated with ammonium acetate in methanol following phenol extraction (Peck *et al.*, 2001) and resuspended in rehydration buffer [7M urea, 2M thiourea, 2% (w/v) CHAPS, 10mM dithiothreitol (DTT), 1% IPG buffer pH 3-11 (GE Healthcare), and traces of bromophenol blue]. Next, about 800 µg total protein was loaded onto IPG strips (pH 3-11, 18 cm, GE Healthcare). The IPG strips were rehydrated overnight according to the manufacturer's instructions. For the first-dimensional separation, isoelectrofocusing (IEF) was performed using the Ettan IPGphor II IEF system (GE Healthcare) at 20°C. The voltage was gradually ramped in a step-and-hold manner to 1000 V in three increasing steps: 1 h 200 V, 1 h 500 V and 1 h 1000 V. The voltage was increased to 4500 V in a linear gradient over the next 9 h. The run was terminated after ~75000 Vh. The focused strips were equilibrated in 10 ml equilibration solution (100 mM Tris- HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS) with 0.5% DTT reducing agent for 12 min, followed by 10 ml of 4.5% iodoacetamide equilibration solution for 5 min. To run the second dimension, strips were soaked in running buffer, loaded on 10% SDS-PAGE gels and covered with 0.5% agarose gel. The running conditions were 30 min at 2W/gel and

subsequently 4 h at 20W/gel, using the Laemmli buffer system. The gels were stained with colloidal CBB (Coomassie Brilliant Blue) (Neuhoff *et al.*, 1985) to visualize total proteins and used for protein identification. Twin gels were run in parallel under identical 2D electrophoresis conditions.

Western blot analysis

One of the twin gels loaded with ¼ total protein (200 µg) was transferred to a PVDF membrane (Amersham Biosciences). Immunodetection of protein-bound DNPH in crude extracts of *P. falciparum* was conducted as previously described (Shacter *et al.*, 1994) with minor modifications as follows: the anti-2,4-DNP antibody (Sigma Ref. D9656) was incubated for 2 h at room temperature at a 1/4000 dilution, followed by HRP-conjugated anti-rabbit IgG (Amersham Biosciences Ref. NA9340V) at a 1/5000 dilution. Detection was performed using the SuperSignal chemiluminescent substrate (Pierce) and exposure to X-ray film. Each experiment (different condition or parasite stage) was repeated tree times.

Tryptic digestion and MALDI-MS

Based on Western blot analysis, the gel spots of interest (stage-specific, predominant or those differently expressed) were manually excised from 2-D gels using biopsy punches. Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin as described (Shevchenko *et al.*, 1996). Briefly, spots were washed twice with water, shrunk 15 min with 100% acetonitrile and dried in a Savant SpeedVac for 30 min. The samples were then reduced with 10mM DTT in 25 mM ammonium bicarbonate for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in 25mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12.5 ng/µl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C. After digestion, the supernatant was collected and 1 µl was spotted onto a MALDI (matrix-assisted laser desorption/ionization) target plate and allowed to air-dry at room temperature. Next, 0.4 µl of a 3mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide spots and allowed, again, to air-dry at room temperature.

MALDI-TOF (time-of-flight) MS (mass spectrometry) analyses were performed in a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) operated in positive reflector mode, with an accelerating voltage of 20000 V. Mass spectra were then collected for Peptide Mass Fingerprinting (PMF). Additionally, proteins ambiguously identified by PMF were subjected to MS/MS (tandem mass spectrometry)

sequencing. Thus, from the MS spectra, suitable precursors were selected for MS/MS analyses with collision-induced dissociation (atmospheric gas was used) in the 1 Kv ion reflector mode and precursor mass windows +/- 10 Da. The plate model and default calibration were optimised for MS/MS spectra processing. The parameters used to analyze the data were: Signal to Noise = 20, resolution >6000.

Protein identification was performed by subjecting trypsin digested peptide masses to three databases (NCBI, Swissprot and EMBL-EBI) separately using the MASCOT 1.9 (http://www.matrixscience.com) through the Global Protein Server v3.5 from Applied Biosystems. The parameters for the combined search were as follows: modification on cysteine residues by carboxyamidomethylation was set as obligate, methionine oxidation was considered as a variable modification; the maximum number of missed tryptic cleavages was one; peptide mass tolerance was set to 100 ppm (parts per million) and MS/MS tolerance to 0.3 Da; and monoisotopic masses were considered. All the identified proteins fulfilled the criteria of being significant ($p<0.05$) according to probability based on Mowse score.

Analysis of the immunoblot images

Immunoblot images, used to compare the carbonyl contents and quantify spots for the different intraerythrocytic stages of non-treated and CQ-treated parasites, were analysed using PDQuest software, version 7.3 (Bio-Rad).

Lipoperoxide-induced protein oxidation analysis

HNE adducts derived from lipid peroxidation were detected by Western blot analysis. 5 µg of protein samples were separated by SDS-PAGE on 10% polyacrylamide gel, and transferred (50 mA, overnight at 4°C) to a PVDF membrane (Amersham Biosciences). After blocking with 10% nonfat dry milk overnight at 4°C, the membrane was incubated overnight with anti-HNE antibody (Calbiochem, Cat. No. 393207) at a 1/1000 dilution, followed by incubation for 1 h at room temperature with peroxidase-linked anti-rabbit IgG antibody (Dakocytomation Ref. P0448) at a 1/1000 dilution. Chemiluminescence signals were developed using the SuperSignal chemiluminescent substrate (Pierce) and exposure to X-ray film.

4. Results

Oxidized proteins in the non-treated and CQ-treated parasites

Protein carbonyl formation is one of the most widely used indicators of oxidative stress (Butterfield, 2004; Cabiscol *et al.*, 2000; Castegna *et al.*, 2002; Poon *et al.*, 2004; Reverter-Branchat *et al.*, 2004). To determine stage specific protein oxidation and the direct effects of CQ, *P. falciparum* Dd2 cultures were synchronized to reach the three main parasite stages of intraerythrocytic development, ring, trophozoite and schizont, in the presence and absence of CQ IC_{50}. Crude extracts from each stage were then derivatized with DNPH and proteins subjected to 2-DE (Fig. 1) followed by Western blotting with anti-DNP (Fig. 2).

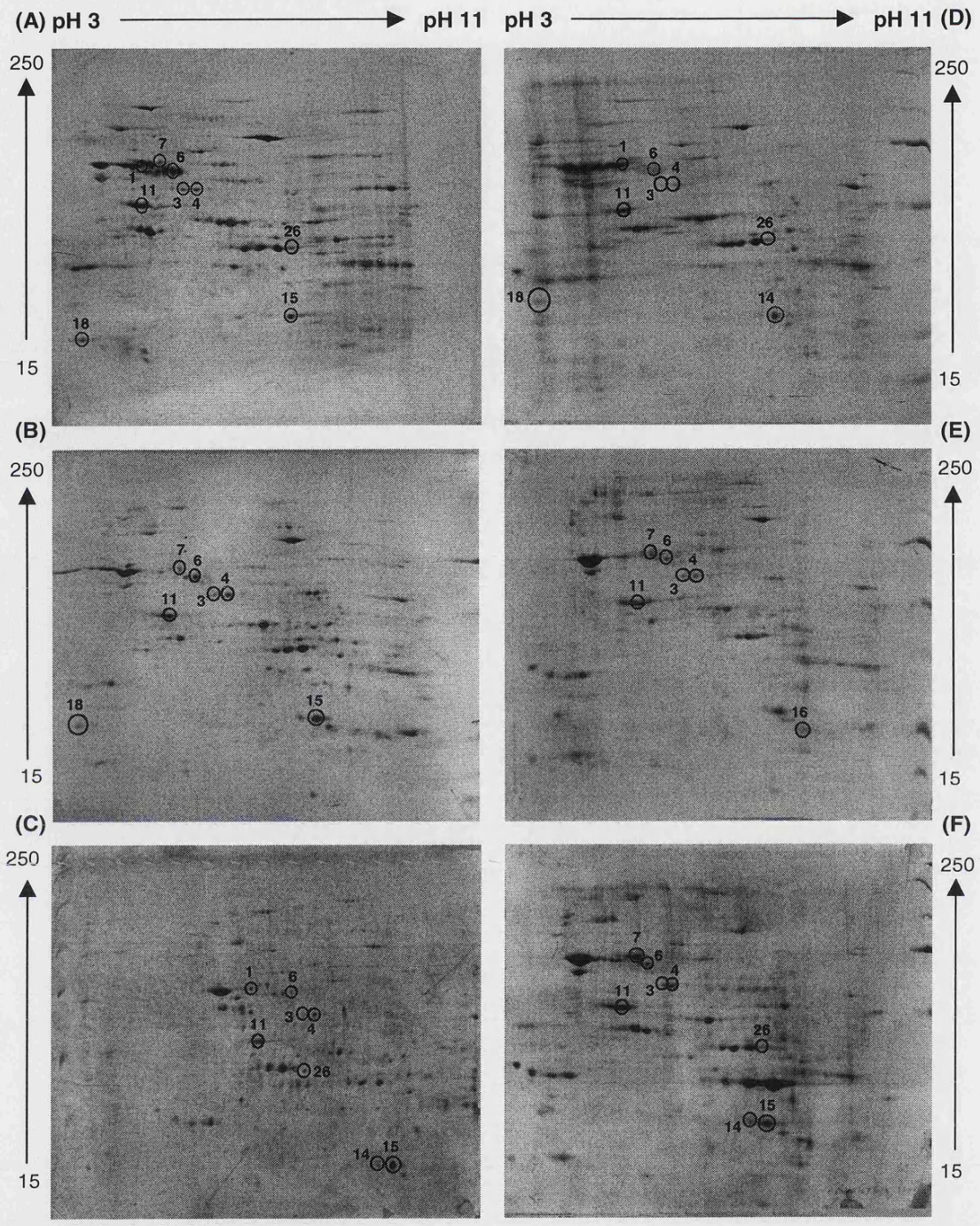

Fig. 1. Colloidal CBB staining of 2-D membranes obtained from intraerythrocytic stages of P. falciparum Dd2 in control untreated and CQ-treated parasites. (A), (B), and (C) ring, trophozoite and schizont stages respectively. (D), (E), and (F) CQ-treated cultures of rings, trophozoites and schizonts respectively. Numbered spots in all plates (3, 4, 6, and 11) are representative of similar protein amounts with a corresponding oxidation signal depending on the stage and/or treatment. Numbered spots not labelled in all plates (1, 7, 14, 15, 18, and 26) are representative spots whose oxidation signal correlates with their presence in a given plate.

