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RESUME

Propriétés redox des indolone-*N*-oxydes et des extraits de *Crinum latifolium* en relation avec leurs propriétés biologiques

Mots clés: anticancéreux, antipaludéens, *Crinum latifolium*, indolone-*N*-oxydes, propriétés redox.

Le travail de thèse porte sur l'examen des liens existants entre les propriétés d'oxydo-réduction de molécules de synthèse (indolone-*N*-oxydes) et d'extraits naturels (*Crinum latifolium*) et leurs activités biologiques, respectivement antipaludiques et anticancéreuses.
Les indolone-*N*-oxydes présentent de fortes activités antipaludiques *in vitro* et *in vivo*. Les molécules sont bioréductibles en milieu biologique et le signal redox qu'elles induisent dans le globule rouge parasité permet de détruire la cellule hôte infectée par *Plasmodium falciparum* sans dégrader le globule sain. Les travaux menés à l'aide, notamment, de méthodes biochimiques, électrochimiques et techniques couplées RPE-électrochimie, ont démontré le lien existant entre le potentiel de réduction et l'activité antiplasmodiale des molécules dans la série indolone-*N*-oxyde ainsi que le rôle joué par différents composants érythrocytaires. Les travaux ont également permis de différencier les mécanismes d'action de ces composés comparativement aux antipaludiques de référence, chloroquine et artémisinine.

Les extraits de *Crinum latifolium* sont largement utilisés en Médecine Traditionelle en Asie, notamment au Viet Nam, pour leurs effets bénéfiques sur la longévité et leurs activités anticancéreuses dans le cas du cancer de la prostate, notamment. Les mécanismes d'action de ces extraits ne sont pas encore bien élucidés. En partant de l'examen des propriétes redox (capacité de réduction, caractère pro-oxydant), les travaux ont permis d'établir que plusieurs extraits sont capables d'activer les macrophages et d'inhiber la prolifération de certaines cellules du lymphome (EL4-luc2). D'autres extraits activent la differenciation des macrophages de type M1.

ABSTRACT

Redox properties of indolone-*N*-oxides and *Crinum latifolium* extracts in relation with their biological properties

Keywords: anticancer, antimalarials, *Crinum latifolium*, indolone-*N*-oxides, redox properties.

The thesis focuses on the examination of the relationship between the redox properties of synthetic molecules (indolone-*N*-oxides) and natural extracts (*Crinum latifolium*) and their biological activities, respectively antimalaria and anticancer.

Indolone-*N*-oxides have strong antimalarial activity *in vitro* and *in vivo*. These molecules are bioreductive in biological medium and induce a redox signal in parasitized red blood cells which destroys host cells infected by *Plasmodium falciparum* without damaging the healthy blood cells. The work with the help biochemical and electrochemical methods and EPR-coupled electrochemistry showed the relation between the reduction potential and the antiplasmodial activities in the indolone-*N*-oxide series and the role played by different erythrocyte components. The work also differentiated mechanisms of action of these compounds compared to the antimalarial references, chloroquine and artemisinin.

Crinum latifolium extracts are widely used in Traditional Medicine in Asia, including Viet Nam, for their beneficial effects on longevity and anticancer activities; particularly in the case of cancer prostate. The mechanisms of action of these extracts are not yet well understood. Based on the examination of the redox properties (capacity reduction, pro-oxidant character), the work has shown that several extracts are capable of activating macrophages and inhibiting the proliferation of lymphoma cells (EL4-luc2). Other extracts activate M1 macrophages differentiation.

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ABBREVIATIONS AND SYMBOLS

1-HH: metabolite of compound **1,** (6-(4-chlorophenyl)-7H-[1,3]dioxolo[4,5-f]indol-7-one-5-oxide)
3D7: chloroquine-sensitive strain of *Plasmodium falciparum*
6HC: 6-hydroxycrinamidine

A

ACT: Artemisinin Combination Therapy
AkEx: alkaloid extract
AM: Artemether
AQ: Amodiaquine
AqEx: aqueous extract.
ART: Artemisinin and derivatives
ARMD: accelerated resistance to multiple
AE1: erythrocyte anion exchanger, band 3 protein

C

Cat: Catalase
CL: *Crinum latifolium*
CDNB: 1-chloro-2,4-dinitrobenzene
Coartem: artemether-lumefantrine
CQ: chloroquine

D

DC: Dendritic cells
DMPO: 5,5-Dimethyl-1-pyrroline-*N*-oxide
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DRE: Digital rectal examination
DV: Digestive vacuole

E

ECM: Extra cellular matrix
eNOS or NOS3: Endothelial NOS

EPR: Electron paramagnetic resonance

F

Fansidar: sulfadoxine-pyrimethamine
FcB1: Chloroquine-resistance strain of *Plasmodium falciparum*
FlEx: flavonoid extract
FrF: fraction F

G

G6PD: Glucose 6 phosphate dehydrogenase
G6PP: Glutathione-6-phosphate dehydrogenase
GMK: Greater Mekong Subregion
GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Glutathione
GSSH: Glutathione disulfide

H

HSA: Human serum albumin

I

IFN- γ: interferon-γ
IL 4: Interleukin 4
IL 10: Interleukin 10
IL 13: Interleukin 13
INOD: Indolone-*N*-oxide
iNOS or NOS2: Inducible NOS
IRS: Indoor residual spraying
ITN: Insecticide-treated bed net

K

K1: Chloroquine and pyrimethamine-resistance strain of *Plasmodium falciparum*
KB: Human carcinoma cell line

L

LC-MS: Liquid chromatography–mass spectrometry
L-cys: L-cysteine
LM: Lumefantrine
LPS: Bacterial lipopolysaccharide

M

MΦ: Macrophages
M1: classically activated MΦ
M2 alternatively activated MΦ
MCF7: Human breast cancer cells
MDR: Multiple drugs resistance
MDSC: Myeloid-derivated suppressor cell
MMV: Medicine formalaria venture
MQ: mefloquine
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NADPH: Nicotinamide adenine dinucleotide phosphate
NEM: *N*-ethylmaleimide
NMCP: National Malarial Control Programme
NO: Nitric oxide
NOS: NO synthase
nNOS or NOS1: Neuronal NOS
NOX: Nicotinamide adenine dinucleotide phosphate oxidase

P

P: parasite
P.: Plasmodium
PCR: Polymerase chain reaction
*Pf*DHPS: *Plasmodium falciparum* dihydropteroate synthetase
*Pf*DHFR: *Plasmodium falciparum* dihydrofolate reductase
*Pf*CRT: *Plasmodium falciparum* chloroquine resistance transporter
*Pf*NHE1: *Plasmodium falciparum* sodium/proton exchanger 1
*Pf*NHE1: *Plasmodium falciparum* sodium/proton exchanger 1
*Pf*MDR1: *Plasmodium falciparum* multidrug resistance transporter 1
*Pf*MRP: *Plasmodium falciparum* multidrug resistance associated protein
PBMC: peripheral blood mononuclear cells
PSA: Prostate specifics antigen

R

RAG2$^{-/-}$: Recombination activation gene 2
RBC: Red blood cell
RDT: Rapid diagnostic test
RESA: ring-infected erythrocyte surface antigen
ROS: Reactive oxygen species
RI: Resistance index

S

SI: Selective index
SOD: Superoxide dismutase
SP: Sulfadoxine-pyrimethamine

T

TAMs: Tumor-associated macrophages
TNF α: Tumor necrosis factor α
TRAIL: TNF related apoptosis-including ligand

W

WHO: World Health Organization

X

XO: Xanthine oxidase

INTRODUCTION GENERALE

Les systèmes redox participent aux processus de signalisation cellulaire et sont essentiels au maintien des fonctions cellulaires durant un stress oxydant qu'il soit d'origine physique, chimique ou biologique. Les espèces réactives de l'oxygène (ROS) sont inhérentes à ces équilibres, en agissant comme réactifs de biosynthèse, messagers cellulaires ou éliminateurs d'agents exogènes. Ces espèces peuvent également infliger directement des dommages aux constituants cellulaires. Le terme "ROS" est commun aux radicaux libres oxygénés ainsi qu'à des espèces non radicalaires telles que le peroxyde d'hydrogène. Compte-tenu du rôle des ROS et des mécanismes d'oxydo-réduction dans diverses maladies, plusieurs approches thérapeutiques ont été développées pour moduler ou activer les réponses redox cellulaires. Si plusieurs stratégies thérapeutiques, le plus souvent à caractère antioxydant, ont été développées pour réduire l'impact des effets néfastes des ROS dans les maladies métaboliques, les stratégies à mettre en œuvre dans le cas des maladies infectieuses et des cancers sont beaucoup plus complexes. Dans le cas des cancers, cibler une cellule cancéreuse, par une approche redox (cytotoxiques pro-oxydants) sans endommager sa voisine, la cellule saine, est un challenge. Dans le cas des maladies infectieuses, la cellule hôte comme l'agent pathogène, ont des voies de signalisation, et des protéines redox, communes pour certaines, distinctes ou partagées pour d'autres. Les stratégies conventionnelles de découverte de nouvelles molécules actives, *via* l'identification de cibles, la conception de nouvelles molécules et la pharmaco-modulation, se révèlent souvent inefficaces à cause de cette imbrication. Le cas du paludisme constitue un tel exemple avec l'intervention de trois acteurs (vecteur, parasite, hôte) dans un cycle à deux niveaux, chez l'homme, exo- et intra-érythrocytaire. Dans ce contexte, pouvoir relier le caractère oxydo-réductible d'une molécule à son activité biologique constitue une étape, dans le processus de découverte, pour comprendre ses mécanismes d'action, améliorer ses propriétés pharmacologiques, voire réorienter ses applications.

Dans ce mémoire, deux sources moléculaires, de synthèse et naturelle, avec des propriétés biologiques avérées, ont été choisies pour étudier les liens entre les propriétés redox et les activités biologiques. Ces deux sources sont issues des travaux des deux équipes environnant le travail de thèse mené en co-tutelle entre Ho Chi Minh City et Toulouse. La série des indolone-*N*-oxydes, obtenue par synthèse organique, à fortes activités antipaludiques *in vitro* et

in vivo, constitue la première source. Les extraits ou substances isolées de *Crinum latifolium*, à réputation traditionnelle sur la longévité et les cancers, constitue la deuxième source.

Les propriétés antipaludiques des indolone-*N*-oxydes ont été démontrées *in vitro* (CI_{50} de l'ordre du nanomolaire) et *in vivo* au sein du laboratoire de Toulouse. Il a été démontré également que ces molécules subissent une bio-réduction au sein du globule rouge dépendante de réducteurs enzymatiques et moléculaires intra-érythrocytaires. Les questions posées dans ce travail portent sur le lien qui pourrait exister entre ces activités antiplasmodiales et le caractère oxydo-réductible, voir le potentiel redox de ces molécules, et les conséquences sur le mécanisme d'action intra-érythrocytaire.

La médecine traditionnelle est d'usage très répandu au Vietnam. *Crinum latifolium* est une plante largement utilisée depuis l'antiquité au Vietnam et dans de nombreux pays, pour traiter différents cancers, dont le cancer de la prostate, et les états inflammatoires. Les effets sur la longévité sont également rapportés. Le laboratoire d'Ho Chi Minh city travaille sur cette plante depuis plusieurs années, notamment pour qualifier les extraits issus de cette plante et pour isoler les molécules à l'origine de ces activités. Dans ce mémoire, il a été choisi d'étudier la capacité des extraits et composés purs isolés de *C. latifolium* à activer et à différencier des macrophages en relation avec les propriétés redox des extraits sur différents modèles. Il s'agit de démontrer l'intérêt de l'usage traditionnel de ces extraits pour améliorer l'état des patients atteints de certains cancers (cancer de la prostate par exemple).

Bien que plusieurs expériences aient été menées conjointement (propriétés redox), il a été choisi de présenter les résultats en deux parties, l'une traitant des indolone-*N*-oxydes (Partie **A**), l'autre traitant de *Crinum latifolium* (Partie **B**). Ainsi la partie **A** comprend une partie bibliographique et deux chapitres de résultats articulés autour de deux publications, l'une parue dans *Bioelectrochemistry*, l'autre acceptée dans *Journal of Inorganic Biochemistry*. La partie **B** comprend un chapitre bibliographique et un chapitre de résultats élaboré autour d'un manuscrit soumis à *Journal of Ethnopharmacology*.
Une conclusion générale présente un bilan des travaux.