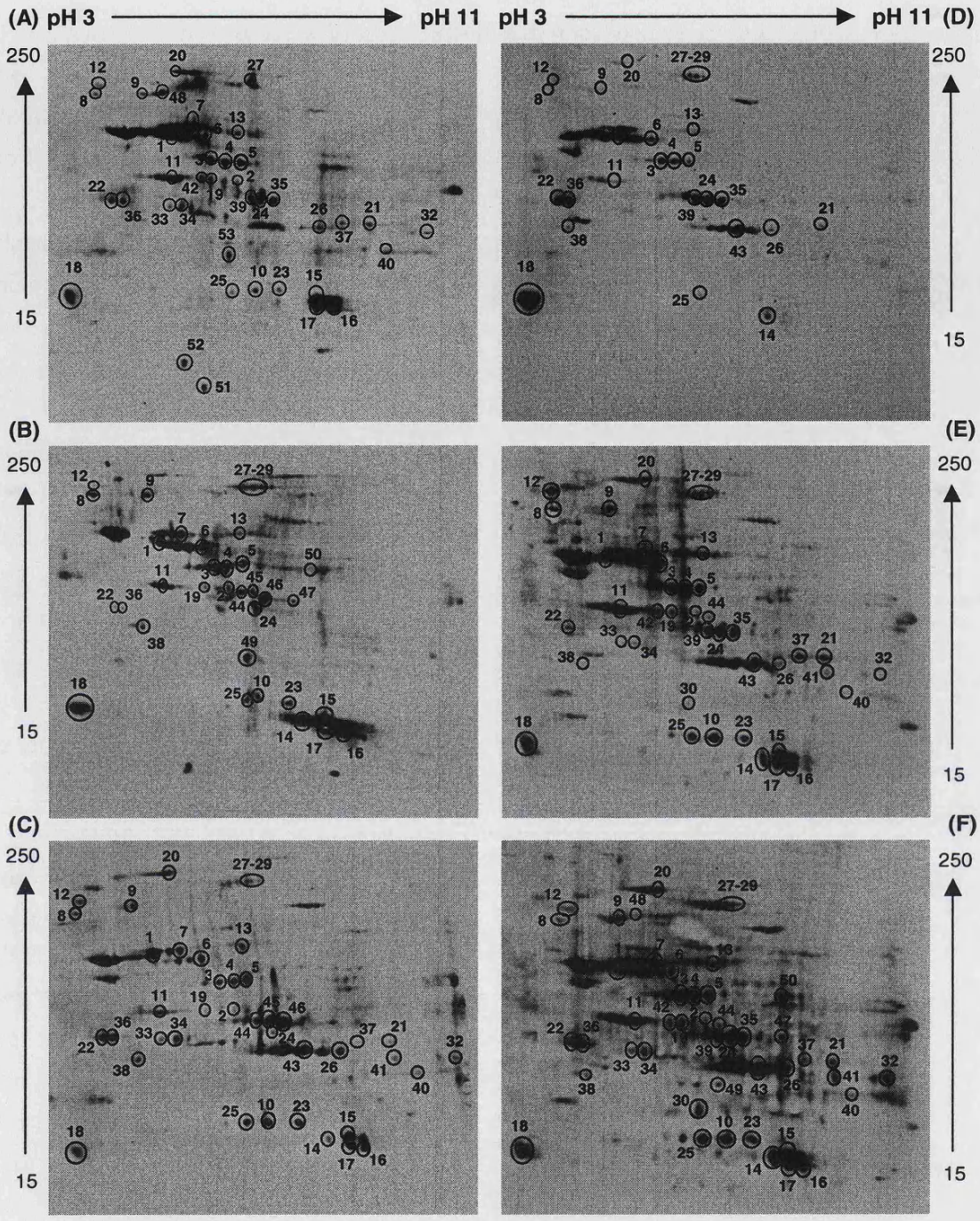

Fig. 2. Redox proteome patterns across the intraerythrocytic stages of *P. falciparum* strain Dd2 in control untreated and CQ-treated parasites. (A), (B), and (C) Comparative carbonyl immunoblots for the stages ring, trophozoite and schizont respectively. (D), (E), and (F) Comparative carbonyl immunoblots for cultures of rings, trophozoites and schizonts, respectively, exposed to CQ for 6 h. Numbered spots show the oxidized proteins identified, which are listed in Tables 2-5. The exposure time of the membrane to the film was 5 min for all blots.

In total, 107 matching spots of oxidized signals in colloidal CBB-stained 2-DE gels were subjected to identification by matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and database searching. Figure 2 shows reference carbonyl immunoblots corresponding to all the non-treated and CQ-treated stages in which a total of 79 spots were identified and assigned to 41 proteins. These results are summarised in Tables 1-6.

By comparing spots containing carbonyl groups and their signal intensities, we were able to observe that the non-treated cultures showed a relative constant number of spots across the stages (range 121-141) while for the CQ-treated cultures, the number of carbonylated spots increased as the parasite cycle progressed (from 80 at the ring to 260 at the schizont stage). This effect was also observed when we compared total spot intensities. Thus, while in the absence of CQ, spot intensities diminished from the ring and trophozoite stages to the schizont stage, in the presence of CQ a reverse effect was noted whereby intensities were 6-fold higher in the schizonts than rings (Table 1). In addition, a large proportion of the carbonylated spots for the control and CQ-treated parasites could be matched across the cycle. Thus, more than 50% of the spots detected for a given stage or treatment were also seen in extracts corresponding to the other stage and/or treatment. An exception was observed for the CQ-treated parasite rings, such that the drug seemed to largely inhibit protein carbonylation rendering the lowest number of spots (80 vs 122 for controls) and total spot intensities ($3x10^3$ vs. $10x10^3$ for controls).

The patterns shown in Figure 2 (D, E & F) also indicate that CQ led to increased protein oxidation in mature stages. Effectively, the number of carbonylated spots for the CQ-treated schizonts (Figure 2F) almost doubled the spots recorded for control schizonts (Fig 2C), with a relatively stronger signal observed in spots appearing in both these groups (see Table 5 for details of the fold increases). Thus, when we compared each non-treated stage with the corresponding CQ-treated stage (Table 1), the mean oxidation intensity was 3.3-fold higher in the presence of CQ for the schizonts, 1.2-fold higher for the trophozoites and 3.4-fold lower for the rings. Individual protein carbonylation analysis (Table 5) revealed a significant proportion of spots (around 25%) only detected for a given stage or treatment.

Table 1. Oxidized spots and oxidation signal intensities for control (C) and CQ-treated intraerythrocytic stages of *P. falciparum* Dd2. PDQuest v.7.3. was used to match spots among the different stages and treatments.

Stage / Treatment		Total No. of spots	Overall spot intensity (Arbitrary Units, AU)	Matched spots					
				Ring		Trophozoite		Schizont	
				C	CQ	C	CQ	C	CQ
Ring	C	122	10480	122					
	CQ	80	3099	57	80				
Trophozoite	C	132	11781	67	50	132			
	CQ	185	13578	94	63	81	185		
Schizont	C	141	6249	94	61	74	115	141	
	CQ	260	20756	107	65	83	147	73	260

The proteomic changes produced by CQ were based on 6 h of transient exposure to IC_{50} to avoid massive parasite damage. The observed effects of sublethal exposure could thus reflect both oxidative damage produced as a consequence of CQ-inhibition of FP IX aggregation and additional protein oxidation damage caused by the specific parasite response to the oxidative stress.

Host cell oxidized proteins

Seven host cell proteins were identified as oxidized (Table 2). Of these, two proteins were members of the α-carbonic anhydrase family (spots 14-17), which were found to be highly oxidized in almost all the non-treated and CQ-treated stages, particularly in the trophozoites and CQ-treated schizonts. Further human proteins detected were: beta-actin (spot 38, more oxidized in treated schizonts), annexin A7 (spot 39, significantly oxidized in both treated rings and schizonts), catalase (spot 50, only in non-treated trophozoites and CQ-treated schizonts), thioredoxin peroxidase B and peroxiredoxin 2 isoform b (spots 52 and 53, respectively, only in non-treated rings).

Table 2. Redox-proteome changes induced by chloroquine across the intraerythrocytic stages of *P. falciparum* Dd2. Ring, R; Trophozoite, T; Schizont, S; CQ, Chloroquine. Accession Nos. correspond to PlasmoDB or NCBI databases. An asterisk indicates that the sequence from NCBI does not appear in PlasmoDB. Two asterisks indicates that the PlasmoDB sequence varies scarcely with respect to the NCBI sequence. The protein with three asterisks was identified by manual sequencing based on mass spectra and database searches. Proteins not detected in a given stage are indicated n/d.

			Spot signal intensity (AU)					
Spot	Protein Description	Accession No.	R	T	S	R+CQ	T+CQ	S+CQ
1	HSP	PFI0875w	317	56	44	72	305	366
2	HSP	PFI0875w	23	88	12	n/d	12	34
3	HSP60	PF10_0153	97	289	50	72	144	215
4	HSP60	PF10_0153	115	236	82	49	210	197
5	HSP60	PF10_0153	84	149	125	11	88	200
6	HSP hsp70 homologue	PF11_0351	57	48	84	30	50	90
7	HSP70	PF08_0054	122	144	88	n/d	310	406
8	HSP 86	PF07_0029	21	88	22	3	147	40
9	HSP, putative	PF14_0417	34	55	26	2	92	101
10	Co-chaperone GrpE, putative	PF11_0258	38	56	97	n/d	165	219
11	Disulphide isomerase precursor, putative	MAL8P1.17	63	56	35	8	163	176
12	Cell division cycle protein 48 homologue, putative	PFF0940c	23	6	39	5	138	56
13	Peptidase, putative	PF14_0517	45	34	52	7	46	366
14	Carbonic anhydrase I [Homo sapiens]	gi|115449	n/d	361	27	28	181	376
15	Carbonic anhydrase I [Homo sapiens]	gi|115449	301	555	55	28	219	322
16	Carbonic anhydrase II [Homo sapiens]	gi|4557395	225	445	49	n/d	55	246
17	Carbonic anhydrase II [Homo sapiens]	gi|4557395	140	404	49	n/d	247	217
18	Hypothetical protein	PFF0190c	364	353	77	664	364	416
19	Erythrocyte membrane protein 1	gi|21886680 *	57	26	9	n/d	94	109
20	Erythrocyte membrane protein 1	gi|19071542 *	65	n/d	51	2	57	153
21	Phosphoglycerate kinase	PFI1105w	35	n/d	8	5	82	69
22	Hypothetical protein	PFA0210c **	80	4	n/d	70	33	116
23	Hypothetical protein PF14_0046	PF14_0046	21	82	64	n/d	64	93
24	Hypothetical protein	PFL1930w	108	118	13	58	122	301
25	GTP-binding nuclear protein ran/tc4	PF11_0183	11	71	75	4	125	222
26	26S Proteasome aaa-ATPase subunit Rpt3, putative	PFD0665c	23	n/d	92	12	21	206
27	M1-family aminopeptidase	MAL13P1.56	72	55	26	18	39	39
28	M1-family aminopeptidase	MAL13P1.56	n/d	73	15	20	47	16
29	M1-family aminopeptidase	MAL13P1.56	n/d	34	5	6	48	11
30	M1-family aminopeptidase	MAL13P1.56	n/d	n/d	n/d	n/d	8	103
31	Proteasome beta-subunit	MAL8P1.142	n/d	361	27	n/d	181	376
32	Fructose-bisphosphate aldolase	PF14_0425 **	14	n/d	26	n/d	3	113
33	Snmp protein, putative	PFE0925c	24	n/d	15	n/d	6	43
34	Snmp protein, putative	PFE0925c	99	n/d	68	n/d	17	125
35	Formin 2, putative	PFL0925w	81	n/d	n/d	42	136	162
36	Hypothetical protein PFB0145c	PFB0145c	67	3	n/d	83	n/d	76
37	Hypothetical protein	MAL13P1.237	26	n/d	3	n/d	74	62
38	Actin, beta [Homo sapiens]	gi|14250401	n/d	2	n/d	10	4	21
39	Annexin A7 [Homo sapiens]	gi|55584155	69	n/d	n/d	99	82	173
40	Glyceraldehyde-3-phosphate dehydrogenase	PF14_0598	34	n/d	12	n/d	4	12
41	Cyclophilin, putative	PFL0120c	n/d	n/d	15	n/d	10	115
42	Hypothetical protein PFA0725w	PFA0725w	95	n/d	n/d	n/d	42	57
43	Elongation factor Tu, putative	MAL13P1.164 ***	n/d	n/d	124	108	167	175
44	Enolase	PF10_0155	n/d	85	80	n/d	11	33
45	Enolase	PF10_0155	n/d	59	148	n/d	n/d	n/d
46	Enolase	PF10_0155	n/d	130	171	n/d	n/d	n/d
47	Enolase	PF10_0155	n/d	21	n/d	n/d	n/d	73
48	Endoplasmin homologue precursor, putative	PFL1070c	73	n/d	n/d	n/d	n/d	15
49	40S ribosomal protein, putative	PF10_0264	n/d	118	n/d	n/d	n/d	32
50	Catalase [Homo sapiens]	gi|179950	n/d	10	n/d	n/d	n/d	42
51	Hypothetical protein	PFE1595c	61	n/d	n/d	n/d	n/d	n/d
52	Thioredoxin peroxidase B [Homo sapiens]	gi|9955016	56	n/d	n/d	n/d	n/d	n/d
53	Peroxiredoxin 2 isoform b [Homo sapiens]	gi|33188452	72	n/d	n/d	n/d	n/d	n/d