GENERAL INTRODUCTION

Redox systems are involved in cell signaling processes and are essential for maintaining cellular functions during oxidative stress whether from physical, chemical or biological origin. The reactive oxygen species (ROS) are inherent in these functions, acting as intermediates in biosynthesis, cellular messengers or scavengers of exogenous agents. These species may also directly inflict damage on cellular components. The term "ROS" is common to oxygen-free radicals, as well as non-radical species such as hydrogen peroxide. Given the role of ROS and redox mechanisms in various diseases, several therapeutic approaches have been developed to modulate or activate cellular redox responses. While several therapeutic strategies, most often with antioxidant characteristics, have been developed to reduce the impact of the adverse effects of ROS in metabolic diseases, in the case of infectious diseases and cancers such strategies are much more complex. In the case of cancer, specifically targeting a cancer cell by a redox approach (cytotoxic pro-oxidant) without damaging the neighboring healthy cells is a challenge. In the case of infectious diseases, the host cell and the pathogen have signaling pathways and redox proteins, some of which are in common, while others are distinct or shared. Conventional approaches to the discovery of new active molecules, through the identification of targets, drug design and pharmacological modulation, are often ineffective because of this nesting. Malaria is such a complex example with the participation of three actors (vector, parasite and host) in a cycle at two levels in humans, exo-and intra-erythrocyte. In this context, establishing a relation between the character of a reducible redox molecule and its biological activity is a step in the discovery process, in order to understand the mechanisms of action and improve its pharmacological properties, even redirect its applications.

In this project, two molecular sources, synthetic and natural, with proven biological properties, were selected to study the relationship between redox properties and biological activities. These two sources are derived from the work of both teams involved in the thesis project, carried out in co-supervision between Ho Chi Minh City and Toulouse. The indolone-*N*-oxide series, obtained by organic synthesis, with high antimalarial activity *in vitro* and *in vivo*, was the primary source, and extracts or substances isolated from *Crinum latifolium* which has a traditional reputation for activity against some cancers and prolonging longivity, was the second source.

The antimalarial properties of indolone-*N*-oxides have been demonstrated *in vitro* (IC_{50} at the nanomolar level) and *in vivo* in the laboratory of Toulouse. It has also been shown that these molecules undergo bio-reduction in red blood cells depending on reducing enzymatic and molecular agents. The questions in this study focus on the possible link between these antimalarial activities and the oxido-reducible character and the redox potential of these molecules, and the consequences on the intra-erythrocytic mechanism of action.

Traditional medicine is widely used in Vietnam. *Crinum latifolium* is a plant that has been used since antiquity in Vietnam and in many countries to treat various cancers, including prostate cancer, as well as inflammatory states. Effects on longevity are also reported. The laboratory of Ho Chi Minh city has been working on this plant for several years, to qualify plant extracts and to isolate molecules behind the therapeutic activities. In this work, it was decided to investigate the ability of extracts and pure compounds isolated from *C. latifolium* to activate and differentiate macrophages in relation with the redox properties of the extracts on different models. This is to demonstrate the value of the traditional use of these extracts to improve the condition of patients with certain cancers (prostate cancer, for example).

Although several experiments have been jointly carried out (redox properties), it has been decided to present the results in two parts, one dealing with indolone-*N*-oxides (Part **A**), the other dealing with *Crinum latifolium* (Part **B**). Part **A** includes a bibliographic chapter and two chapters of results, organized around two publications, one published in *Bioelectrochemistry*, and another accepted in the *Journal of Inorganic Biochemistry*. Part **B** includes a bibliographic chapter and a chapter of results developed around a manuscript submitted to the *Journal of Ethnopharmacology*.

A general conclusion provides an overview of the results.

PART A.

REDOX PROPERTIES OF INDOLONE-*N*-OXIDE (INODs) IN RELATION TO THEIR ANTIMALARIAL PROPERTIES

I. BIBLIOGRAPHY

1. Malaria

a. Description and distribution

Malaria is a life-threatening disease caused by parasite *Plasmodium (P.)* including five species: *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi*. There are at least 225 millions infection cases and 781,000 deaths per year (WHO, 2010). Among them, *P. falciparum* is the most dangerous species and lead to 90% of the deaths in malaria (Petersen *et al*, 2011). It is transmitted by infected female Anopheles mosquito. Malaria is one of important causes of morbidity and mortality in children in Africa (WHO, 2010). During pregnancy, infection by malaria may lead to low birth weight or mortality or adverse consequences in the longer term (Tarning *et al*, 2012).

Malaria is distributed in sub-tropical or tropical regions (Figure 1), especially in poor areas and the poverty accelerates the spread of the disease. The major malaria burden is in sub-Saharan Africa (Cui *et al*, 2012). Southeast Asia is considered as one of the most dangerous foci with high risk of new drug resistance due to various ecological systems together with diverse breeding habitats, creating a good condition for multiple mosquito vector species (Cui *et al*, 2012).

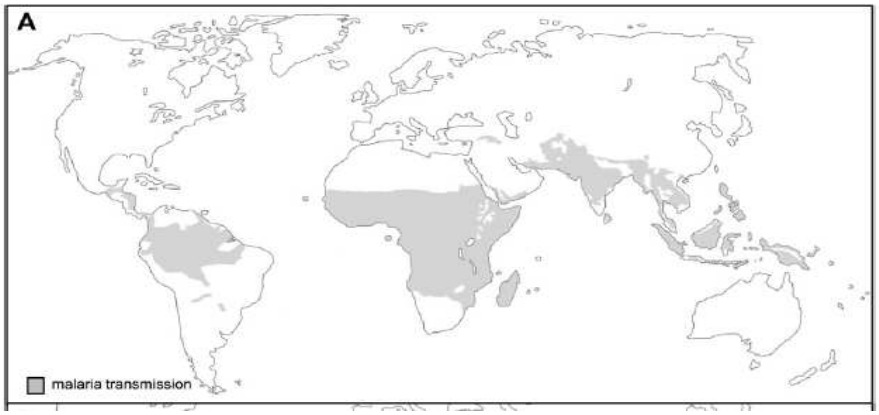


Figure 1. Distribution of malaria (Petersen *et al*, 2011)

b. The cycle of transmission

Plasmodial sporozoites enter human blood stream when infected female Anophele mosquito is having a blood meal. They rapidly migrate to the liver where they infect and proliferate. There are no symptoms or any abnormal liver function in this state. About a week after, merozoites

are released into the circulation from the hepatocytes and invade erythrocytes. It initiates intraerythrocytic cycle. Merozoites feed on hemoglobin, develop and replicate asexually to become schizont. This process is called schizogony. When schizonts are mature in erythrocyte, they rupture out of it and infect other healthy erythrocytes (Figure 2). Complete asexual replication cycle lasts every 72 hours (*P. malariae*), 48 hours (*P. falciparum*, *P. vivax*, *P. ovale*) and 24 hours (*P. knowlesi*). Clinical symptoms are developed as the result of rupture of schizont.

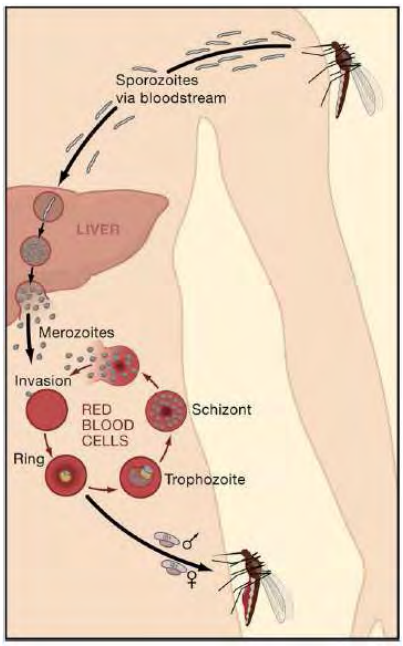


Figure 2. Life cycle of *Plasmodium falciparum* in human host and Anopheles mosquito vector (Cowman *et al*, 2006).

c. The clinical forms

Malaria doesn't cause any specific symptoms. In general, it includes fever, running nose, cough, diarrhoea/dysentery, burning micturition and/or lower abdominal pain, skin rash/infections, abscess, painful swelling of joints, ear discharge, lymphadenopathy (WHO, 2009). Most important clinical feature in malaria bases on fever, often irregular or continuous at one set. This lack of specific symptoms leads to over-treatment because there are many possible causes of fever. To get a good treatment, it is recommended to do parasitological diagnosis (WHO, 2009).

d. Diagnostic

The early diagnostic helps to reduce the chance of death. Diagnosis is performed either by rapid diagnostic test (RDT) or by microscopy (WHO, 2011). These two methods which are

inexpensive which provide information about species and number of infected erythrocytes. Microscopy requires well-trained employees to avoid making error in species identification and parasite density estimation. While thick blood films are used to identify Plamsodium species, thin blood films is used for checking morphologic parasites (Wongsrichanalai *et al*, 2007). In order to overcome the disadvantages of blood smear test, RDT was developed. RDT is applied to detect malaria parasite antigen in finger-prick blood samples which require minimal skill to perform and interpret. Most of RDTs detect *P. falciparum* with high sensitivity (>95%) (Wongsrichanalai *et al*, 2007). Beside RDT and microscopy, polymerase chain reaction (PCR) provides high sensitivity and specificity but is expensive and requires well-trained employees (Suh *et al*, 2004). Finally, diagnosis is extremely important to select the appropriate treatment to cure patients and contribute to malaria prevention and control.

e. Drug resistance and treatments

Drug resistance

Control of malaria has become a big challenge since drug resistance was found in current antimalarials (Figure 3). Drug resistance is influenced by many factors i) the mutation rate of the parasite, ii) the fitness costs associated with the resistance mutation, iii) the overall parasite load, iv) the strength of drug selection, v) the treatment compliance (Petersen *et al*, 2011). Mutation allows parasites to adapt to environmental change. Mutation rate in *P. falciparum* dihydropteroate synthetase (PfDHFR) gene is relatively low (10^{-9}) (Paget-McNicol *et al*, 2001). However, accelerated resistance to multiple drugs (ARMD) phenotype in South East Asia leads to new resistance which is able to fight against the new drug effect (Rathod *et al*, 1997). Therefore, mutation in parasite is capable to change drugs' active site so that drugs are not able to reach their targets. Nevertheless, mutation causes not only new resistances but also vital dysfunction. It is called fitness cost which plays an important role in drug resistance selection. In addition, a strong drug selection pressure creates fast environmental change that evidently accelerates drug resistance. Finally, improper treatment (inadequate drugs, dosing problem) is one important reason of drug resistance in malaria (Muller, 2011).

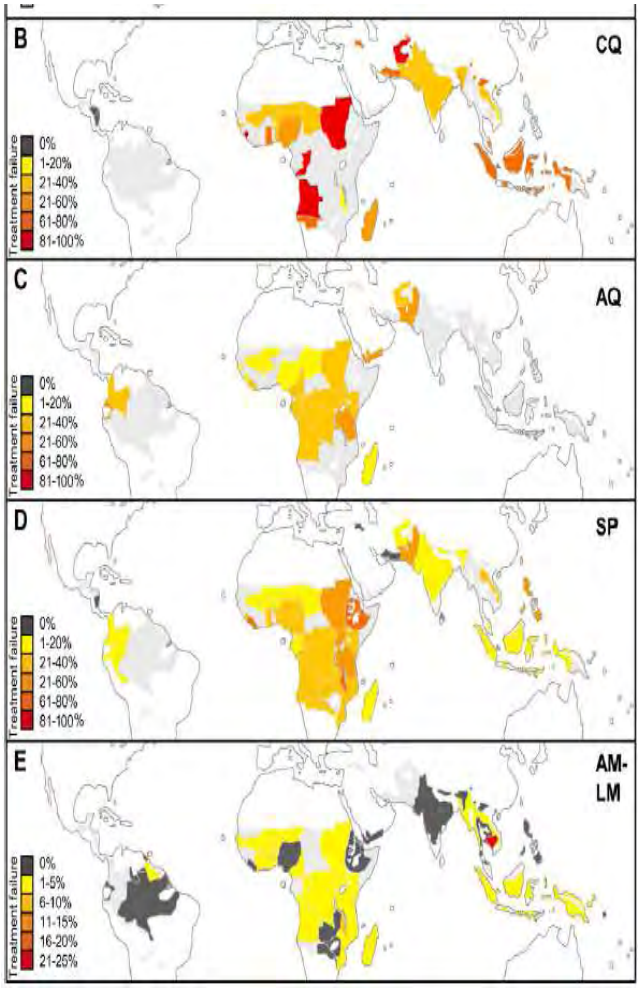


Figure 3. Drug resistance distribution in malaria. (B) Chloroquine treatment failure, (C) amodiaquine treatment failure, (D) sulfadoxine–pyrimethamine (Fansidar) treatment failure, (E) artemether–lumefantrine (Coartem) treatment failure. (CQ: chloroquine; AQ: amodiaquine; SP: sulfadoxine–pyrimethamine; AM: artemether; LM: lumefantrine) (Petersen *et al*, 2011).

Drug resistance in antimalarials is mainly due to mutation in seven genes: *P. falciparum* chloroquine resistance transporter (*Pf*CRT), *P. falciparum* multidrug resistance transporter 1 (*Pf*MDR1) and *P. falciparum* multidrug resistance-associated proteine (*Pf*MRP), *P. falciparum* Na^+/H^+ (*Pf*NHE), dihydropteroate synthase (*Pf*DHPS), dihydrofolate reductase-thymidylate synthase (*Pf*DHFR), ubiquinol binding site of cytochrome bc_1 complex. *Pf*CRT and *Pf*MDR1 are located within the digestive vacuole (DV) membrane (Cowman *et al*, 1991). *Pf*CRT acts as an active transport to pump drugs out from DV (Sanchez *et al*, 2007). *Pf*MDR1 is active transport that facilitates the accumulation of drugs in DV (Rohrbach *et al*, 2006), (van Es *et al*, 1994). *Pf*MRP resides at the parasitic plasma membrane and function as a drug efflux (Raj *et al*, 2009). *Pf*NHE functions as a source of energy, a pH control by anaerobic glycolysis and by an active transport of protons in the parasite (Bosia *et al*, 1993). The two genes *Pf*DHPS and

*Pf*DHFR encode for two important enzymes in folate pathway which are essential for DNA replication and metabolism of amino acids. *Pf*DHPS is responsible for folate precursors formation. *Pf*DHFR reduces dihydrofolate into tetrahydrofolate (Hyde *et al*, 2005). Cytochrome bc_1 complex is the complex III in electron transport chain responsible for ATP formation.
Although resistance occurs in current antimalarials, they are still administered as part of combination therapy, base on their different targets within parasite.

Quinine which is an active alkaloid with an aryl-amino alcohol structure is the oldest antimalarial. It was first isolated in 1820 from the bark of cinchona tree (Butler *et al*, 2010). Its mechanism of action is not fully understood. It accumulates in parasites DV and then inhibits the detoxification of heme (Fitch, 2004). It has short half-life with 8-10 hours. The resistance to quinine is complex including multiple mutations in three genes *Pf*CRT, *Pf*MDR1 and *Pf*NHE1 (Sidhu *et al*, 2005), (Nkrumah *et al*, 2009), (Cooper *et al*, 2002), (Cooper *et al*, 2007). Nowadays, quinine is used in second line of malarial treatment and in combination with antibiotics (Petersen *et al*, 2011).

Chloroquine (CQ), a synthesized 4-aminoquinoline derivative, was first introduced in the 1940s. It was successfully used for ten years after first introduction based on its highly efficacy, affordability and safety, especially in pregnancy (Alkadi, 2007). It is useful for chemoprophylactic effect because of 60 days long half-life (Stepniewska *et al*, 2008). The mechanism of action of CQ was intensively studied (Figure 4). CQ is a weak base, pKa 8.1-10.2 that remains uncharged in neutral condition of blood (Martin *et al*, 2009). It can freely cross membrane by passive diffusion and becomes diprotonated in acidic medium within DV. In charged form, CQ is not able to pass the membrane (Martin *et al*, 2009). Therefore, it binds to hematin, resulting in prevention of heme detoxification and death of parasite (Fitch, 2004). CQ resistance began at the Thai-Cambodian border and in Colombia in the 1950s (Mita *et al*, 2009). Then the resistance spread to Africa in the 1970s (Mita *et al*, 2009). Cross resistance between CQ and amodiaquine was reported due to their similarity in chemical structure and mechanism of action. Polymorphisms in *Pf*CRT and *Pf*MDR1 mainly cause resistance to the two drugs (Sa *et al*, 2009) (Figure 4).

Amodiaquine is a 4-aminoquinoline derivative that has been used as an antimalarial for 70 years. Even though its half-life is short ($t_{1/2}$ = 3 hours), its primary metabolite,

monodesethylamodiaquine, has a longer half-life ($t_{1/2}$ = 9 - 18 days) (Stepniewska *et al*, 2008). The mechanism of action and resistance is similar to CQ (Figure 4).

Mefloquine was first introduced in the 1970s (Trenholme *et al*, 1975). It is a 4-methanolquinoloneline with $t_{1/2}$ = 14 - 18 days (Stepniewska *et al*, 2008). Its mechanism remains unclear. Its target properly locates outside DV. One of its actions could be inhibition the transportation of some solutes to DV. Mefloquine resistance arises by overexpression of *Pf*MDR1 or *Pf*MRP (Rohrbach *et al*, 2006). While *Pf*MDR1 facilitates the accumulation of mefloquine inside DV and prevents the interaction between the drug and its target molecule, *Pf*MRP prevents the drug to enter inside the parasitic cellular space (Figure 4).

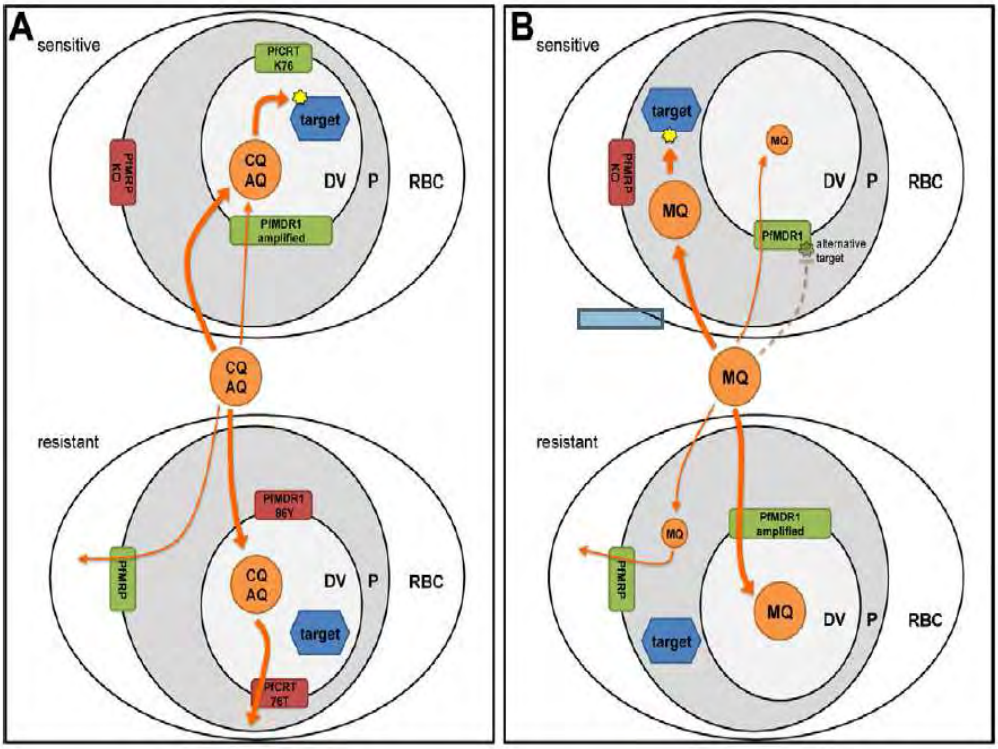


Figure 4. Pathway of action and drug resistance. (A) Chloroquine (CQ) and amodiaquine (AQ), (B) mefloquine (MQ), red blood cells (RBC), parasite (P), digestive vacuole (DV), *P. falciparum* chloroquine resistance transporter (*Pf*CRT), *P. falciparum* multidrug resistance transporter 1 (*Pf*MDR1) and *P. falciparum* multidrug resistance-associated proteine (*Pf*MRP) (Petersen *et al*, 2011).

Piperaquine belongs to bis-4-aminoquinoline derivatives with a long half-life $t_{1/2}$ = 5 weeks. The mechanism of action is not fully understood. Resistance to piperaquine rose due to intensive monotherapy in China in the late 1970s. Mutation in PfCRT particularly causes resistance to piperaquine (Muangnoicharoen *et al*, 2009). It is used now in artemisinin based combination therapy (Davis *et al*, 2005).

Lumefantrine is a hydrophobic aryaminoalcohol antimalarial. The mechanism of action is unknown. It has a half-life $t_{1/2}$ = 3 - 5 days (Ezzet *et al*, 1998). Polymorphisms in PfMDR1 (variant N86) reduce sensitivity of parasites to the drug (Figure 5) (Sisowath *et al*, 2007).

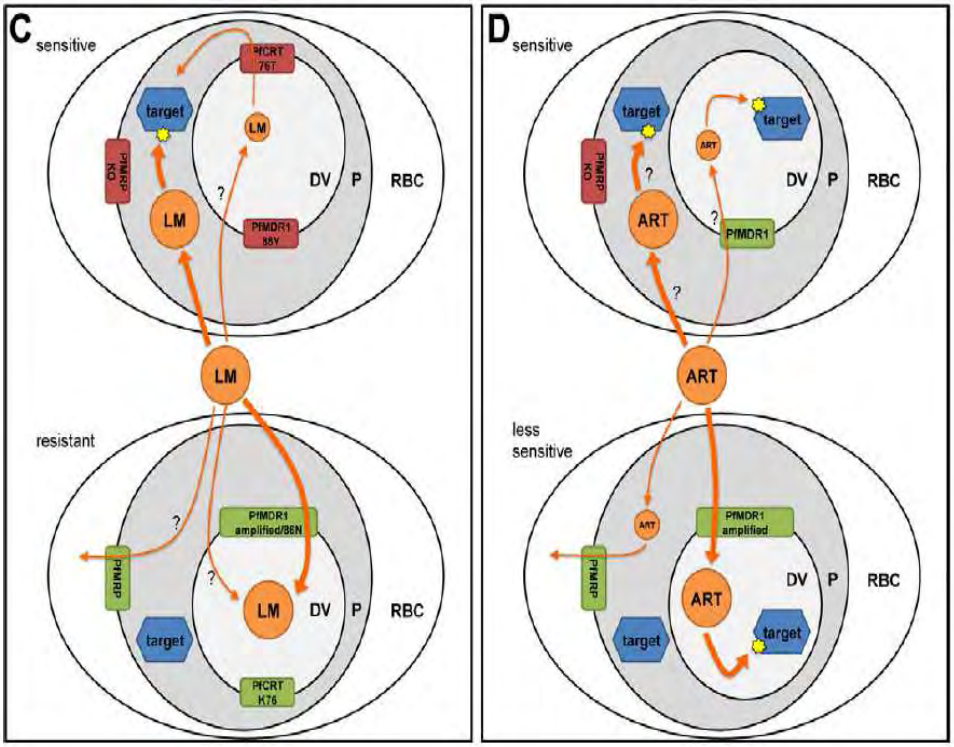


Figure 5. Pathway of action and drug resistance. (C) lumefantrine (LM), (D) artemisinin (ART) and its derivatives, red blood cells (RBC), parasite (P), digestive vacuole (DV), *P. falciparum* chloroquine resistance transporter (*Pf*CRT), *P. falciparum* multidrug resistance transporter 1 (*Pf*MDR1) and *P. falciparum* multidrug resistance-associated proteine (*Pf*MRP) (Petersen *et al*, 2011).

Primaquine is a 8-aminoquinoline with $t_{1/2}$ = 6 h (Edwards *et al*, 1993). It is used for *P. vivax* hypnozoite liver stages. Unfortunately, it causes hemolytic anemia in glucose-6-phosphate dehydrogenase (G6PD) deficiency. It shows synergistic action with CQ by binding to *Pf*CRT and by inhibiting of CQ efflux (Sanchez *et al*, 2004).

Atovaquone inhibits electron transport chain in mitochondria due to its chemical similarity to ubiquinol, a liphophilic hydroxynaphthoquinone. Its target molecule is cytochrom bc_1. Half-life is $t_{1/2}$ = 2 - 3 days (Hughes *et al*, 1991). It is indicated as an anti-parasitical drug for Plasmodium, Toxoplasma, Theileria, Babesia (Srivastava *et al*, 1997). Since atovaquone resistance appeared because of mutation at the ubiquinol binding site of cytochrom bc_1, atovaquone is used in combination with proguanil, in prophylactic medication for tourists (Gil *et al*, 2003).

Antifolate drugs including sulfadoxine and dapson inhibit dihydropteroate synthetase (*Pf*DHPS), while pyrimethamine and proguanil inhibit dihydrofolate reductase (*Pf*DHFR). *Pf*DHPS is responsible for folate precursors production. The function of *Pf*DHFR is to reduce dihydrofolate to tetrahydrofolate. After chloroquine resistance, sulfadoxine-pyrimethamine ($t_{1/2}$ = 4 - 5 days) was introduced as highly effective, cheap and well-tolerated antimalarials. Resistance also happened due to point mutation in these two enzymes *Pf*DHPS and *Pf*DHFR (Uhlemann *et al*, 2005). Antifolate drugs are still used in malaria for prevention during pregnancy. Combination of dapson-proguanil was withdrawn from the market since there was high risk of hemolysis in G6PD deficiency (Luzzatto, 2010).