Table 3. Redox-proteome changes in the intraerythrocytic stages of the control *P. falciparum* Dd2 cultures. The schizont stage showed the highest number of spots and was thus used as reference to calculate fold changes in oxidation intensity (values from Table 2) given as the ratios ring/schizont (R/S) and trophozoite/schizont (T/S). Accession Nos. are from the PlasmoDB or NCBI databases.

Spot	Protein Description	Accession No.	R/S	T/S
1	Heat shock protein	PFI0875w	7.2	1.3
2	Heat shock protein	PFI0875w	2.0	7.6
3	HSP60	PF10_0153	1.9	5.7
4	HSP60	PF10_0153	1.4	2.9
5	HSP60	PF10_0153	0.7	1.2
6	HSP hsp70 homologue	PF11_0351	0.7	0.6
7	HSP70	PF08_0054	1.4	1.6
8	Heat shock protein 86	PF07_0029	1.0	4.1
9	Heat shock protein. putative	PF14_0417	1.3	2.1
10	Co-chaperone GrpE. putative	PF11_0258	0.4	0.6
11	Disulfide isomerase precursor. putative	MAL8P1.17	1.8	1.6
12	Cell division cycle protein 48 homologue. putative	PFF0940c	0.6	0.1
13	Peptidase. putative	PF14_0517	0.9	0.7
14	Carbonic Anhydrase I [Homo sapiens]	gi|115449	Only in S	13.4
15	Carbonic Anhydrase I [Homo sapiens]	gi|115449	5.5	10.1
16	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	4.6	9.1
17	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	2.8	8.2
18	Hypothetical protein	PFF0190c	4.7	4.6
19	Erythrocyte membrane protein 1	gi|21886680	6.6	3.0
20	Erythrocyte membrane protein 1	gi|19071542	1.3	Only in S
21	Phosphoglycerate kinase	PFI1105w	4.4	Only in S
22	Hypothetical protein	PFA0210c	Only in R	Only in T
23	Hypothetical protein PF14_0046	PF14_0046	0.3	1.3
24	Hypothetical protein	PFL1930w	8.0	8.8
25	GTP-binding nuclear protein ran/tc4	PF11_0183	0.1	0.9
26	26s Proteasome aaa-ATPase subunit Rpt3. putative	PFD0665c	0.2	Only in S
27	M1-family aminopeptidase	MAL13P1.56	2.7	2.1
28	M1-family aminopeptidase	MAL13P1.56	Only in S	5.0
29	M1-family aminopeptidase	MAL13P1.56	Only in S	6.7
30	M1-family aminopeptidase	MAL13P1.56	no	no
31	Proteasome beta-subunit	MAL8P1.142	Only in S	13.4
32	Fructose-bisphosphate aldolase	PF14_0425	0.5	Only in S
33	Snrnp protein. putative	PFE0925c	1.6	Only in S
34	Snrnp protein. putative	PFE0925c	1.4	Only in S
35	Formin 2. putative	PFL0925w	Only in R	no
36	Hypothetical protein PFB0145c	PFB0145c	Only in R	Only in T
37	Hypothetical protein	MAL13P1.237	8.8	Only in S
38	Actin. beta [Homo sapiens]	gi|14250401	no	Only in T
39	Annexin A7 [Homo sapiens]	gi|55584155	Only in R	no
40	Glyceraldehyde-3-phosphate dehydrogenase	PF14_0598	2.8	Only in S
41	Cyclophilin.putative	PFL0120c	Only in S	Only in S
42	Hypothetical protein PFA0725w	PFA0725w	Only in R	no
43	Elongation factor Tu, putative	MAL13P1.164	Only in S	Only in S
44	Enolase	PF10_0155	Only in S	1.1
45	Enolase	PF10_0155	Only in S	0.4
46	Enolase	PF10_0155	Only in S	0.8
47	Enolase	PF10_0155	no	Only in T
48	Endoplasmin homolog precursor. putative	PFL1070c	Only in R	no
49	40S ribosomal protein. putative	PF10_0264	no	Only in T
50	Catalase [Homo sapiens]	gi|179950	no	Only in T
51	Hypothetical protein	PFE1595c	Only in R	no
52	Thioredoxin Peroxidase B [Homo sapiens]	gi|9955016	Only in R	no
53	Peroxiredoxin 2 isoform b [Homo sapiens]	gi|33188452	Only in R	no

Table 4. Redox-proteome changes in the intraerythrocytic stages of the CQ-treated *P. falciparum* Dd2 cultures. The schizont stage showed the highest number of spots and was thus used as reference to calculate fold changes in oxidation intensity (values from Table 2) given as the ratios ring/schizont (RCQ/SCQ) and trophozoite/schizont (TCQ/SCQ). Accession Nos. are from the PlasmoDB or NCBI databases.

Spot	Protein Description	Accession No.	RCQ/SCQ	TCQ/SCQ
1	Heat shock protein	PFI0875w	0.2	0.8
2	Heat shock protein	PFI0875w	Only in SCQ	0.3
3	HSP60	PF10_0153	0.3	0.7
4	HSP60	PF10_0153	0.2	1.1
5	HSP60	PF10_0153	0.1	0.4
6	HSP hsp70 homologue	PF11_0351	0.3	0.6
7	HSP70	PF08_0054	Only in SCQ	0.8
8	Heat shock protein 86	PF07_0029	0.1	3.7
9	Heat shock protein. putative	PF14_0417	0.0	0.9
10	Co-chaperone GrpE. putative	PF11_0258	Only in SCQ	0.8
11	Disulfide isomerase precursor. putative	MAL8P1.17	0.0	0.9
12	Cell division cycle protein 48 homologue. putative	PFF0940c	0.1	2.4
13	Peptidase. putative	PF14_0517	0.0	0.1
14	Carbonic Anhydrase I [Homo sapiens]	gi|115449	0.1	0.5
15	Carbonic Anhydrase I [Homo sapiens]	gi|115449	0.1	0.7
16	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	Only in SCQ	0.2
17	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	Only in SCQ	1.1
18	Hypothetical protein	PFF0190c	1.6	0.9
19	Erythrocyte membrane protein 1	gi|21886680	Only in SCQ	0.9
20	Erythrocyte membrane protein 1	gi|19071542	0.0	0.4
21	Phosphoglycerate kinase	PFI1105w	0.1	1.2
22	Hypothetical protein	PFA0210c	0.6	0.3
23	Hypothetical protein PF14_0046	PF14_0046	Only in SCQ	0.7
24	Hypothetical protein	PFL1930w	0.2	0.4
25	GTP-binding nuclear protein ran/tc4	PF11_0183	0.0	0.6
26	26s Proteasome aaa-ATPase subunit Rpt3. putative	PFD0665c	0.1	0.1
27	M1-family aminopeptidase	MAL13P1.56	0.4	1.0
28	M1-family aminopeptidase	MAL13P1.56	1.2	2.9
29	M1-family aminopeptidase	MAL13P1.56	0.5	4.3
30	M1-family aminopeptidase	MAL13P1.56	Only in SCQ	0.1
31	Proteasome beta-subunit	MAL8P1.142	Only in SCQ	0.5
32	Fructose-bisphosphate aldolase	PF14_0425	Only in SCQ	0.0
33	Snrnp protein. putative	PFE0925c	Only in SCQ	0.1
34	Snrnp protein. putative	PFE0925c	Only in SCQ	0.1
35	Formin 2. putative	PFL0925w	0.3	0.8
36	Hypothetical protein PFB0145c	PFB0145c	1.1	Only in SCQ
37	Hypothetical protein	MAL13P1.237	Only in SCQ	1.2
38	Actin. beta [Homo sapiens]	gi|14250401	0.5	0.2
39	Annexin A7 [Homo sapiens]	gi|55584155	0.6	0.5
40	Glyceraldehyde-3-phosphate dehydrogenase	PF14_0598	Only in SCQ	0.4
41	Cyclophilin.putative	PFL0120c	Only in SCQ	0.1
42	Hypothetical protein PFA0725w	PFA0725w	Only in SCQ	0.7
43	Elongation factor Tu, putative	MAL13P1.164	0.6	1
44	Enolase	PF10_0155	Only in SCQ	0.3
45	Enolase	PF10_0155	no	no
46	Enolase	PF10_0155	no	no
47	Enolase	PF10_0155	Only in SCQ	Only in SCQ
48	Endoplasmin homolog precursor. putative	PFL1070c	Only in SCQ	Only in SCQ
49	40S ribosomal protein. putative	PF10_0264	Only in SCQ	Only in SCQ
50	Catalase [Homo sapiens]	gi|179950	Only in SCQ	Only in SCQ
51	Hypothetical protein	PFE1595c	no	no
52	Thioredoxin Peroxidase B [Homo sapiens]	gi|9955016	no	no
53	Peroxiredoxin 2 isoform b [Homo sapiens]	gi|33188452	no	no

Table 5. Redox-proteome changes induced by CQ at each intraerythrocytic stage in *P. falciparum* Dd2. The fold change in oxidation intensity (values from Table 2) appears as the ratio of the value obtained for the CQ-treated stage to that for its corresponding control stage. Accession Nos. are from PlasmoDB or NCBI databases.