Artemisinin is a natural compound originated from *Artemisia annua* plant. In order to improve its poor solubility, semi-synthetic artemisinin derivatives (artemether, artesunate, dihydroartemisinin) were developed and applied in malaria. It contains one endoperoxide bond which is essential for antimalarial activity (Eastman *et al*, 2009). Endoperoxide bond is cleaved by ferrous iron present in heme or by specific protein which can be found in DV and cytosolic compartment. This endoperoxide cleavage results in the formation of reactive radicals, following by death of parasites (Bray *et al*, 2005). Partial artemisinin resistance is caused by *Pf*MDR1 amplification, suggesting accumulation of artemisinin in DV (Figure 5). To prolong life span of artemisinin, a strong drug selection pressure must be avoided and artemisinin based combination therapy should be applied (artemether - lumefantrine, artesunate - mefloquine, artesunate - modiaquine, artesunate – sulfadoxine - pyrimethamine, dihydroartemisinin-piperaquine and artesunate-pyronaridine) (Muller *et al*, 2009)

Table 1. Pathway of action and resistance of antimalarial drugs.

Drugs	Pathway of action	Resistance mechanism (Mutation)
Chloroquine Amodiaquine Quinine Lumefantrine	Entering to DV to inhibit haem polymerization	Mutation in *Pf*CRT. Result: drug is directly pumped out of DV via an active transport (mutated *Pf*CRT)
Lumefantrine Artemisinin Quinine Mefloquine Halofantrine (Increase resistance of CQ)		Mutation in *Pf*MDR1 at amino acid position 86, 184, 1034, 1042 and 1246. Result: drug is indirectly pumped out of DV by affecting intracellular ion gradients (Cl^-) or pH.
Chloroquine Quinine Artemisinin Piperaquine Primaquine		Mutation in *Pf*MRP. Result: drugs and other metabolites are pumped out of parasite.
Quinine	Entering to DV to inhibit haem polymerization	Mutation in *Pf*NHE1. Result: Quinine cannot reach target molecule
Sulfadoxin and dapson	Inhibition of folate pathway.	Mutation in *Pf*DHPS. Result: folate pathway is not inhibited
Pyrimethamine and cycloguanil	Inhibition of folate pathway	Mutation in *Pf*DHFR. Result: folate pathway is not inhibited
Sulfadoxine-pyrimethamine	Inhibition of folate pathway	Mutation point in PfDHPS and PfDHFR. Result: folate pathway is not inhibited
Atovaquone	Inhibition of electron transport chain by inhibiting transfers electron from ubiquinol to cytochrom *c*	Altering atovaquone binding site of cytochrom bc_1. Result: Atovaquone cannot reach target molecule

Treatment

For uncomplicated malaria, artemisinin should never be given as monotherapy which could rapidly cause resistance. Artemisinin Combination Therapy (ACT) is a combination between artemisinin derivatives and other antimalarials (amodiaquine, lumefantrine, mefloquine or sulfadoxine-pyrimethamine). Some combinations have been used in resistance area, such as artesunate + sulfadoxine-pyrimethamine (SP), artemether + lumefantrine, artesunate + mefloquine. ACTs could be safely given during the second and third trimester of pregnancy. For the first trimester of pregnancy, quinine is recommended (WHO, 2009).

To treat complicated malaria, artemisinin derivatives (artesunate, artemether, arteether) and quinine are used via intravenous or intramuscular administration at the beginning. Patients then take oral drugs, quinine and doxycycline are recommended for following 7 days (WHO, 2009). The method of preventing malaria for travelers is also mentioned in Table 2.

Table 2. Treatments against malaria (recommendation from WHO, 2009)

Clinical forms	Parasites/ Administration	Medicines	Dosages
Uncomplicated malaria	*P. vivax*	Chloroquine	25 mg/kg for 3 days
		Primaquine (for prevention)	0.25 mg/kg for 14 days
	P. falciparum	Chloroquine (in sensitive area)	25 mg/kg for 3 days
		Artemisinin Combination Therapy (ACT)	Artesunate: 50 mg x 4/day for 3 days Sulfadoxine: 500 mg x 3 for 1st day
	Mixed infected	Treated as *P. falciparum*	
Severe malaria	Intravenous (i.v.) Intramascular (i.m.)	Artesunate	2.4 mg/kg i.v. or i.m. at t=0, then at 12 h and at 24 h, then once a day
		Quinine	20 mg quinine salt/kg i.v. after every 4 hours
		Artemether	3.2 mg/kg i.m. at t=0, then 1.6 mg/kg per day.
		Arteether	150 mg daily i.m. for 3 days
	Oral	Quinine + doxycycline	Quinine: 10 mg/kg x3/day for 7 days Doxycycline: 3 mg/kg/ day for 7 days
Chemoprophylaxis		Doxycycline (less than 6 weeks travelling)	100 mg daily, start 2 days before travel, 4 weeks after leaving malarious area
		Mefloquine (more than 6 weeks travelling)	5 mg/kg daily, start 2 weeks before travel, 4 weeks after leaving malarious area

f. Malaria in South East Asia

Although many countries in Asia such as Indonesia, Malaysia, Philippine are free from malaria, the Greater Mekong subregion (GMK) is still affected by malaria. Resistance to chloroquine and antifolates was first developed here and spread to other parts of the world. Resistance to quinine and mefloquine is also rising. Since partial resistance to artemisinin was reported in 2008 in Cambodia (Noedl *et al*, 2008), artemisinin resistance gradually spreads to different area out of the GMK including six countries Viet Nam, Cambodia and Thailand, Laos, Mynamar, China (Yunnan province) (Figure 6). There are various distributions of malaria over South East Asia. The common and major endemic regions are in forest, cross-border, small community with poor health infrastructure (Cui *et al*, 2012). Monitoring and controlling malaria are difficult in these areas because of some common reasons.

- Poor diagnosis without parasitologic confirmation leads to inadequate treatment.
- Artemisinin monotherapy by self-treatment is still common although WHO recommends ACTs as the first line treatment in malaria (Butler, 2009).
- Many antimalarial drugs are substandard or counterfeit (Vijaykadga *et al*, 2006), (Lon *et al*, 2006), (Dondorp *et al*, 2004). It results in reduction of efficacy and the

development of resistance as well as life-threatening. Counterfeit can be substandard or expired and degraded or can contain very small amount of active compounds. They are freely sold in private markets in these developing countries. It is difficult to combat the counterfeit because government agencies (the customs or the police) are not well-trained enough to recognize the counterfeits.

- Malaria burden is heaviest in borders between these countries due to several socio-cultural, economic, environmental and political factors. The heavy population flows between these borders because of many reasons such as finding better jobs, escaping from political fighting, wars and diseases (Martens *et al*, 2000). Especially, border line along with forest is an ideal environment for mosquitoes living and malarial epidemic. In these areas, many ethnic minorities stay in small isolated community with poor health care system and health education. Borders between these countries are the biggest reservoir of malarial infection which makes the control of the disease even more difficult. The cross-border malaria control could be feasible but it is slowly established.

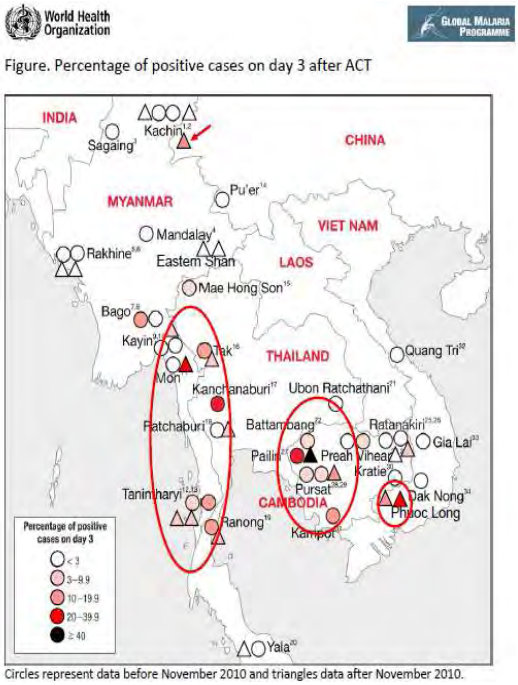


Figure 6. Resistance of artemisinin and its derivatives in Mekong area (WHO, 2012).

Cambodia is the epicenter of drug resistance in malaria since resistance to chloroquine, antifolates and artemisinin were first reported here and spread through the world at accelerated rate (Anderson *et al*, 2005), (Noedl *et al*, 2009), (Noedl *et al*, 2010). Malarial incidence is

highest in western region of the country where is the border line between Cambodia and Thailand. Multiple Drugs Resistance (MDR) is the most dangerous problem in this area, causing high risk to develop drug resistance to new antimalarials (Rathod *et al*, 1997).

In **China**, highest malarial incidence areas are in Central China, Hainan Island and Yunnan provinces (border lines with Myanmar, Laos and Viet Nam). Malarial transmission peaks occur in May-July and October-November. *P. vivax* is the most dominant species. *P. falciparum* is only found in subtropical Hainan and Yunnan province. Due to China's national malaria control program, malaria cases dropped from 6.79 million cases in 1954 (Yip, 1998) to 29 039 cases in 2000 (Cui *et al*, 2012). Prevention of malaria reintroduction and management of drugs resistance are challenges because of the 4000 km border line with three malarious countries (Lin *et al*, 2009). Resistance to artemisinin and its derivatives is rising in Yunnan province where artemisinin has been used for the longest time (Cui *et al*, 2012).

In **Laos**, malarial situation is getting better. The number of cases was reduced from 63 736 cases in 2000 to 18 740 in 2008 (WHO, 2009). The endemic area is in the south part of the country (Delacollette *et al*, 2009). The management of drug resistance is difficult since the rate of self-medication is high and counterfeit antimalarials are out of control (Cui *et al*, 2012).
Myanmar is considered as the heaviest malaria burden since there is the highest death rate in GMK, for example 75% all of death in 2007 (WHO, 2008). The highest malarial incidence areas include forest and forest-fringe areas of Kachin, Rakhine states and Sagaing Division. *P. falciparum* is the most abundance species. Malarial outbreaks often occur. The most serious one, about a thousand deaths, was reported in November 2003 in Shan and Kachin (Li *et al*, 2005). Poverty and inadequate health care system are the major reasons that make a huge chanllenge in malaria control, especially in the border areas with minorities and mobile workers.

In **Thailand**, malaria mostly accumulates in Yala, Narathiwat and Sa Kaeo province. All of the five different Plasmodium species have been reported in Thailand (Sattabongkot *et al*, 2004), (Zhou *et al*, 1998) (Putaporntip *et al*, 2009). The most common species is *P. vivax* (Sattabongkot *et al*, 2004). High level of *P. falciparum*, *P. malariae* and *P.ovale* are also observed. There are only few reported cases of *P. knowlesi*. Two annual endemic peaks occur in raining season. Malaria situation has been dramatically improved in the country, for example 63 528 infected cases in 2001 reduce to 26 150 cases in 2008 (WHO, 2009). The

problems in malaria control in Thailand are pretty similar to the neighbor countries such as heavy population migration between border with Myanmar and Cambodia, life style and behavioral factors of minorities. In addition, high proportion of multiple drugs resistance (MDR) exists in border area which contributes to the difficulty of the control program and accelerates the resistance to ACTs (Cui *et al*, 2012).

In **Viet Nam**, malaria was the major cause of morbidity and mortality. There are two dominant species, *P. falciparum* and *P. vivax* and a few amount of *P. knowlesi*. Malaria mainly accumulates in Central and in South of Viet Nam especially in remote area, central highland near to the border of Viet Nam-Cambodia and Viet Nam-Laos (Figure 7.). In the central highland, nearly 80 % of the severe cases have been observed each year. The country is also known to undergo annually natural disasters such as drought, typhoons, floods which are favorable to trigger malarial epidemics. In general, the malarial epidemic peak occurs in raining season, between June and December (Thang *et al*, 2009). In 1991, there were 144 epidemics of malaria that caused 1 672 000 uncomplicated cases and 32 000 severe malaria with 4 650 deaths (Hung *et al*, 2002). At the same year, National Malaria Control Programme (NMCP) was launched, together with fast economic development and strong international support. This resulted in dramatical reduction of malaria. In order to control malaria, some important strategies have been done:

- The national insecticide-treated bed net (ITN) campaign: Impregnated-bed nets with permethrin were freely supplied. Bed nets were pre-impregnated twice a year (Hung *et al*, 2002).
- Indoor residual spraying (IRS) to prevent people from mosquitoes biting (Hung *et al*, 2002).
- Health education through media (television or radio) or directly to locals by volunteer health workers that educate people to prevent mosquitoes at home and to act when someone is infected by malaria (Hung *et al*, 2002).
- Producing high quality and low cost of artemisinin (from Thanh Hao tree, *Artemisia annua* traditionally used in Chinese and Viet Nam) and its derivatives (WHO, 2010).
- Free diagnosis and treatment of malaria (Hung *et al*, 2002).

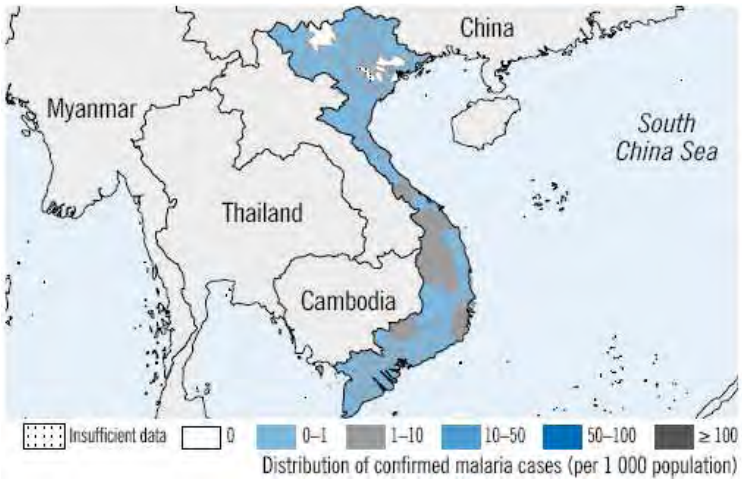


Figure 7. Distribution of malaria in Viet Nam (WHO, 2010b).

Within five years 1992-1997, the number of deaths by malaria was reduced by 97% (Figure 8) (WHO, 2010). There were only a few small outbreaks of malaria in 1997 (WHO, 2010). There was not any epidemic peaks in 2006 (Thang *et al*, 2009). Nowadays, malaria is under control in Viet Nam but it can't be totally eliminated due to remote area, ethnic minorities (without using insecticide-treated bed net and indoor spray) and migrate workers between Viet Nam - Cambodia, Laos and China (active carriers).

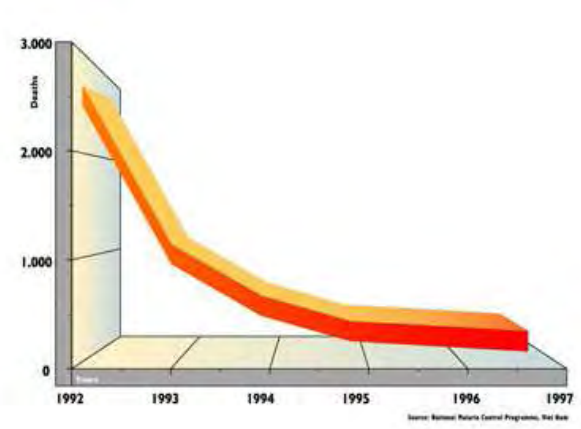


Figure 8. Free treatment and insecticide-treated nets reduce malaria deaths in Viet Nam (WHO, 2010).

In Viet Nam, ACTs was applied as the first line treatment in all malarial area since 2003 when resistance to chloroquine and sulfadoxine–pyrimethamine was already widespread. A ten-year (1998-2008) study showed a stable sensitivity of *P. falciparum* to artemisinin in Viet Nam (Thanh *et al*, 2010). It is important to prevent resistance to artemisinin by cross-border malaria control, discouraging monotherapy, self-therapy, inadequate treatment and usage of poor quality medicines.

Together with increased government investment and international supports, malaria situation in GMK has been improved and controlled. Unfortunately, there are many obstacles that these countries should face before reaching malaria elimination. These common obstacles include border malaria, counterfeit antimalarials and MDR *P. falciparum*. In addition, resistance to artemisinin and its derivatives has been developed and spread among these nations. In one hand, it requires to coordinate work between these countries to control border malaria, improve the diagnosis and treatment regimen, and in order to slow down resistance spread. In another hand, it is urgent to develop new antimalarials until the end of this decade.

2. Indolone-*N*-oxides

At a time when there is an unprecedented global effort to control and potentially eliminate malaria, antimalarial resistance remains the single most important obstacle to success. Therefore new alternative therapeutic agents with novel chemical scaffolds and new mechanisms of action are strongly and continuously needed. The series of indolone-*N*-oxides (INODs) (Figure 9) with strong antimalarial properties at low nanomolar concentration in vitro has been developed in our group in this context (Nepveu et al, 2010).

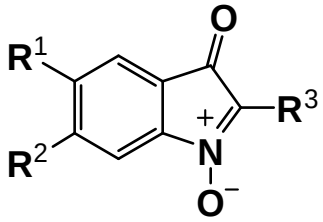


Figure 9: Structure of the indolone *N*-oxides (INODs)

Several new chemical classes with potent blood schizontocidal activity *in vitro* have been identified in the two past decades. These include endoperoxides (Pandey *et al*, 1999), (Gemma *et al*, 2009) inhibitors of phospholipid biosynthesis (Vial *et al*, 2003), ferroquines (Domarle *et al*, 1998), trioxaquines (Coslédan *et al*, 2008), imidazole-piperazines (A. Nagle *et al*, 2012), pyrazino-indole-diones (Beghyn *et al*, 2012), stilbene-chalcone hybrids (Sharma *et al*, 2012), and spiroindolones (Rottmann *et al*, 2010). folate anatagonist (Martyn *et al*, 2010), antibiotics (Rosenthal, 2003), iron chelators (Ferrer *et al*, 2012), new natural products (Muiva *et al*, 2009). According to Medicines for Malaria Venture (www.mmv.org), new drug candidates are ACTs (artemether + lumefantrine, dihydroartemisinin + piperaquine), combination of different antimalarials (sulfadoxine-pyrimethamine + amodiaquine), combination of antimalarial and antibiotics (azithromycin + chloroquine), synthesized current antimalarial derivatives (primaquine and artemisinin derivatives). Thus, the new coming antimalarials are ACTs or developed analogs of existing agents. We are then wondering “how long do these new agents successfully fight against malarial parasites when they have the same mutated target as their origin analogs?”. Therefore, it is necessary to have new agents with new mechanism of action before ARTs resistance totally arises and resistant parasites spread to higher transmission areas such as Africa. Among new chemical groups with antimalarial activity, indolone-*N*-oxide (INODs) (Figure 9) developed in our research group show potent and high promising antimalarials properties with low toxicity (Nepveu *et al*, 2010).

Indolone-*N*-oxide derivatives belong to a novel chemical class of potent antimalarial activity without any chemical similarities to aminoquinolines and sesquiterpene lactones.

a. Physico-chemical, biochemical and biological properties

Indolone-*N*-oxides, (3-oxo-3*H*-indole-1-oxides), commonly known as isatogens, were described more than 100 years ago. The first isatogen (3-oxo-1-oxy-3h-indole-2-carboxylic acid ethyl ester) was reported by Baeyer as early as 1881 (Baeyer, 1881). These compounds are brightly colored solids that do not occur naturally (Slatt and Bergman, 2002). After the synthesis of the first isatogen, the research on this family was continued by Pfeiffer and Hooper and other groups to find that isatogens can readily undergo nucleophilic attack in the 2-position (Pfeiffer, 1916), (Hooper *et al*, 1965). Later on, various biological activities have been attributed to this interesting chemical scaffold. In mammalian systems, isatogens are known as inhibitors of the synthesis of adenosine triphosphate (ATP) in mitochondria (Sweetman *et al*, 1971), (Hooper *et al*, 1974). Anti-tuberculosis (anti-mycobacterial) activities have been reported for some indolone-*N*-oxides (Sahasrabudhe *et al*, 1980), (Ibrahim *et al*, 2012). In *in vitro* screening tests, isatogens showed antibacterial (Hooper *et al*, 1965), antifungal (Hagen *et al*, 1982) and antileishmanial (Ibrahim *et al*, 2012) potencies. Moreover, they elicit smooth muscle relaxation (Spedding and Weetman, 1978), (Foster *et al*, 1983), and have neuro-protective (Menton *et al*, 2002) and hypertensive activities (Wihlborg *et al*, 2003).

The *N*-oxide redox functional group has shown its importance to generate new bio-active compounds in various pharmacological fields. This can be illustrated by the chlordiazepoxide introduced in therapy as tranquilizer in the 1960's and more recently the aliphatic or heteroaromatic *N*-oxides bioreductive drugs to treat hypoxic cancer cells (McKeown *et al*, 2007), (Solano *et al*, 2007). Recently, indazole *N*-oxide derivatives have been reported as antiprotozoal agents with a reduction mechanism (Gerpe *et al*, 2006).

The indolone-*N*-oxides contain the nitrone moiety, a strong dipole, ($C=N^{+}-O^{-}$), which is highly reactive. This nitrone group is the chemical scaffold of radical spin traps. This led the laboratory to study the spin trapping properties of some indolone derivatives revealing that the phenylisatogen derivatives are capable of trapping hydroxyl and superoxide radicals (Figure

10) (Nepveu *et al*, 1998), (Boyer *et al*, 2004). Additionally, 2-aryl-indolone-*N*-oxides gave very stable spin adducts by trapping oxygen or carbon-centered free radicals chemically or enzymatically produced, while it was not the case for 2-alkyl derivatives.

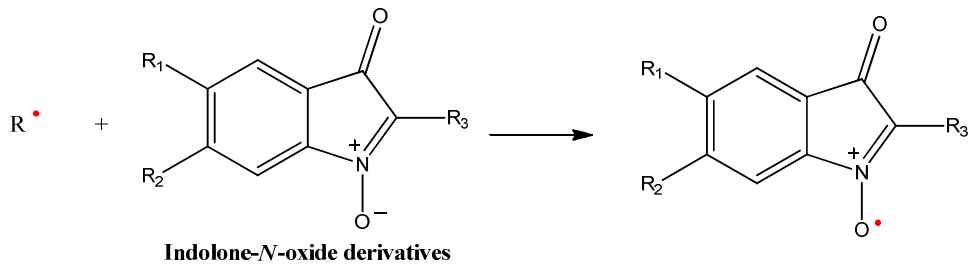


Figure 10. Spin adduct formation by indolone-*N*-oxide.

The 2-alkyl-indolone-*N*-oxides were rapidly reduced in solution leading to radical nitroxide intermediates while 2-aryl-indolone-*N*-oxides were stable in solution. Moreover, alkyl nitrones are instantly decomposed by ethanol while aryl nitrones are commonly recrystallized from ethanol (Hamer and Macaluso, 1964). Under certain conditions (Lunazzi *et al*, 1967), very long-lived radicals were generated via a proton attack from the solvent by heating up these indolones. In addition, indolone derivatives can be easily reduced by thiols (Danieli and Maccagnani, 1965). Considering these physicochemical properties, especially the indolone-N-oxide reducible character, their ability to generate stable radical intermediates and their reactivity with thiols our team has hypothesized that this may be critical for parasite growth within red blood cells (RBCs) (Nepveu *et al*, 2010). RBCs contain high levels of reductases and reductants, such as glutathione, and display an increase in reactive oxygen species during parasite maturation. This hypothesis was tested, the antimalarial activities of INODs were demonstrated and a drug research program was launched.

It was mentioned earlier that isatogens may exert their biological action by oxidizing molecules which are present in natural redox systems (Bunney *et al*, 1970) and inhibit activity of transhydrogenase enzyme by reacting with nucleophiles such as thiols (Figure 11) (Sweetman *et al*, 1974).

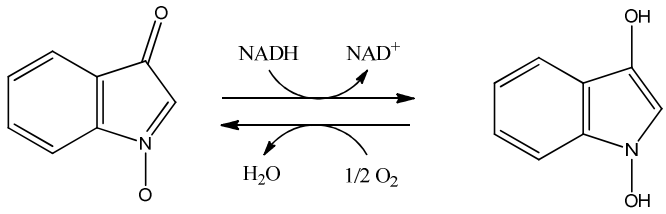


Figure 11. Reaction between indolone-*N*-oxide and transhydrogenase (Katrizky, 1978).