Spot	Protein Description	Accession No.	RCQ/R	TCQ/T	SCQ/S
1	Heat shock protein	PFI0875w	0.2	5.4	8.3
2	Heat shock protein	PFI0875w	Only in R	0.1	2.9
3	HSP60	PF10_0153	0.7	0.5	4.3
4	HSP60	PF10_0153	0.4	0.9	2.4
5	HSP60	PF10_0153	0.1	0.6	1.6
6	HSP hsp70 homologue	PF11_0351	0.5	1.0	1.1
7	HSP70	PF08_0054	Only in R	2.2	4.6
8	Heat shock protein 86	PF07_0029	0.1	1.7	1.8
9	Heat shock protein. putative	PF14_0417	0.1	1.7	3.8
10	Co-chaperone GrpE. putative	PF11_0258	Only in R	2.9	2.3
11	Disulfide isomerase precursor. putative	MAL8P1.17	0.1	2.9	5.0
12	Cell division cycle protein 48 homologue. putative	PFF0940c	0.2	23.5	1.4
13	Peptidase. putative	PF14_0517	0.2	1.4	7.1
14	Carbonic Anhydrase I [Homo sapiens]	gi|115449	Only in RCQ	0.5	13.9
15	Carbonic Anhydrase I [Homo sapiens]	gi|115449	0.1	0.4	5.8
16	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	Only in R	0.1	5.0
17	Carbonic Anhydrase II [Homo sapiens]	gi|4557395	Only in R	0.6	4.4
18	Hypothetical protein	PFF0190c	1.8	1.0	5.4
19	Erythrocyte membrane protein 1	gi|21886680	Only in R	3.6	12.4
20	Erythrocyte membrane protein 1	gi|19071542	0.0	Only in TCQ	3.0
21	Phosphoglycerate kinase	PFI1105w	0.2	Only in TCQ	8.7
22	Hypothetical protein	PFA0210c	0.9	8.7	Only in SCQ
23	Hypothetical protein PF14_0046	PF14_0046	Only in R	0.8	1.4
24	Hypothetical protein	PFL1930w	0.5	1.0	22.3
25	GTP-binding nuclear protein ran/tc4	PF11_0183	0.4	1.8	3.0
26	26s Proteasome aaa-ATPase subunit Rpt3. putative	PFD0665c	0.5	Only in TCQ	2.2
27	M1-family aminopeptidase	MAL13P1.56	0.2	0.7	1.5
28	M1-family aminopeptidase	MAL13P1.56	Only in RCQ	0.6	1.1
29	M1-family aminopeptidase	MAL13P1.56	Only in RCQ	1.4	2.2
30	M1-family aminopeptidase	MAL13P1.56	no	Only in TCQ	Only in SCQ
31	Proteasome beta-subunit	MAL8P1.142	no	0.5	13.9
32	Fructose-bisphosphate aldolase	PF14_0425	Only in R	Only in TCQ	4.3
33	Snmp protein. putative	PFE0925c	Only in R	Only in TCQ	2.9
34	Snmp protein. putative	PFE0925c	Only in R	Only in TCQ	1.8
35	Formin 2. putative	PFL0925w	0.5	Only in TCQ	Only in SCQ
36	Hypothetical protein PFB0145c	PFB0145c	1.2	Only in T	Only in SCQ
37	Hypothetical protein	MAL13P1.237	Only in R	Only in TCQ	21.1
38	Actin. beta [Homo sapiens]	gi|14250401	Only in RCQ	2.4	Only in SCQ
39	Annexin A7 [Homo sapiens]	gi|55584155	1.4	Only in TCQ	Only in SCQ
40	Glyceraldehyde-3-phosphate dehydrogenase	PF14_0598	Only in R	Only in TCQ	1.0
41	Cyclophilin.putative	PFL0120c	no	Only in TCQ	7.8
42	Hypothetical protein PFA0725w	PFA0725w	Only in R	Only in TCQ	Only in SCQ
43	Elongation factor Tu, putative	MAL13P1.164	Only in RCQ	Only in TCQ	1.4
44	Enolase	PF10_0155	no	0.1	0.4
45	Enolase	PF10_0155	no	Only in T	Only in S
46	Enolase	PF10_0155	no	Only in T	Only in S
47	Enolase	PF10_0155	no	Only in T	Only in SCQ
48	Endoplasmin homolog precursor. putative	PFL1070c	Only in R	no	Only in SCQ
49	40S ribosomal protein. putative	PF10_0264	no	Only in T	Only in SCQ
50	Catalase [Homo sapiens]	gi|179950	no	Only in T	Only in SCQ
51	Hypothetical protein	PFE1595c	Only in R	no	no
52	Thioredoxin Peroxidase B [Homo sapiens]	gi|9955016	Only in R	no	no
53	Peroxiredoxin 2 isoform b [Homo sapiens]	gi|33188452	Only in R	no	no

Parasite oxidized proteins

Oxidized proteins in non-treated and CQ-treated cultures were sorted into main functional classes (Fig. 3) according to the Munich Information Centre for Protein Sequences (MIPS) catalogue (Ruepp *et al.*, 2004) and the proteomic categories defined previously for *P. falciparum* (Florens *et al.*, 2002). The carbonylated proteins identified (Fig. 3 and Table 6) were mostly assigned to the category protein fate (15), followed by a second large group of hypothetical proteins (8) and a third group of metabolic proteins (4).

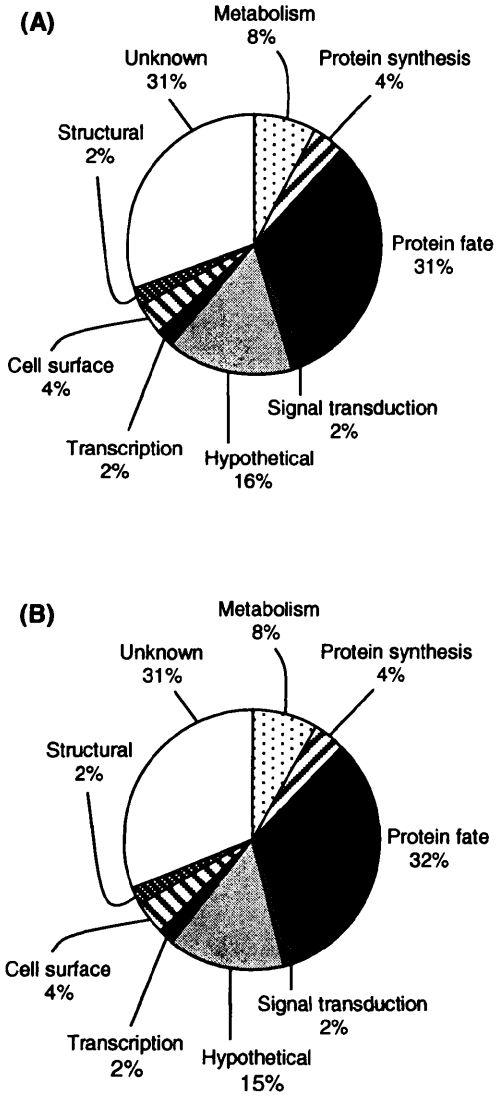

Fig. 3. Functional profiles of the parasite carbonylated proteins identified. Proteins identified in control untreated and CQ-treated parasites are classified according to their functional definition in the MIPS catalogue (Ruepp *et al.*, 2004). To avoid redundancy, only one class was assigned per protein. (A): Functional categories for control parasites. (B): Functional categories for CQ-treated parasites.

In the protein fate class, chaperones were the most frequently appearing oxidized proteins (Table 2) in almost all the non-treated and CQ-treated stages (remarkably in the CQ-treated schizonts, details in Table 5) and included species of the heat shock protein (HSP) 70 (spots 1, 2, 6 and 7), HSP60 (spots 3-5), HSP86 (spot 8, noticeably oxidized in treated trophozoites), a putative HSP (spot 9) and putative endoplasmin homologue precursor (spot 48, significantly oxidized in non-treated rings); the latter two are members of the HSP90 family. Further chaperones identified were a putative co-chaperone GrpE (spot 10), a putative disulphide isomerase precursor (spot 11), and a putative cyclophilin (spot 41).

Other identified oxidized proteins of the protein fate class were: a putative cell division cycle protein 48 homologue, significantly oxidized in the CQ-treated trophozoites, with proteolysis, peptidolysis and chaperone-like functions (spot 12); a putative peptidase, mainly oxidized in treated schizonts (spot 13); a putative 26S proteasome aaa-ATPase subunit Rpt3, mostly oxidized in CQ-treated schizonts (spot 26); an M1-family aminopeptidase (spots 27-30), and the 20S proteasome beta-subunit, which was highly oxidized in non-treated trophozoites and treated schizonts (spot 31).

In the metabolic protein class, four glycolytic enzymes were found to be oxidized (Table 2 and Table 6): phosphoglycerate kinase (spot 21, significantly oxidized in treated trophozoites), fructose-bisphosphate aldolase (spot 32, remarkably oxidized in treated schizonts), glyceraldehyde-3-phosphate dehydrogenase (spot 40, markedly oxidized in non-treated rings), and four species of enolase (spots 44-47, non-detectable in rings).

Within the cell surface class (Table 6), two proteins from the *var* gene family were identified, both designated erythrocyte membrane protein 1 (PfEMP1) (spots 19 and 20, more oxidized in CQ-treated schizonts).

Two oxidized proteins of the protein synthesis class (Table 6) were identified, the putative elongation factor Tu (spot 43, oxidized in all CQ-treated stages and in non-treated schizonts) and a putative 40S ribosomal protein (spot 49, oxidized in non-treated trophozoites and CQ-treated schizonts).

Other oxidized proteins of the classes signal transduction, transcription, and structural (Table 2 and 6) detected were: the GTP-binding nuclear protein ran/tc4 of the Ras family (spot 25, markedly oxidized in treated schizonts); a putative Snrnp protein (spots 33 and 34, significantly oxidized in treated schizonts), and a putative formin 2 (spot 35, more oxidized in treated schizonts) respectively (Table 2).

For each of the stages, some spots identified were designated as hypothetical parasite proteins (Table 2, spots 18, 22, 23, 24, 36, 37, 42, 51) and therefore ascribed to the hypothetical protein class (Table 6) with unknown functional roles and cellular

localization. Among these, spot 18, the most striking oxidized protein in treated rings, was the most carbonylated protein in all the non-treated and CQ-treated stages.

It should be noted that approximately 25% of the spots could not be identified by MALDI-TOF/TOF MS analysis and database searches. These unidentified spots are not included in Tables 2 and 6.

Table 6. Functional classification and protein description of identified oxidized spots. Accession Nos. are from the PlasmoDB or NCBI databases.