The nitrone group can hence be called a *redox pharmacophore* because, from the pharmacological point of view, reducible chemical groups may be able to disrupt cellular redox homeostasis. Isatogens have properties similar to those of quinones (Pfeiffer, 1916), (Bunney *et al*, 1970) and they form adducts analogous with quinhydrones (Hiremath and Hooper, 1978). The reaction between INODs and transhydrogenase leads to the inhibition of the ATP synthesis. This reaction was also used to identify the location of the enzyme, in the outside of the inner membrane of mitochondria (Katrizky, 1978). INODs are able to modify the mitochondrial metabolism, resulting in smooth muscle relaxation, ATP receptor antagonism and inhibition of ATP synthesis (Foster et al, 1983). These effects are not specific (Foster et al, 1983). The role of mitochondrial modification due to nitrone is not fully understood. It could be involved in all pharmacological properties since the mitochondria are important energy suppliers and the cell-death regulators at cellular level.

b. Antimalarial properties

The series of indolone-*N*-oxides (INODs), developed in our group as part of search for new antimalarial molecular scaffolds, has shown *in vitro* antiplasmodial activity in the nanomolar range against *Plasmodium falciparum* strains sensitive and resistant to current antimalarial agents (chloroquine and pyrimethamine) (Nepveu et al, 2010). Certain members of this family are even more active than chloroquine and artesunate with an IC_{50} less than 3 nM on FcB1 and equal to 1.7 nM on 3D7 (INOD-3) along with a very satisfactory selectivity index (CC_{50} MCF7/IC_{50} FcB1 = 14 623; CC_{50} KB/IC50 3D7 = 198 823) (Table 3). Initial screening of 64 derivatives have demonstrated that 15 of these derivatives inhibit *P. falciparum* growth at 50 % inhibitory concentration (IC_{50}) ≤ 100 nM (2 derivatives at IC_{50} ≤ 3 nM) and exhibit low cytotoxicity against MCF-7 and KB human cell lines. These promising results led the group to evaluate the *in vivo* antimalarial activity on *P. berghei*-infected murine model. However, on this model the best antimalarial activity did not exceed 62.1 % inhibition of parasitemia at 30

mg/kg/4 days by intraperitoneal (ip) route of administration and 14.5 % inhibition of parasitemia by oral (po) route (INOD-1) (Table 3). No acute toxicity was observed up to 140 mg/kg (ip) (Nepveu et al, 2010). Higher doses were not tested due to the limit of solubility of INOD-1 in DMSO. Three conclusions were drawn from these assays, i) no good correlation was seen between the in vitro and in vivo results; ii) the poor solubility of these compounds is a limiting factor in the assays of biological activity and ADME/Tox profiling and iii) the half-life of this compound in liver microsomes is very short.

Currently, INOD-1 has been identified as the best hit of the indolone-*N*-oxide series (Figure 12).

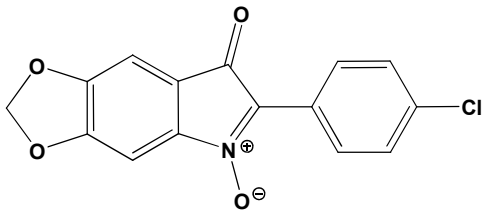
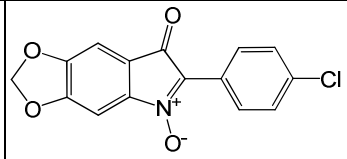
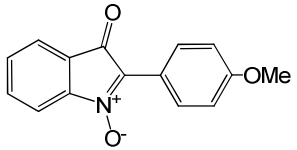
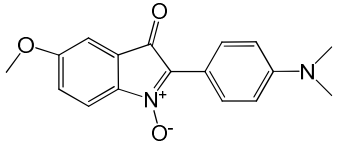


Figure 12. 6-(4-chlorophenyl)-7H-[1,3]dioxolo[4,5-f]indol-7-one-5-oxide (INOD-1)

Table 3 summarizes the main physicochemical and biological findings of the most promising compounds of the indolone-*N*-oxide series (Nepveu *et al*, 2010).

Table 3. Main physicochemical and biological characteristics of INOD-1, INOD-2 and INOD-3 (Nepveu *et al*, 2010)

Compound	Structure	LogP[a]	IC_{50} *P. falciparum* (nM)			CC_{50} (µM)		Selectivity index		$t_{1/2}$ (min)[b]	% Parasitemia inhibition (30 mg/kg/day)	
			FcB1	3D7	K1	MCF7	KB	MCF7/ FcB1	KB/ 3D7		**Po[c]**	**Ip[d]**
INOD-1		2.07	75 ± 63	58 ± 17	88 ± 17	15.9	27.0	212	450	**< 1**	**14.5**	**62.1**
INOD-2		2.01	195 ± 20	101 ± 32	124 ± 44	> 39.5	457	> 202	4525	**16**	**32.4**	**40.5**
INOD-3		2.30	< 3	1.7	nd[e]	43.9	338	> 14623	198823	**6**	**20.9**	**15.3**

[a] LogP calculated with VCCLAB *(http://www.virtuallaboratory.org/lab/alogps/start.html)*

[b] half-life in mouse liver microsomes, verapamil, 6 min; [c] *po:* per oral; [d] *ip*: intraperitoneal; [e] nd: not determined

Two selected compounds (INOD-1 and INOD-2) were tested on fresh clinical isolates of *P. falciparum* collected from symptomatic patients. Geometric mean 50 % inhibitory concentration (IC_{50}) of INOD-1 is 48.6 nM. Correlation between INOD-1 and CQ is not observed (r = 0.015; $P > 0.05$). In contrast, there is significant correlation between INOD-1 and dihydroartemisinin (r = 0.444; $P< 0.05$) (Tahar *et al*, 2011).

There is no significant drug interaction between INODs and different current antimalarials (artesunate, artemisinin, dihydroartemisinin, chloroquine, amodiaquine, mefloquine, quinine). However, quinine and INOD-1 showe slight antagonism interaction against 3D7. Quinine and INOD-2 exert slight synergistic interaction against 3D7. Therefore, their mechanisms of action are slightly different (Tahar *et al*, 2011).

In the stage specific study, blood samples were taken at different times (6, 12, 24, 30, 36, 48 h) of life cycle of parasite, incubated with INOD-1 and INOD-2 and then compared with untreated control ring cultures. INOD-1 and INOD-2 stop the maturation of parasite at ring stage. Their effect is only obtained when the compounds are added before 30 hours of the cycle. After 30 hours, both compounds are not able to remove entire number of new rings (Tahar *et al*, 2011).

There are some compounds relatively have higher IC_{50} than INOD-1 *in vitro*, but they couldn't be selected to further tests due to their low aqueous solubility or/and chemical instability. Therefore, these results are useful for future molecular design in order to improve chemical stability and pharmacological properties of the drug candidate. In addition, INODs exhibited antiplasmodial activity within a highly active range (IC_{50} < 100 nm). Their activity is not influenced by the level of chloroquine resistance (Tahar *et al*, 2011). These finding confirms that indolone-*N*-oxide group is a highly promising antimalarial group in near future.

c. Nanoformulation and *in vivo* assays

It was first necessary to overcome the unfavorable physicochemical (solubility) and pharmacokinetic (rapid hepatic metabolism) properties of the INODS series so as to render them bioavailable, an essential prerequisite for reproducible and accurate *in vivo* results. It was clear that the development of this series should first go through the preparation of suitable formulations that enhance the solubility of the indolone-*N*-oxides and hence enable the intravenous route of administration to be used. This evades the rapid and extensive first-pass effect and ensures direct delivery of antimalarial drug to the site of infection (blood).

However, the development of a suitable formulation of the hydrophobic, rapidly acting indolone-*N*-oxides was not an easy task. An appropriate preparation that respects the rapid antimalarial effect and the mechanism of action of this class of compounds was sought.

A nano-formulation process, innovative for antimalarials, has been developed in our group (N. Ibrahim, 2012).and applied with success to the INODs leads, first to have water soluble forms of those compounds available, second to increase their antimalarial efficacy by a right choice of a biocompatible polymer used to prepare the nano-particles. In fact, all the significant efforts undertaken during the past two years to increase bioavailability with a series of traditional methods failed (oils, microemulsions, liposomes, inclusion in cyclodextrins) gave no satisfying results. Beyond the effect of wettability brought by this polymer, it appeared that it was a vector of choice, in the context of malaria, for transporting INODs to parasitized erythrocytes. The assays of our laboratory (Table 5) strongly suggest that the use of these nanoparticles to target erythrocytes constitutes a very innovative approach in the context of malaria drug therapy.

Table 4. *In vivo* antimalarial activity of unformulated and formulated drug tested on the murine model (*Plasmodium berghei*) with Peters' test as compared with artemisinin, chloroquine and artesunate (NPs = Nanoparticles), (N. Ibrahim, 2012).

Compound	Dose	% Inhibition of parasitemia at D4	Mean survival time (days)
Unformulated drug [a]	30 mg/kg/4 days, *po*	14.5	-
Unformulated drug [a]	30 mg/kg/4 days, *ip*	62.1	-
Drug/ NPs	25 mg/kg/4 days, *iv*	99.1	> 34
Chloroquine[b]	10 mg/kg/4 days	99	17
Artesunate[c]	10 mg/kg/3 days, *po*	99.1	8.2
Commercial artesunate[d]	3.2 mg/kg/4 days, *iv*	58	11.7
Commercial artesunate[d]	6.4 mg/kg/4 days, *iv*	87	12.4

[a] Nepveu *et al*, 2010; [b] Musonda *et al*, 2009 ; [c] Chadwick *et al*, 2011 ; [d] Pail *et al*, 2012

The reduction in parasitemia is calculated after examining blood smears taken on day 4 (96 hr post-infection). Additional smears were examined on subsequent days to confirm absence of relapse and revealed that 100 % inhibition of parasitaemia was achieved on day 7.

Table 5. *In vivo* antimalarial activity of drug/ NPs (NPs = nanoparticles) tested on the 'humanized' mouse model (*Plasmodium falciparum*) (N. Ibrahim, 2012)

Compound	Dose	% Inhibition of parasitaemia
Chloroquine	15 mg/kg/4 days, *po*	100
Artesunate	4 mg/kg/4 days, *po*	28
Drug/ NPs	25 mg/kg/4 days, *iv*	97.5

With these nanotechnologies, an organic solvent-free formulation of the practically water insoluble antimalarial indolone-*N*-oxides was obtained. A thousand-fold increase in aqueous solubility was attained, allowing the administration of higher doses by the intravenous route, which enables the antimalarial compound to reach the systemic circulation (with 100 % bioavailability) and to be exposed directly to parasitized erythrocytes (cellular target). The method described for producing the nanoparticles achieved an excellent entrapment of the drug with a satisfactory particle size along with good homogeneity and successfully liberated the drug rapidly preserving the flash antimalarial activity of the indolone-*N*-oxides (N. Ibrahim, 2012), (N. Ibrahim *et al*, 2010).

e. Chemical synthesis

The indolone-*N*-oxide derivatives were synthesized by three different methods as reported before (Nepveu *et al*, 2010).

Method **1** (Figure 13) is based on 1,2-diketone intermediates b, that are cyclised *via* nitro group reduction to generate the corresponding indolone-*N*-oxides (Génisson et al, 2001), (Nepveu *et al*, 2003). The 1,2-diketones were prepared by permanganate oxidation of a styrene precursor **a**, which is easily obtained by Wittig olefination of nitrobenzaldehydes with phosphonium ylides.

Method **2** (Figure 14) (Rosen *et al*, 2000) is a one-pot procedure based on Sonogashira coupling of *o*-iodo nitro-aryles with terminal alkynes, and concomitant cyclisation of *o*-(arylalkynyl)-nitroaryles intermediates into indolone-*N*-oxides (Sonogashira *et al*, 1975), (A. Elangovan *et al*, 2003).

Method **3 (** (Figure 15) derives from optimization of method **2** and was divided into two sub-steps: the first involved Sonogashira coupling as in method **2** as well as intermediate extraction, followed by an intramolecular cyclisation using 4-dimethyl amino pyridine (4-DMAP) in refluxing pyridine (Kim, 2007), (Bond and Hooper, 1969), (Suvilo *et al*, 2003),

(Berry *et al*, 2001). Preparations of INODs were strongly improved in our group. For example, INOD-1 (Scheme 1, Method 1) obtained with a yield of 17.4% in (Nepveu *et al*, 2010) is now obtained with a yield of 65%.