Functional Class	Accession No.	Protein Description	Annotation Swiss Prot
Protein fate	PFI0875w	HSP	Cellular component: endoplasmic reticulum. Molecular function: ATP binding. Biological process: protein folding, response to unfolded protein. Pfam accession number: PF00012.
Protein fate	PF10_0153	HSP60	Molecular function: ATP binding, unfolded protein binding. Biological process: protein folding. Comments: FUNCTION: Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions (By similarity). SIMILARITY: Belongs to the chaperonin (HSP60) family. Pfam accession number: PF00118.
Protein fate	PF11_0351	HSP hsp70 homologue	Molecular function: ATP binding, unfolded protein binding. Biological process: protein folding, response to unfolded protein. Comment: SIMILARITY: Belongs to the heat shock protein 70 family. Pfam accession number: PF00012.
Protein fate	PF08_0054	HSP70	Molecular function: ATP binding. Biological process: protein folding, response to unfolded protein. Comment: SIMILARITY: Belongs to the heat shock protein 70 family. Pfam accession number: PF00012.
Protein fate	PF07_0029	HSP 86	Molecular function: ATP binding, unfolded protein binding. Biological process: protein folding, response to unfolded protein. Comment: FUNCTION: Molecular chaperone. Has ATPase activity. Pfam accession numbers: PF02518, PF00183.
Protein fate	PF14_0417	HSP, putative	Molecular function: ATP binding, unfolded protein binding. Biological process: protein folding, response to unfolded protein. Comment: FUNCTION: Molecular chaperone. Has ATPase activity (By similarity). Pfam accession numbers: PF02518, PF00183.

Table 6. Continued

Functional Class	Accession No.	Protein Description	Annotation Swiss Prot
Protein fate	PF11_0258	Co-chaperone GrpE, putative	Cellular component: mitochondrion. Molecular function: adenyl-nucleotide exchange factor activity, chaperone binding, protein homodimerization activity. Biological process: protein folding. Pfam accession number: PF01025.
Protein fate	PFL1070c	Endoplasmin homolog precursor, putative	Molecular function: ATP binding, unfolded protein binding. Biological process: protein folding. Pfam accession numbers: PF02518, PF00183.
Protein fate	MAL8P1.17	Disulfide isomerase precursor, putative	Cellular component: endoplasmic reticulum. Molecular function: Electron carrier activity, protein disulfide isomerase activity, protein disulfide oxidoreductase activity. Biological process: cell redox homeostasis. Pfam accession number: PF00085.
Protein fate	PFL0120c	Cyclophilin,putative	Molecular function: peptidyl-prolyl cis-trans isomerase activity. Biological process: protein folding. Comments: FUNCTION: PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (By similarity). SIMILARITY: Belongs to the cyclophilin-type PPIase family. SIMILARITY: Contains 1 PPIase cyclophilin-type domain. Pfam accession number: PF00160.
Protein fate	PFD0665c	26s Proteasome aaa-ATPase subunit Rpt3, putative	Official Name: Proteasome ATPase. Cellular component: cytosol, nucleus, protein complex. Molecular function: ATP binding, nucleoside-triphosphatase activity. Biological process: protein catabolic process . Comments: Belongs to the AAA-type superfamily and, like EC 3.6.4.5, is involved in channel gating and polypeptide unfolding before proteolysis in the proteasome. Six ATPase subunits are present in the regulatory particle (RP) of 26S proteasome. Pfam accession number: PF00004.
Protein fate	PF14_0517	Peptidase, putative	Molecular function: metalloexopeptidase activity. Biological process: proteolysis. Pfam accession number: PF00557.

Table 6. Continued

Functional Class	Accession No.	Protein Description	Annotation Swiss Prot
Protein fate	MAL13P1.56	M1-family aminopeptidase	Molecular function: membrane alanyl aminopeptidase activity, zinc ion binding. Biological process: proteolysis. Pfam accession number: PF01433.
Protein fate	MAL8P1.142	Proteasome beta-subunit	Official name: Proteasome endopeptidase complex. Cellular component: cytosol, proteasome core complex. Molecular function: threonine endopeptidase activity. Biological process: ubiquitin-dependent protein catabolic process. Comments: FUNCTION: The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity (By similarity). CATALYTIC ACTIVITY: Cleavage of peptide bonds with very broad specificity. SUBCELLULAR LOCATION: Cytoplasm. Nucleus (By similarity). SIMILARITY: Belongs to the peptidase T1B family. Comments on structure: A 20-S protein composed of 28 subunits arranged in four rings of seven. The outer rings are composed of alpha subunits, but the beta subunits forming the inner rings are responsible for peptidase activity. In eukaryotic organisms there are up to seven different types of beta subunits, three of which may carry the N-terminal threonine residues that are the nucleophiles in catalysis, and show different specificities. The molecule is barrel-shaped, and the active sites are on the inner surfaces. Terminal apertures restrict access of substrates to the active sites. Inhibited by mercurial reagents and some inhibitors of serine endopeptidases. Pfam accession number: PF00227.
Protein fate	PFF0940c	Cell division cycle protein 48 homologue, putative	Molecular function: ATP binding, ATP-dependent peptidase activity, nucleoside-triphosphatase activity, serine-type endopeptidase activity. Biological process: cell division, proteolysis. Pfam accession numbers: PF00004, PF02359.

Table 6. Continued

Functional Class	Accession No.	Protein Description	Annotation Swiss Prot
Metabolism	PF14_0425	Fructose-bisphosphate aldolase	Comments: CATALYTIC ACTIVITY: D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate. PATHWAY: Carbohydrate degradation; glycolysis; D-glyceraldehyde 3-phosphate and glycerone phosphate from D-glucose: step 4/4. SUBUNIT: Homotetramer (By similarity). SIMILARITY: Belongs to the class I fructose-bisphosphate aldolase family. Pfam accession number: PF00274.
Metabolism	PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase	Molecular function: glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity, NAD binding. Biological process: glycolysis. Comment: SIMILARITY: Belongs to the glyceraldehyde-3-phosphate dehydrogenase family. Pfam accession numbers: PF02800, PF00044.
Metabolism	PFI1105w	Phosphoglycerate kinase	Comments: CATALYTIC ACTIVITY: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate. PATHWAY: Carbohydrate degradation; glycolysis; pyruvate from D-glyceraldehyde 3-phosphate: step 2/5. SUBUNIT: Monomer. SIMILARITY: Belongs to the phosphoglycerate kinase family. Pfam accession number: PF00162.
Metabolism	PF10_0155	Enolase	Official name: Phosphopyruvate hydratase. Comments: CATALYTIC ACTIVITY: 2-phospho-D-glycerate = phosphoenolpyruvate + H2O. COFACTOR: Magnesium. Required for catalysis and for stabilizing the dimer (By similarity). PATHWAY: Carbohydrate degradation; glycolysis; pyruvate from D-glyceraldehyde 3-phosphate: step 4/5. SUBUNIT: Homodimer (By similarity). SUBCELLULAR LOCATION: Cytoplasm. SIMILARITY: Belongs to the enolase family. Pfam accession numbers: PF00113, PF03952.
Hypothetical	PFL1930w	Hypothetical protein	Pfam accession number: PF02524.
Hypothetical	PFF0190c	Hypothetical protein	No information available

Table 6. Continued

Functional Class	Accession No.	Protein Description	Annotation Swiss Prot
Hypothetical	PFA0210c	Hypothetical protein	No information available
Hypothetical	PF14_0046	Hypothetical protein PF14_0046	No information available
Hypothetical	PFB0145c	Hypothetical protein PFB0145c	Pfam accession number: PF02524.
Hypothetical	MAL13P1.237	Hypothetical protein	No information available
Hypothetical	PFA0725w	Hypothetical protein PFA0725w	No information available
Hypothetical	PFE1595c	Hypothetical protein	No information available
Cell surface	gi|21886680	Erythrocyte membrane protein 1	Pfam accession number: PF05424.
Cell surface	gi|19071542	Erythrocyte membrane protein 1	Pfam accession numbers: PF05424, PF03011.
Transcription	PFE0925c	Snrnp protein, putative	Molecular function: ATP binding, ATP-dependent helicase activity, nucleic acid binding. Pfam accession numbers: PF00270, PF00271.
Signal transduction	PF11_0183	GTP-binding nuclear protein ran/tc4	Cellular component: exosome (RNase complex), nucleus. Molecular function: GTP binding, Biological process: intracellular protein transport, small GTPase mediated signal transduction. Pfam accession number: PF00071.
Protein synthesis	PF10_0264	40S ribosomal protein, putative	Cellular component: small ribosomal subunit. Molecular function: structural constituent of ribosome. Biological process: translation. Comment: SIMILARITY: Belongs to the ribosomal protein S2P family. Pfam accession number: PF00318.
Protein synthesis	MAL13P1.164	Elongation factor Tu, putative	Cellular component: intracellular. Molecular function: GTP binding, GTPase activity, translation elongation factor activity. Biological process: translational elongation. Pfam accession numbers: PF00009, PF03144 and PF03143.
Structural	PFL0925w	Formin 2, putative	Molecular function: actin binding. Biological process: actin cytoskeleton organization and biogenesis. Pfam accession number: PF02181.

4-Hydroxynonenal-modified proteins

As shown in Figure 4, protein carbonylation caused by HNE was limited to two major bands of 69 and 42 kDa detected at the different intraerythrocytic stages in the non-treated and CQ-treated parasites. Interestingly, although CQ slightly increased oxidation of the 69 kDa band in mature rings and schizonts, it clearly inhibited lipoperoxide-induced oxidation of both bands in young rings and trophozoites. This effect was particularly noticeable in trophozoites, at which stage greatest HNE-protein modification was observed in the absence of CQ and practically no modifications were detected in both bands in the presence of an IC_{50} of CQ.

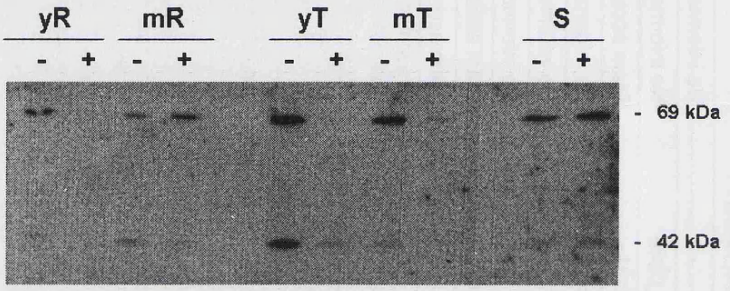

Fig. 4. HNE-modified proteins at different intraerythrocytic stages of *P. falciparum* Dd2. yR, young ring; mR, mature ring; yT, young trophozoite; mT, mature trophozoite and S, schizont of control (-) and IC_{50} CQ-treated (+) cultures. The exposure time of the membrane to the film was 30 min.

5. Discussion

Although the malaria parasite is susceptible to oxidative stress during its erythrocytic life stages given the red blood cell environment contains oxygen and iron, factors required for the formation of ROS, it is also well adapted to such oxidative stress through specialized systems (Becker et al., 2004) Thus, toxic FP IX (Fe^{+3}), which is released upon haemoglobin degradation by the parasite, is mostly (up to 90%) detoxified by biomineralisation (Egan et al., 2002) to form inert haemozoin. Notwithstanding, it has been reported that a considerable amount of FP IX (possibly as much as 50%) escapes biomineralisation and must be detoxified by alternative pathways (Muller, 2004). In addition, low intracellular levels of ROS are maintained by a number of redox and antioxidant systems described for the erythrocytic stages of *P. falciparum* such as those comprised of superoxide dismutases, mitochondrial redox and antioxidant systems, and enzymatic control of intracellular glutathione levels by redox cascades including glutathione reductase, glutaredoxins, glyoxalases, glutathione S-tranferases, thioredoxins, peroxiredoxins and glucose-6-phosphate dehydrogenase (Muller, 2004). Effectively, the

induction of several antioxidant enzymes upon transient silencing of glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (Crooke *et al.*, 2006) has revealed the key role of such enzyme cascades in maintaining oxidative homeostasis.

Despite functioning FP IX detoxification systems and a number of enzymatic and non-enzymatic antioxidant systems, the high number of oxidatively modified proteins in *P. falciparum* relative to numbers reported for eukaryotic organisms (Poon *et al.*, 2004; Poon *et al.*, 2005; Reverter-Branchat *et al.*, 2004; Singh *et al.*, 2007; Sultana *et al.*, 2006) suggests a highly oxidative environment of the parasite, probably as a consequence of toxic haem escaping detoxification, increasing ROS generation and causing damage to intracellular proteins (Ginsburg *et al.*, 1998; Loria *et al.*, 1999). Our present observation of increased protein oxidation induced by CQ in the mature stages of the parasite's intraerythrocytic cycle, which is when most haemoglobin is digested, suggests that CQ interferes with haem detoxification, in agreement with current hypotheses on the antimalarial mechanism of CQ action (Egan *et al.*, 2000; Egan and Ncokazi, 2005; Ginsburg *et al.*, 1998; Loria *et al.*, 1999).

Although protein carbonylation produced by HNE adducts only represents a fraction of the oxidative damage derivatized by DNPH, the lower number of oxidized spots and reduced spot intensities detected in CQ-treated parasite rings could reflect the inhibition by CQ of protein-HNE adducts in early stages of parasite development. Thus, lipoperoxides could be a main driving component of protein oxidation in early development stages. Subsequent to this, when haemoglobin degradation by trophozoites reaches a significant rate, the main source of oxidation would switch to free haem. CQ-induced inhibition of haem biomineralisation would then enhance protein oxidation, as indicated by our results. Indeed, increased haem production in mature stages has been also described as a source for HNE formation in parasitized erythrocyte membranes (Arese and Schwarzer, 1997); (Skorokhod *et al.*, 2007). This would also explain the slight increase in HNE adducts also detected in our CQ-treated schizonts.

Protein carbonyl detection as a measure of oxidative damage indicated that some groups of proteins such as molecular chaperones, proteases and glycolytic enzymes were more susceptible to oxidation than others. Interestingly, some of these oxidation targets or their isozymes, such as HSP60, HSP70, HSP86, 26S proteasome subunit, glyceraldehyde-3-phosphate dehydrogenase, enolase, fructose-1,6-bisphosphate aldolase, carbonic anhydrase isoenzymes, beta-actin, and catalase have been identified as oxidatively-modified proteins in eukaryotic cells subjected to oxidative stress, from yeast to human cells (Butterfield, 2004; Cabiscol *et al.*, 2000; England *et al.*, 2004; Ishii *et al.*, 2005; Poon *et al.*, 2004; Reverter-Branchat *et al.*, 2004; Sultana *et al.*, 2006). The significant sequence divergence between *P. falciparum* proteins and their functional

homologues in other organisms, suggests a common oxidative signalling mechanism controlling protein turnover across this wide evolutionary scale (Dalle-Donne *et al.*, 2006).

Although great care was taken to only extract parasite proteins using our differential lysis and treatment method, not all the proteins detected were of plasmodial origin, around 1/6 were derived from the human erythrocyte host. This effect has also been detected in other malaria proteomic studies (Florens *et al.*, 2002; Nirmalan *et al.*, 2004) and suggests that some of the host proteins detected correspond to erythrocyte proteins internalised by the parasite, or proteins arising from host-parasite interaction or from cross-contamination during erythrocyte lysis. Thus, in practically all the parasite stages, erythrocyte cytosolic carbonic anhydrase I and II were among the most oxidized proteins in both non-treated and CQ-treated parasites. Particularly high oxidation signals were observed in non-treated trophozoites followed by treated schizonts. Carbonic anhydrase has been identified as a major intracellular peroxidation target in erythrocytes (Uchida *et al.*, 1997). High concentrations of carbonic anhydrase have been found in the erythrocyte cytosol (Ozensoy *et al.*, 2004). Also, carbonic anhydrase III, the main oxyradical scavenger cytosolic isoenzyme expressed at high levels in skeletal muscle (Raisanen *et al.*, 1999), is strongly carbonylated independently of endogenous oxidative stress (Barreiro *et al.*, 2005). Hence, the oxidized carbonic anhydrases detected in all the parasite stages examined could be attributed to oxidative stress in the parasitized erythrocytes irrespective of the presence CQ. Thus, if oxidative damage to an essential red blood cell enzyme also occurs during *in vivo* infection, this could modify the phagocytosis rate of parasitized cells and consequently the course of malaria-associated anaemia, as described in oxidative red cell defects (Cappadoro *et al.*, 1998).

A further three important redox-related enzymes from the human host were also found to be oxidized in our experiments. Thus, peroxiredoxin 2 isoform b and thioredoxin peroxidase B were detected in non-treated rings, and catalase in non-treated trophozoites and CQ-treated schizonts. Although *Plasmodium* lacks the antioxidant enzymes catalase and glutathione peroxidase, high H_2O_2 concentrations released during haemoglobin digestion in the parasite vacuole are directed towards its own cytoplasmic peroxidases and thioredoxins (Nickel *et al.*, 2006) with the additional participation of the host cell antioxidant system, including catalase (Atamna *et al.*, 1994). Host catalase imported into the food vacuole (Fairfield *et al.*, 1988) has been reported to remain active at pH 5 and achieve H_2O_2 detoxification until it is also digested by vacuolar proteases (Tilley, 2001). Thus, the four-fold increase in oxidized catalase in CQ-treated schizonts suggests an ancillary effect of the drug against host-imported antioxidant proteins, which could enhance oxidative damage in the parasite.

The vast majority of the carbonylated proteins identified were of plasmodial origin (83%). Strikingly, nearly one-third of these plasmodial proteins belonged to chaperone families or exhibited chaperone properties. Our results revealed that chaperones from different families became carbonylated in almost all non-treated and CQ-treated parasites, but carbonylation was especially intense in CQ-treated schizonts. Several carbonylated HSPs of 60, 70, 82 and 86 kD have been identified under different oxidative stress conditions in eukaryotic cells (Butterfield, 2004; Poon *et al.*, 2004; Reverter-Branchat *et al.*, 2004; Sultana *et al.*, 2006). Molecular chaperones in eukaryotes both contribute to protein folding, assembly and translocation and regulate the activities of protein kinases and transcription factors, inducing their expression under several types of cellular stress (Parsell, 1994). HSP members of families 60, 70 and 90 are expressed throughout the intraerythrocytic cycle of the parasite (Banumathy *et al.*, 2003). Additionally, HSP90 has been identified as the target of the antimalarial activity of geldanamycin (Kumar *et al.*, 2003). The high propensity for oxidation revealed by this class of proteins in *P. falciparum* might suggest their rapid turnover in quickly replicating parasite cells. In addition, the increased carbonylation of HSPs observed in the mature stages of CQ-treated parasites at IC_{50} could indicate interference with the cellular chaperone protective role giving rise to the build-up of harmful misfolded proteins (Dalle-Donne *et al.*, 2006), thus further affecting cell survival at the mature stages, in agreement with an observed inhibitory effect of CQ on schizonts in culture (Yayon *et al.*, 1983).

In the cell, chaperone and degradation systems are functionally linked (Berke and Paulson, 2003). Two proteins involved in protein catabolism, the 20S proteasome beta-subunit and a putative 26S proteasome aaa-ATPase subunit Rpt3, were also identified in our experiments as carbonylated proteins. In both these proteins, most oxidation was detected in CQ-treated schizonts (up to a 14-fold increase with respect to non-treated schizonts) although the former was also notably oxidized in non-treated trophozoites. Oxidative modification of proteasome subunits under different oxidative stress conditions has been reported in neuroblastoma cells (Ishii *et al.*, 2005), T cells (Ponnappan *et al.*, 2007), and myocardiocytes (Bulteau *et al.*, 2001). On the other hand, oxidatively modified proteins are degraded in the proteasome (Bulteau et al., 2001), whose activity gradually diminishes as a consequence of oxidative damage (Dalle-Donne *et al.*, 2006). Thus, the increased oxidation by CQ observed in the parasite proteasome could modify the overall protein turnover machinery, also compromised by the intense oxidation of chaperones mentioned above. Recently, a critical mutation associated with resistance to CQ in *P. chabaudi* has been found in a deubiquinating enzyme involved in the ATP-dependent protein degradation pathway (Hunt *et al.*, 2007). Adaptation to CQ of these proteins with common cellular functions, whether by mutation or chemical modification, suggests that

the parasite protein fate machinery has a physiological role in fighting against the oxidative stress caused by sublethal concentrations of the drug. This hypothesis is also supported by the identification of a deubiquinating protease as the carbonylated target of the oxidative stress induced in mutant superoxide dismutase deficient mice (Poon *et al.*, 2005). Thus, targeting the *P. falciparum* proteasome complex, as reported for lactacystin (Certad *et al.*, 1999), may be an alternative or a complementary therapeutic strategy in combination with antimalarials that trigger oxidative stress in the parasite.

Besides processing proteases, the proteolytic M1-family aminopeptidase responsible for haemoglobin digestion and erythrocyte invasion (Allary *et al.*, 2002), also appeared carbonylated in almost all non-treated and treated stages, with the most intense carbonylation signal observed in treated schizonts. Up to four species of the enzyme were detected: three, showing slight size differences, which were probably generated through autoproteolysis and a fourth species, which was largely trimmed. The latter species was seen oxidized only in CQ-treated schizonts suggesting differences in conformational state or posttranslational modifications induced by CQ-related oxidative stress. Although post-translational modifications in a variety of *P. falciparum* proteins have been observed by quantitative proteomic analysis (Nirmalan *et al.*, 2004), clues about their nature have not yet emerged. On the other hand, the antimalarial activity of inhibitors of the M1-family aminopeptidase (Flipo *et al.*, 2003) suggest an essential role for this protease in the parasite's life cycle. Given that blocking FP IX aggregation is a main effect of CQ (Egan and Ncokazi, 2005), parallel increased ROS formation and oxidative inactivation of this essential protease could promote damage in the parasite as a secondary effect (Becker *et al.*, 2004; Orjih *et al.*, 1994).