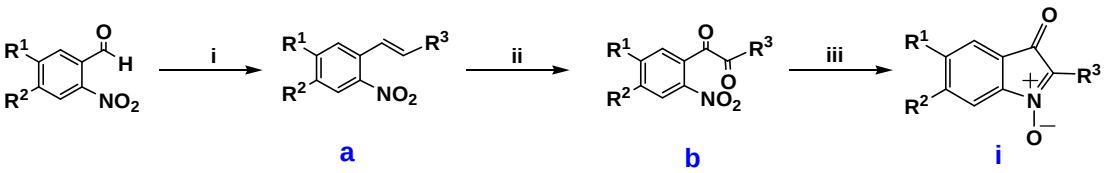


Figure 13. Method **1** used to synthesize indolone *N*-oxide derivatives (R^3 = alkyl). Reagents and conditions: (i) $Ph_3PCH_2R^3Br$, NaOH, CH_2Cl_2, $Bu_4N^+Br^-$; (ii) $KMnO_4$, acetic anhydride, 0 °C; (iii) Zn, NH_4Cl, tetrahydrofuran, CH_2Cl_2 (Nepveu *et al*, 2010).

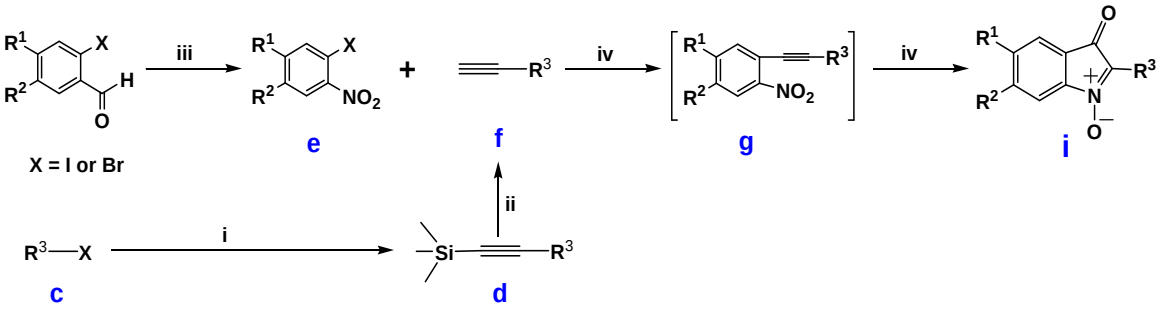


Figure 14. Method **2** used to synthesize indolone *N*-oxide derivatives (R^3 = aryl). Reagents and conditions: (i) $(CH_3)_3Si\text{-}C{\equiv}CH$, $Pd[(C_6H_5)_3P]_4$, CuI, Et_3N; (ii) CH_3OH, K_2CO_3; (iii) CH_3COOH, HNO_3 fuming; (iv) $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, N_2, r. t (Nepveu *et al*, 2010).

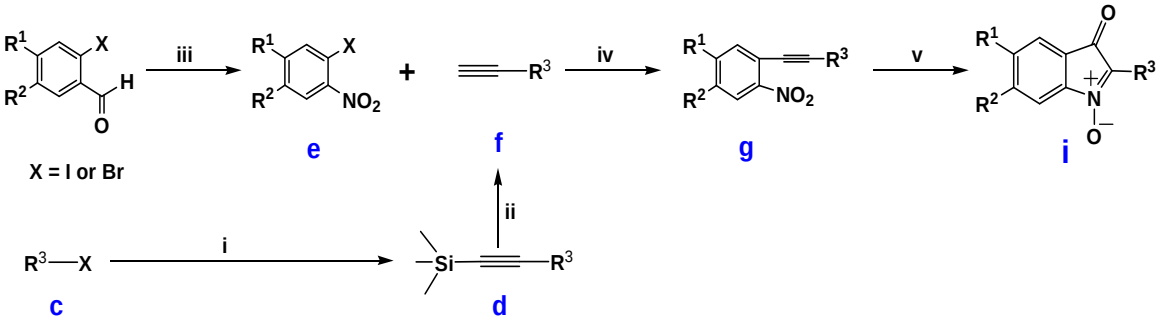


Figure 15. Method **3** used to synthesize indolone-*N*-oxide derivatives (R^3 = aryl). Reagents and conditions: (i) $(CH_3)_3Si\text{-}C{\equiv}CH$, $Pd[(C_6H_5)_3P]_4$, CuI, Et_3N; (ii) CH_3OH, K_2CO_3; (iii) CH_3COOH, HNO_3 fuming; (iv) $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, N_2, r. t.; (v) pyridine, 4-dimethylaminopyridine, reflux 140 °C (Nepveu *et al*, 2010).

Pharmacomoluation studies showed that substitution at position 2 has a great influence on the antiplasmodial IC_{50} values while variation at positions 5 and 6 of the substituants did not modify strongly the IC_{50} values (Figure 16). At position 2 (R3) chemical groups having mesomeric effect such as either +M: F, Cl, OCH_3, OPh or –M: NO_2, enhance the activity (Figure 16). In contrast, chemical group having inductive effect for instance +I: CH_3, C_2H_5, CH_2OH or –I: CF_3 decreased the activity (Figure 16). When a phenyl ring is attached at position 2, the full structure is almost coplanar (X-ray crystallographic studies). The planarity is disturbed by steric effect due to the substitution at position 2.

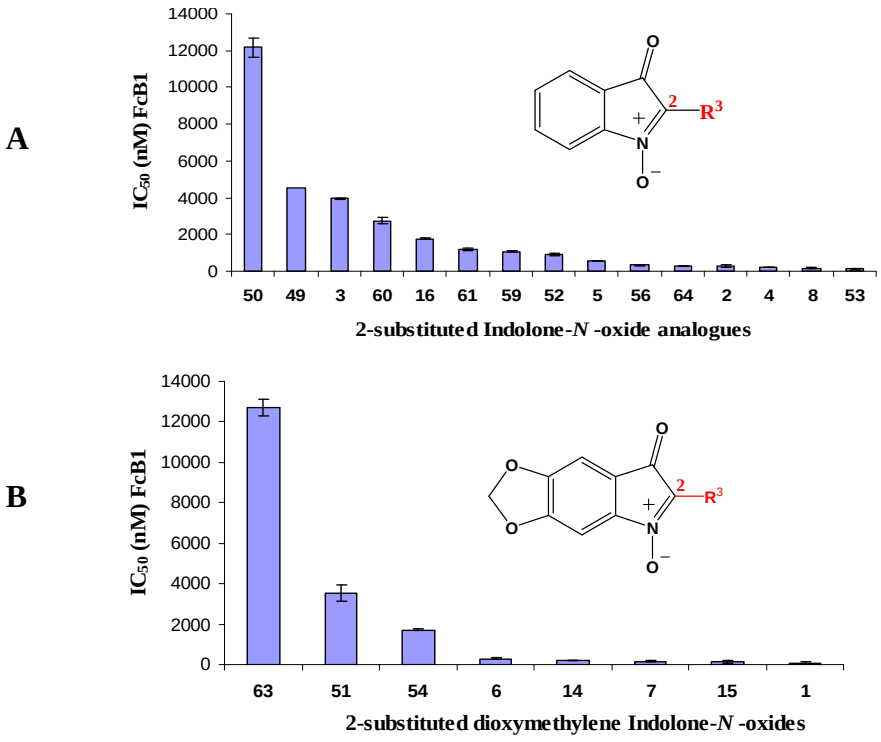


Figure 16. Comparison of antiplasmodial activity (IC_{50} [nM], strain FcB1) of A) 2-substituted indolone-*N*-oxides, B) Dioxymethylene derivatives of indolone-*N*-oxides (Nepveu *et al*, 2010).

Table 6. Structure of indolone-*N*-oxide analogues (Figure 9) obtained, and their antiplasmodial and cytotoxic activities *in vitro* (Nepveu *et al*, 2010).

Compound	R^1	R^2	R^3	$LogP_{calc}$ [a] (VCCLAB)	IC_{50} (nM) FcB1 strain	CC_{50} (μM) [b] MCF7	Selectivity index MCF-7/FcB1
1	O-CH_2-O		4′-Chlorophenyl	2.07	75 ± 63	15.9	212
2	H	H	4′-Phenoxyphenyl	3.51	264 ± 60	11.1	42
3	H	H	4′-Hydroxymethyl phenyl	1.29	3,950 ± 50	39.5	10
4	H	H	4′-Methoxyphenyl	2.01	195 ± 20	> 39.5 [c]	> 202
5	H	H	4′-6′-Methoxy-naphthalen-2-yl	3.03	560 ± 15	15.3	66
6	O-CH_2-O		4′-6′-Methoxy-naphthalen-2-yl	2.51	288 ± 30	25.6	89
7	O-CH_2-O		4′-Phenoxyphenyl	3.00	165 ± 40	12.2	74
8	H	H	3′, 4′-Dichlorophenyl	3.14	155 ± 20	13.7	88
14	O-CH_2-O		3′, 4′-Dichlorophenyl	2.66	195 ± 2	7.4	38.1
15	O-CH_2-O		4′-Ethoxyphenyl	1.98	156 ± 57	51.9	332
16	H	H	4′-Tolyl	2.23	1,770 ± 20	15	8.5
49	H	H	*i*-Butyl	1.61	> 4,500 [c]	43.1	<9
50	H	H	*n*-Propyl	1.41	12,160 ± 500	26.4	2
51	O-CH_2-O		Ethyl	0.62	3,560 ± 400	59.0	16
52	H	H	Phenyl	1.96	889 ± 88	19.5	22
53	H	H	4′-Fluorophenyl	2.06	120 ± 29	8.7	72.5
54	O-CH_2-O		4′-Fluorophenyl	1.58	1,710 ± 30	n. d.	n. d.
56	H	H	4′-Nitrophenyl	1.96	56 ± 4	2.8	8.4
59	H	H	4′-Chloro-3′-trifluoromethylphenyl	3.31	1,045 ± 35	2.0	1.9
60	H	H	4′-Ethylphenyl	2.81	2,750 ± 150	> 39.8 [c]	> 14
61	H	H	4′-Trifluoromethylphenyl	2.71	1,170 ± 80	34.3	29
63	O-CH_2-O		Phenyl	1.5	12,720 ± 400	n. d.	n. d.
64	H	H	4′-Chlorophenyl	2.54	272 ± 16	17.8	66
Chloroquine				4.63	151 ± 6	19.4	167
Sodium artesunate				2.41	6 ± 3	9.8	1,633

d. Mechanisms of action

INODs bind to site **I** on albumin (Ibrahim *et al*, 2010). The interaction between INODs and albumin spontaneously occurs (negative ΔG) due to hydrophobic interaction between conjugation system of INODs and hydrophobic regions of HSA (Ibrahim *et al*, 2010). This phenomenon is explained by the rigid coplanar of indolone analogue which reduces the number of possible conformations or any possibility of other bindings. This interaction alternates the HSA conformation by slightly inducing unfolding of α-helix domain (Ibrahim *et al*, 2010). INOD-HSA complex with binding affinities $K = 10^4$ (Ibrahim *et al*, 2010) is in the range of weak or nonspecific (Tajmir-Riahi *et al*, 2007). Low drug-protein binding leads to high therapeutic index which is favorable to continue further INODs study such as in administration, distribution, metabolism and elimination.

As demonstrated by the research done in our laboratory and in collaboration with Pr P. Arese and Pr F. Turrini (University of Torino, Italy), INODs and derivatives have an original

mechanism of action: i) they interfere with regulatory mechanisms of the host cell membrane without affecting non-parasitized erythrocytes; iii) erythrocyte membrane modifications determine its destabilization and intense vesiculation that leads to parasite death; iv) the mechanism leading to the destabilization of the membrane in parasitized erythrocytes is triggered by the activation of a stress-sensitive phosphorylation pathway which effects the membrane-cytoskeleton interactions (Ferru, 2011) v) marked hyperphosphorylation of AE1 (band 3) appears to be the hallmark of the process (Pantaleo *et al*, 2012.). As a result, overload oxidative stress is created which is critical for survival of parasites such as in sickle cell disease, G6PD deficiency, α- and β-thalassemias and oxidizes drugs (Pantaleo *et al*, 2012). In overload oxidative stress environment, membrane vesiculation is accelerated at very early stage of parasite maturation, so that parasit is killed before it reaches full maturation. Therefore, INODs are able to destabilize the membrane of malaria infected erythrocyte cells by the action of redox signaling pathway (Pantaleo *et al*, 2012).

In parallel, the biotransformation of INODs in red blood cells was studied because the human erythrocytes are the cellular target of Plasmodium; compound **1** was selected for these studies in our group.