Four glycolytic enzymes, phosphoglycerate kinase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and enolase were identified as oxidized parasite proteins. It should be stressed that all have already been identified as oxidatively-modified proteins associated with oxidative stress in other cells (Butterfield, 2004; Cabiscol *et al.*, 2000; England *et al.*, 2004; Poon *et al.*, 2004; Reverter-Branchat *et al.*, 2004; Sultana *et al.*, 2006). Since high concentrations of a given protein favour its oxidation and turnover (Dalle-Donne *et al.*, 2006), the identification of these oxidized glycolytic enzymes in the parasite suggests the need for relatively high concentrations to sustain the major energy dependence on glycolysis of the parasite (Lang-Unnasch and Murphy, 1998). Further, the increase in glucose flux in parasitized erythrocytes related to the parasitaemia and peak levels in trophozoites (Mehta *et al.*, 2005) are in agreement with the elevated oxidative turnover found in parasite glycolytic enzymes and clarifies their parasite origin (Roth *et al.*, 1988). Phosphoglycerate kinase and fructose-bisphosphate aldolase were significantly oxidized in mature stages of CQ-treated parasites while

glyceraldehyde-3-phosphate dehydrogenase showed little change between treated and non-treated parasites. Enolase deserves a more detailed discussion given the four species identified of close molecular weights with slightly different isoelectric points. Thus, while all four enolase forms were highly oxidized in mature stages, none of the forms appeared oxidized in rings. Strikingly, three of these enolase species– the most acidic– were highly oxidized in non-treated parasites but not in CQ-treated cultures. In contrast, the most basic enolase species was highly oxidized in CQ-treated schizonts and only very slightly in not-treated trophozoites. This different susceptibility of enolase species points to different roles under CQ pressure and could reflect different conformational states caused by oxidative stress. This was also observed here for the M1- family aminopeptidase (see above) and has been described in another *P. falciparum* proteomic analysis (Nirmalan *et al.*, 2004).

Phosphoglycerate kinase and fructose-bisphosphate aldolase were found to be highly oxidized in mature stages of CQ-treated parasites. Given the importance of glycolysis as a source of cellular ATP, this has key implications for cell function, and is thus consistent with reports indicating that mature parasites are considerably more sensitive to CQ than ring-stage parasites following glycolysis inhibition by CQ (Yayon *et al.*, 1983).

Further to the previous rationale on the secondary effect of CQ on aminopeptidase, CQ-induced binding of FP IX to parasite glycolytic enzymes could also inhibit glycolysis as a secondary effect, as previously suggested (Campanale *et al.*, 2003; Famin and Ginsburg, 2003).

Only two cell surface proteins appeared oxidized. These two proteins are the products of two separate genes of the highly polymorphic *var* family that code for PfEMP1 harbouring Duffy-binding-like domains. Both proteins were found highly carbonylated in CQ-treated schizonts. These two PfEMP1s are encoded by genes of the highly polymorphic *var* family which, coincidentally, are commonly occurring and expressed in placental field isolates of *P. falciparum* (Fried and Duffy, 2002; Rowe *et al.*, 2002). PfEMP1s have been linked to the adhesion properties of parasitized erythrocytes (Howard and Gilladoga, 1989; Singh *et al.*, 2006) and implicated in many features of severe malaria and its pathogenesis, particularly malaria in pregnancy (Kirchgatter and Del Portillo, 2005). Carbonylation of these proteins could –albeit very tentatively– indicate a change in the cytoadhesion properties of CQ-treated parasites related to the non-prevention of *P. falciparum* infection in pregnant women on regular CQ prophylaxis, who nevertheless exhibit lower densities and rates of placental infection (McDermott *et al.*, 1988).

In this first redox proteomic profiling of CQ-resistance in *P. falciparum* across its intraerythrocytic stages, the proteins identified as oxidized are key instruments of the parasite's main functions, such as protein folding, protein fate, energy metabolism, signal transduction and pathogenesis. Our findings provide new insight into the molecular mechanisms triggering the parasite's response to CQ, along with information on normal protein-oxidation modifications that could play a role in its development and pathophysiology. Our results also indicate that redox proteomics is an appropriate tool for identifying CQ-response systems, which if targeted might render new ways of fighting against CQ-resistant strains. Among these, HSPs, the parasite's specific proteolytic machinery and glycolytic enzyme systems are the best candidates, given the long stretches of identity loss between their protein sequences and those of their homologues in the host (Fig. 5).

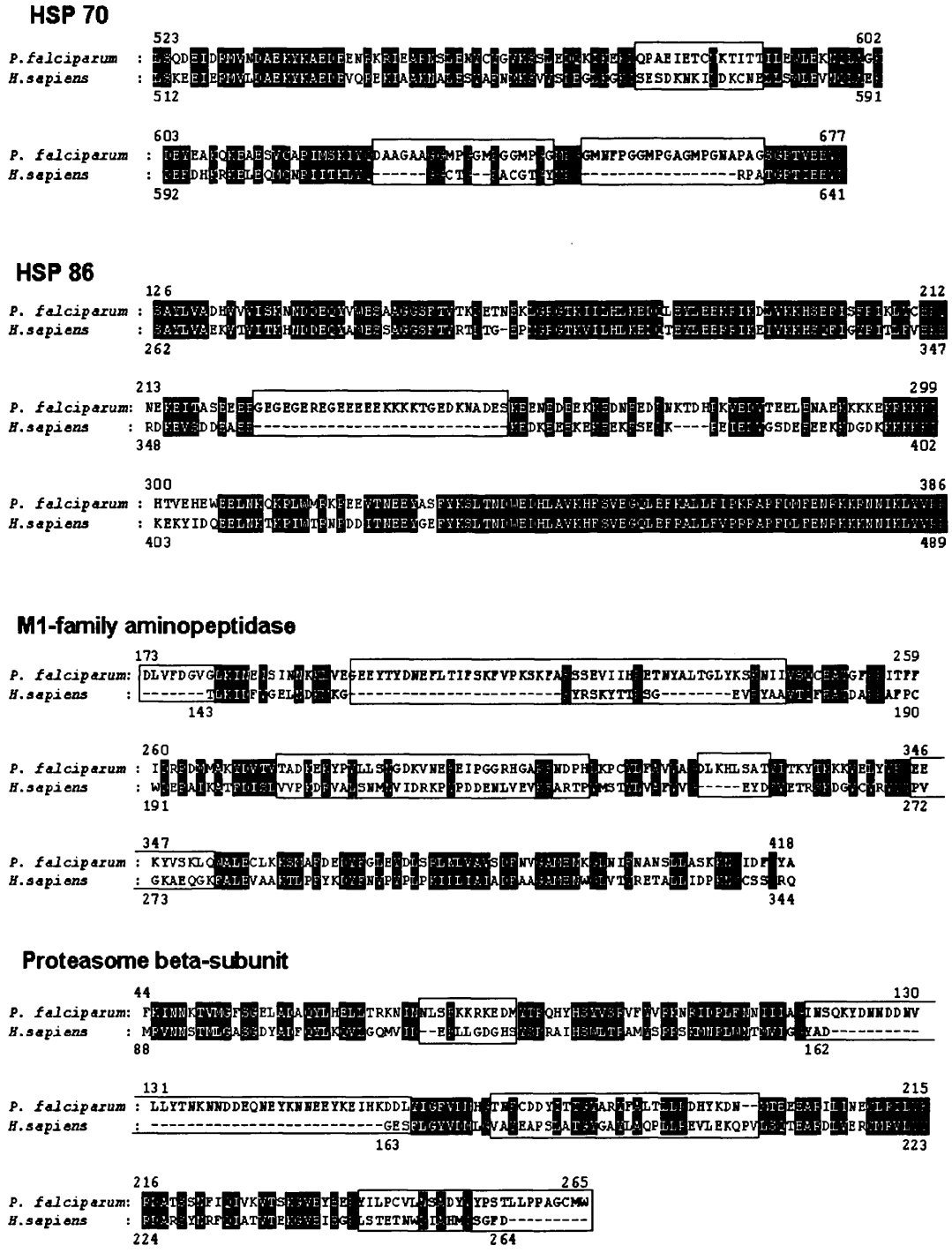

Fig. 5. Protein alignments of HSP70 (PF08_0054), HSP86 (PF07_0029), M1-family aminopeptidase (MAL13P1.56) and proteasome beta-subunit (MAL8P1.142) from *P. falciparum* with their corresponding host homologues showing the long stretches of identity loss between the parasite and host protein sequences (in boxes).

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Conclusions

5. CONCLUSIONS

The results presented in this thesis represent new perspectives for biology of the parasite and antimalarial chemotherapy. The following conclusions can be raised from the results obtained:

1- The high expression levels of PfNna1 gene and transcriptional up-regulation by PCI in the mature-stage parasites suggest the involvement of this putative protein in essential activities for the parasite survival.

2- The unique bifunctional *Plasmodium* G6PD-6PGL protein varies in size depending on the parasite developmental stage. In fact, a controlled pattern of PfG6PD-6PGL processing during parasite maturation can be hypothesized. Detecting two different band sizes using the anti-G6PD and anti-6PGL antibodies implies that the bifunctional protein could mature to render two different polypeptides with separate enzyme activities.

3- Proteomic profiling of oxidation is dependent on the time course of the intraerythrocytic stages of *P. falciparum* either in non-treated or in chloroquine-treated parasites.

4- Oxidative damage in *P. falciparum* is identified in the following key components of the parasite cellular functions: protein folding, protein turnover and proteolytic processing, energy metabolism, signal transduction and pathogenesis.

5- Detection of specific 4-hydroxy-2-nonenal adducts indicates that protein lipoperoxidation is inhibited by chloroquine, particularly at early stages of development.

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Annex

7. ANNEX I

Protocols for *Plamodium falciparum* culture

1. Preparation of Culture Medium

Nomenclature of culture medium:

- COMPLETE MEDIUM (blue medium) → RPMI 1640 + HEPES + ALBUMAX I + HYPOXANTHINE + GENTAMICINE + BICARBONATE. Prepare just before using it.
- INCOMPLETE MEDIUM → Like complete medium **without BICARBONATE.** Prepare weekly (7-10 days) *(LASTS UP TO 7 DAYS, 4°C; 10 DAYS, -20°C).*
- WASHING MEDIUM (yellow medium)→ Like complete medium **without ALBUMAX I.** Prepare every 15 - 21 days. *(LASTS UP TO 14-20 DAYS, 4°C).*

Reagents:

. 50mg Gentamicine (bottle) + 1ml distilled water → Final concentration = 50 mg/ml

. 0.1M Hypoxhantine: 1.361g in 100ml 1M NaOH [MW Hypoxhantine: 136.1].

. 1M NaOH: 4g in 100ml destilled water [MW NaOH: 40].

. 5% Sodium bicarbonate: 5 g sodium bicarbonate in 100 ml distilled water [MW = 84.01] (Lasts up to 5 days at 4°C).

STOCK SOLUTIONS: All stock solutions should be stored at 4°C.

Reagent	Company	Reference
RPMI 1640	Sigma	R6504-1L (10 shachets)
HEPES 1M	Sigma	H0887 (100 ml)
SODIUM BICARBONATE	Sigma	S 5761 (500g)
HYPOXANTHINE	Sigma	H9636 (1g)
ALBUMAX I	Gibco	11020-021 (25g)
GENTAMICINE	Sigma	G-1264 (50 mg)

INCOMPLETE CULTURE MEDIUM (1 L):

1. Add 800 ml of milliQ or bidistilled water in a beaker.
2. Add one sachet of RPMI 1640 to the water and constantly shake the mix on a shaker.
3. Add 25 ml 1M HEPES (pH =7.4)
4. Transfer 150 ml (approximately) of this medium to another beaker and add the following:
   1 ml hypoxanthine from the 0.1M stock solution, shake and
   5g Albumax. Mix well.
5. Add that mix into the general RPMI medium.
6. Add 250 µl Gentamicine.
7. Adjust pH = 7.25 (The culture medium pH has to be 7.4 but when we filter the medium, the pH increase).
8. Add milliQ or bidistilled water until the total volume is 1 L.
9. Filter the culture medium with sterile 500 ml micropore bottles (pore Ø = 0.22 µm).
10. Label the bottle, mentioning name and date. Store at 4°C.

The freeze incomplete culture medium is valid for 10 days at -20°C (MR4). At 4°C it is valid for approximately **7 days**.

INCOMPLETE MEDIUM (1L)	**Doses**	Final concentration	Initial concentration
RPMI 1640	**10.4 g**	10.4 g/l	
HEPES buffer solution	**25 ml**	25 mM	1 M
ALBUMAX	**5 g**	0.5%	
HYPOXANTHINE 0.1M	**1 ml**	100 µM	0.1 M
GENTAMICINE	**250 µl**	12.5 µg/ml	50 mg/ml

COMPLETE CULTURE MEDIUM (100 ml):

According to MR4 protocol, the complete culture medium works well for 3D7, Dd2, W2, and HB3 strains. In our laboratory only strains of 3D7, Dd2 and a clinical isolate from Guinea have been cultured with this medium.

All manipulations of cultures should be carried out in a laminar flow hood, using standard aseptic techniques. Gloves must always be worn.

The medium should be warmed up at 37°C before use.

Prepare this medium just before using it. We need a molarity of 23.8 mM for Sodium bicarbonate in complete culture medium. Add 4 ml of Sodium bicarbonate 5% (0.59 M) to 100 ml of incomplete medium. The colour of culture is very important; avoid using culture medium with purple colour.

An initial culture with 1% haematocrit and 10% parasitaemia can grow well without any need to change the medium for 24h.

2. Extraction human whole blood

Normal Human RBC (Type O^+ or A^+ or B^+) are obtained from donors malaria no immune.

Reagents:

. CPD anticoagulant (100 ml)

Reagents	Amount	Company	Reference
Citrate acid (monohydride) or Citrate acid (anhydride)	0.327 g or 0.288 g	Panreac	131808
Sodium citrate (dihydrate) or Sodium citrate (anhydride)	2.630 g 2.255 g	Panreac	141653
Sodium phosphate monobasic	0.222 g	Panreac	131965
Dextrose anhydride (D-glucose)	4.630 g	Panreac	131341
MilliQ water	100 ml		

Final concentration citrate acid: 0.015M.

1. Add 80 ml (preferably sterile) milliQ water to a beaker. We should add the reagents keeping the order indicated in the table above. Dissolve while shaking for 5-10 min.
2. Add (sterile) milliQ water till 100ml.
3. Filter by syringe and aliquote them in sterile tubes. Filtering should be done in a laminar flow hood.
4. Store at 4°C.

Extraction Material

- Rack with vacuum tubes (7 per extraction)
- Pippet (blue) and pippet-sampler
- Plastic band, pair of scissors, gauze and plaster

Method

1. Extract whole blood
2. Add 1.4 ml CDP anti-coagulant solution **RAPIDLY** in 10 ml whole blood. Mix it by inverting the tubes 2-3 times, do not mix with pippet.
3. Cover tubes with Parafilm (totally) and store at 4°C.

This blood is **valid up to 20 days.**

3. Preparation of Red Blood Cells

Blood only should be washed when it is needed and only the necessary amount, because without washing it can be kept longer.

Method (White cell removal with Lymphoprep)

1. Centrifuge the whole blood at 2300 rpm, 10 min (blood should be stored at 4°C at least one day after blood extraction).
2. Discard the upper serum layer (approximately half the tube).
3. Mix 5 ml of RBCs with 5 ml of washing medium and add the mixture carefully onto 5 ml of Lymphoprep.
4. Centrifuge at 2300 rpm, 20 min. Discard the supernatant.
5. Wash RBCs with 10 ml of washing medium.
6. Centrifuge at 2300 rpm, 5 min. Discard the supernatant.
7. Store RBCs by 50% diluting the packed cells with incomplete culture medium.

4. Thawing of glycerol-frozen parasites with NaCl

Reagents:

- Solution A → Sterile 12% NaCl
- Solution B → Sterile 1.6% NaCl

Pre-warm for 15 minutes at 37 ℃ water bath before using.

Method

1. Take a vial containing malaria parasites from -80°C/liquid nitrogen and thaw it during 2-3 min in a 37°C water bath or keeping it in hand to warm it.
2. Spray the vial tube with 70% ethanol.
3. Transfer the vial contents to a 50-ml centrifuge tube using a 1ml serological pipette Measure the **total volume (V)**.
4. Add **0.1X V of solution A slowly**, dropwise, while swirling the tube.
5. Let the tube stand for **5 min at room temperature**.
6. Add **10X V of solution B slowly**, dropwise at first, while swirling the tube.
7. **Centrifuge** at 1800 rpm (240 [g]), (≈ 37°C), **5 min,** brake 4.
   When we work with infected RBC, never use centrifugation speed > 1500 rpm.
8. Discard the supernatant. Resuspend the **pellet in 10 ml washing medium** and transfer to a sterile tube.
9. **Centrifuge**, and remove the supernatant.
10. **Estimate the pellet volume and add equal amount of fresh RBC, mix well.** Resuspend the mixture in **complete medium** to keep **haematocrit 1-2%**.

11. Flush flasks for 60 sec or less with gas mixture (1% O_2, 3% CO_2, 96% N_2) at 150 Bar pressure (15°C).
12. Incubate the flask at 37°C for 48 hours.
13. **Assess viability** (count ring-stage parasites).

Example:

V = 600 µl (300 µl infected RBC + 300 µl freezing solution)

1. *Solution A = 0.1 * 600 = 60 µl solution A*
2. *Solution B → 10 * 600 = 6000 µl solution B*
3. *Fresh RBC → 300 µl (*)*
4. *Complete medium →*

   1% haematocrit → 60 ml complete medium (little flask).

   2% haematocrit → 60 ml complete medium + RBC. Total RBC should be 1200 µl (300 µl infected RBC (*) + 900 µl fresh RBC).

5. Parasite synchronization

We use sorbitol synchronization method in our laboratory.

5% Sorbitol: To keep the parasites synchronized, the sorbitol treatment must be performed once a week. However, it can be done after each cycle (48 h) if a very synchronized culture is needed. Objective: eliminate or lysis of mature stages (trophozoites and schizonts) thus selecting ring-stage parasites.

Never freeze your parasite just after sorbitol synchronization. Grow them one more cycle before storing in the liquid N_2 bank.

Sorbitol-synchronization

The most important thing for synchronization is to be sure that you have enough ring-stage parasites **(more than 5%).** The rings must **not be later than 10 to 12 h postinvasion** to treatment with sorbitol. Do not synchronize more than 3 flasks.

To deposit the parasites in the bank, synchronize one flask and divide it into 3 flasks to grow one more cycle.

Reagents:

- Sorbitol: Sterile 5% sorbitol

Add 80 ml milliQ water to a beaker. Dissolve the sorbitol while shaking for 5-10 min. Add water till 100 ml. Filter with syringe (0.22 µ) and store at 4°C

Method

1. Take out 5% sorbitol from 4°C one hour before use and warm up at room temperature (or 5 min at 37°C).
2. Remove some medium from the flasks with a pump and mix the rest (approximately 15 ml medium or more) with RBC. Transfer the contents to 15ml centrifuge tubes.
3. Spin down the parasite culture for 5 min in swing-out centrifuge at 2000rpm; brake 4. When working with infected RBC, never use centrifugation speed over 1500-2000 rpm.
4. Discard the supernatant, estimate the pellet volume and add **9-10X 5% sorbitol.**
5. **Vortex 15 sec.**
6. **Incubate at 37°C, 8 min (better, in agitation).**
7. **Vortex 15 sec.**
8. Centrifuge 5 min, 2000rpm and remove supernatant (we should see haemolysis).
9. **Add 10X washing medium** → centrifuge for 5 min at 2000 rpm.
10. Discard supernatant and **repeat step 9.**
11. Make a smear to assess the presence of ring forms.
12. **Resuspend the pellet adding complete medium** to reach 1-2% haematocrit. Transfer to a sterile flask, gas the flasks, seal them and incubate at 37°C incubator, as usual.

6. Store-freezing infected RBC in liquid nitrogen

- The minimum parasitaemia required to freeze the parasites cultures is 5% and the cycle stage must be young rings (< 12h).
- Take the flasks from the incubator and aspirate the medium with a pump. Resuspend the RBCs in 3-4 ml of washing medium and transfer to a 15 ml flask.
- Centrifuge 1500-2000 rpm for 5 min 35°C.
- Discard the supernatant.
- Estimate the volume of RBC pellet and add equal amount of freezing solution dropwise while swirling the tube and mix them with the help of the pippet.
- Aliquote the mixture in 1 ml N_2-vials (the minimum amount in each vial must be: 300 µl).
- Store at liquid nitrogen as soon as possible.

7. Parasite harvest

Reagents:

. Freezing solution

Reagent	Fv = 100 ml	Fv = 250 ml
NaCl	0.65 g	1.63 g
Sorbitol	3.02 g	7.55 g
Bidistilled sterile water	72.0 ml	180.0 ml
Glycerine	28.0 ml	70.0 ml

1. Add all the reagents in the same order than indicated in the table above.
2. Filter with syringe (Ø=0.22µm) and put on sterile tube. Filter should be carried out in a laminar flow hood.
3. Store at 4°C.

Method

1- Take the culture and aspirate the medium with a pump.
2- Transfer the monolayer of parasites to 50 ml tubes with a 10 ml pippet.
3- Centrifuge at 2700-2800 rpm, 7 min, 37°C.
4- Discard supernatant.
5- Reduce the number of tubes to one with the help of washing medium.
6- Centrifuge 2700-2800 rpm, 7 min.
7- Transfer to a 15 ml Falcon. Centrifuge 2700-2800 rpm, 7 min at 37°C.
8- Discard supernatant and keep the parasites pellet at –80°C for further analysis (for RNA expression analysis the use of RNAlater solution is recommended).