Compound **1** (INOD-1) is not only found to have a rapid diffusion from plasma to red blood cells (RBCs) but also it has rapid metabolism in cytoplasmic of RBCs, resulting in bio-transformation to a fluorescent product, the dihydroanalogue, **1-HH** (INOD-1-HH) (Figure 17). Two possible metabolic chemical pathways are suggested via deoxygenation followed by hydration or epoxide formation followed by hydration (Figure 17) (Ibrahim *et al*, 2011). Furthermore, this biotransformation depends on the enzyme process since glutathion or thiol-containing compounds are found necessary for the bio-reductive transformation of compound **1** (Ibrahim *et al*, 2011). In addition, haemoglobin could be essential in co-subtrate in an enzymatic redox cycle. RBCs are not inert to compound **1** *in vitro*, the transformation of compound **1** in RBC could play an privotal role in parasite redox balance and antiplasmodial activity. More studies are required to confirm these suggestions

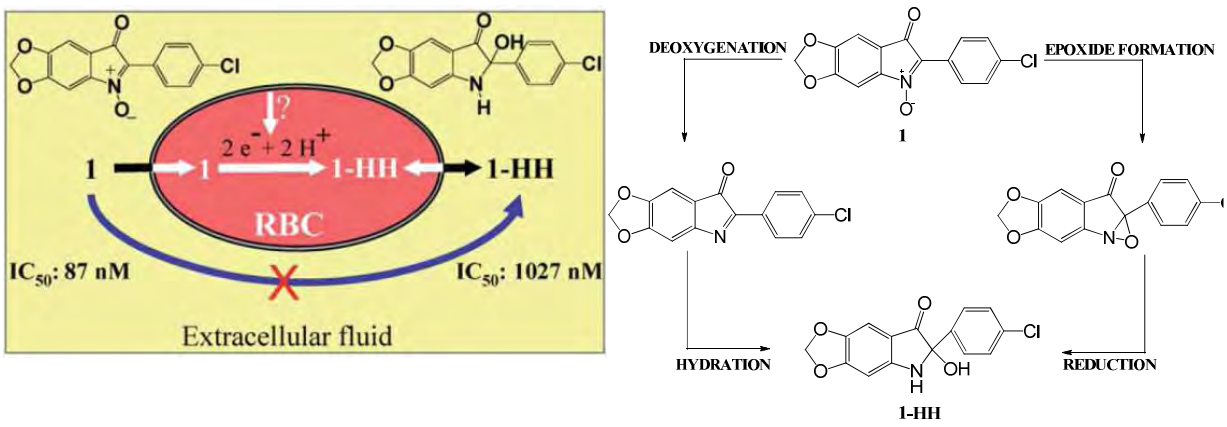


Figure 17. Biotransformation model of compound **1** (Ibrahim *et al*, 2011).

Table 7. Comparison of the biological, chemical properties of compound **1** (INOD-1) with its metabolite (**1-HH**)

Characters/Assay	**Compound 1** (INOD-1)	**Its metabolite (1-HH)** (INOD-1-HH)	**Conclusion**
Planarity	Coplanar	Loss planarity	Loss conjugated sytem,
Color	Deep pink	Greenish yellow	Loss conjugated sytem, loss planarity
Fluorescence property	No	Yes	Loss nitron function
Conjugated system	Yes	No	
Hydrophobicity	Liphophilic	Hydrophilic	Difference in distribution
Distribution	Mainly in intracellular	Both in intracellular and in extracellular	Effect on IC_{50} value
IC_{50}	87 nM	1027 nM	Both active against Plasmodium
Interact with microsome	Half-life: less than 1 min.	Half-life: 10 min.	**1-HH** is more stable than compound **1**

Table 8. Factors affecting biotransformation of compound **1** (INOD-1) in the erythrocyte.

Factors/ incubation with	**Biotransformation**	**Conclusion**
Temperature	reaction rate at 37°C > at 4°C	Temperature dependence
Purified heamoglobin	No	Reaction takes place in cytoplasmic of RBC
Ghost fraction	No	
Heat-denatured RBC	No	Enzyme depend process
1-chloro-2,4-dinitrobenzene (CDNB)- or N-ethylmaleimide (NEM)-RBCs (CDNB, NEM deplete glutathione)	Lower	
Pf-infected RBC containing haemozoin	Yes *	No depend on haemoglobin process
β-thalassemic RBCs containing abnormal haemoglobin		
Phenylhydrazine (PHZ) -treated RBC (PHZ induces $HbFe^{III}$, hemichrom formation)	Yes*	No depend on heme-iron process
Liver microsome	No	Microsome doesn't participate in this biotransformation

*: No significant different from incubated sample of compound **1** (INOD-1) and normal RBC

In summary, INODs have low affinity with HSA which allows enough free INODs to reach infected-RBCs. When INODs penetrate to infected-RBCs, they undergo an enzyme and thiol-dependant biotransformation. According to these results, redox properties of INODs are evidently essential for their antimalarial activity.

These first data on the mechanism of action of the INODs, introducing the concept of oxidative stress and bio-reducibility of the bio-active molecules, require to study in more details the redox character of the INODs, by physico-chemical approaches, and their interactions with the biochemical components of the erythrocyte. It is also important to conduct these studies in comparison with chloroquine and artemisinin to highlight the similarities and/or differences between these three chemical families to evaluate the capacity of INODs to address the problem of the resistance of the parasite to current antimalarials.

II. ELECTROCHEMICAL BEHAVIOR OF INDOLONE-*N*-OXIDE: RELATIONSHIP TO STRUCTURE AND ANTIPLASMODIAL ACTIVITY

1. Introduction

Les indolone-*N*-oxydes (INODs) constituent une nouvelle famille de molécules antipaludiques prometteuses. Les INODs présentent des activités antiplasmodiales avec des activités inhibitrices (CI_{50}) proches du nanomolaire contre *Plasmodium falciparum* et une faible toxicité sur cellules humaines *in vitro* (Nepveu *et al*, 2010). Sur la base de ces activités biologiques et des études de relations structure-activité, le groupement *N*-oxyde réductible ne devient biologiquement actif que s'il est introduit dans des systèmes conjugués, tels que des composés hétérocycliques et pseudo-quinoïdes. Par conséquent, le système conjugué entre le groupe nitrone et la fonction cétonique est essentielle à l'activité antipaludique (Nepveu *et al*, 2010). Au cours de sa biotransformation au sein du globule rouge, nous avons montré précédemment que les INODs subissent une bio-réduction, enzym- et thiol- dépendantes (Ibrahim et al, 2011). Etant donné la présence de trois différentes fonctions réductibles dans ces molécules, le groupement *N*-oxyde, le carbone hautement électrophile dans le groupement nitrone et la fonction cétone, il apparait indispensable d'étudier les propriétes redox des INODs au niveau moleculaire.

En utilisant la voltampérométrie cyclique, la résonance paramagnétique électronique (RPE) et la spectroélectrochimie, les propriétés redox de 37 représentants de la famille INODs ont été étudiées. Dans ce contexte, les relations entre substituants, potentiels redox, lipophilie et activités antipaludiques ont été analysées. Les études ont été menées pour répondre aux questions suivantes: Quel est le comportement redox des INODs en solution? Y a-t-il formation de radicaux libres? Y a-t-il une relation entre le comportement redox et l'activité antipaludique? Les résultats sont importants pour la conception de nouvelles structures chimiques visant à obtenir des molécules antipaludiques plus actives.

1. Introduction

The indolone-*N*-oxides (INODs) are a new promising antimalarial family. INODs exert antimalarial activity in nanomolar range against *Plasmodium falciparum* with low toxicity for mammalian cells *in vitro* (Nepveu *et al*, 2010). Based on the biological evaluation and structure-activity relationship studies, the reducible *N*-oxide moiety only becomes biological active when it is inserted into conjugated systems such as heterocyclic and pseudo-quinoid. Therefore, the conjugated system between the nitrone group and ketonic function is essential for antimalarial activity (Nepveu *et al*, 2010). In cell based metabolism study, INODs undergo an enzyme and thiol-dependent bioreductive transformation in RBCs (Ibrahim *et al*, 2011). Since there are three different reducible functions presenting in an INOD analogue: the *N*-oxide moiety, the highly electrophilic carbon in the nitrone moiety and the ketone function, it is definitely necessary to study the redox properties of INODs at the molecular level to better explain their mechanism of action.

By using cyclic voltammetry and electron spin resonance (EPR) spectroelectrochemical measurement, the redox properties of thirty seven INODs were investigated. In this context, the relationship between the substituents, redox potentials, lipophilicity and antiplasmodial activities were analysed. The results of this study wish to answer for these questions: What is redox behavior of INODs? Is there any free radical formation? Is there any relationship between redox behavior and antimalarial activity of INODs? The results are important for chemical structure design to obtain more potent antimalarial molecules.

2. Electrochemical behavior of indolone-*N*-oxides: relationship to structure and antiplasmodial activity.

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Electrochemical behavior of indolone-*N*-oxides: Relationship to structure and antiplasmodial activity

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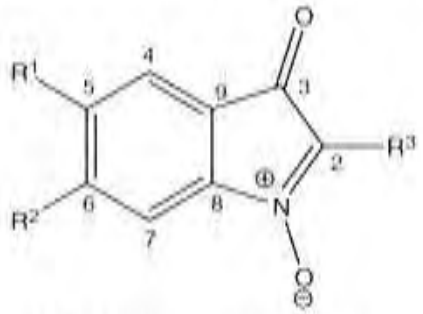
ABSTRACT

Indolone-*N*-oxides exert high parasiticidal activity at the nanomolar level *in vitro* against *Plasmodium falciparum*, the parasite responsible for malaria. The bioreductive character of these molecules was investigated using cyclic voltammetry and EPR spectroelectrochemistry to examine the relationship between electrochemical behavior and antimalarial activity and to understand their mechanisms of action. For all the compounds (17 compounds) studied, the voltammograms recorded in acetonitrile showed a well-defined and reversible redox couple followed by a second complicated electron transfer. The first reduction (-0.88 V < $E_{1/2}$ < -0.50 V vs. SCE) was attributed to the reduction of the *N*-oxide function to form a radical nitroxide anion. The second reduction (-1.65 V < $E_{1/2}$ < -1.14 V vs. SCE) was assigned to the reduction of the ketone function. By coupling electrochemistry with EPR spectroscopy, the EPR spectra confirmed the formation of the nitroxide anion radical. Moreover, the experiments demonstrated that a slow protonation occurs at the carbon of the nitrone function and not at the NO function. A relationship between electrochemical behavior and indolone-*N*-oxide structure can be established for compounds with R^1 = $-OCH_3$, R^2 = H, and electron-withdrawing substituents on the phenyl group at R^3. The results help in the design of new molecules with more potent *in vivo* antimalarial activity.

1. Introduction

The *N*-oxide moiety is a reducible function that leads to biologically active compounds when introduced into conjugated systems such as heterocyclic and pseudo-quinoid compounds. These derivatives are thought to exert their biological action by the generation of reactive oxygen species through a redox cycling process that causes oxidative stress. This may explain how these compounds act as anti-infective agents towards a large number of microorganisms. This is the case of quinoxaline-1,4-di-*N*-oxide derivatives acting as anti-infectious [1–4], or mono-*N*-oxides such as benzofuroxan, nitrofurane and nitroimidazole, derivatives with antiprotozoal activities [5–7], and of indolone-*N*-oxides with various antimicrobial activities [8–10]. The precise mechanism by which these drugs act in different biological models has not yet been fully elucidated [11]. Recently we reported the antimalarial activity of a series of indolone-*N*-oxides (Scheme 1). The biological evaluation and structure–activity relationship (SAR) studies proved the efficient antimalarial activity of these derivatives *in vitro* and *in vivo* against *Plasmodium falciparum* (*Pf*) [12,13]. Proteomic studies that we have reported suggest that these indolone-*N*-oxides cause a profound destabilization of the membrane of the *Pf* infected red blood cells (RBC) through a mechanism apparently triggered by the activation of a redox signaling pathway, which could be initiated by a chemical redox reaction in the RBC [14]. These results gave



Scheme 1. Structure of the indolone-*N*-oxide series.

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Table 1
Structure, cyclic voltammetric data, log P and antiplasmodial activity of indolone-*N*-oxide derivatives.

Compounds	R^1	R^2	R^3	$Log P^c_{calc}$ (VCCLAB)	IC_{50}/nM FcB1 strain[a]	$E_{1/2}$/V[d,e] (vs. SCE)	Rip[f]	ΔEp/mV[g]
1	O—CH_2—O		4′-Chlorophenyl	2.07	75 ± 63	−0.705	0.27(1)	68
						−1.312	0.35	145
2	H	H	4′-Phenoxyphenyl	3.51	264 ± 60	−0.749	0.60(0.86)	77
						−1.510	0.10	275
4	H	H	4′-Methoxyphenyl	2.01	195 ± 20	−0.744	0.89(1)	68
						−1.264	0.48	113
5	H	H	4′-6′-Methoxy-naphthalen-2-yl	3.03	560 ± 15	−0.748	0.70(0.86)	71
						−1.425	0.17	116
7	O—CH_2—O		4′-Phenoxyphenyl	3.00	165 ± 40	−0.788	0.50(0.84)	80
						−1.405	0.46	198
8	H	H	3′,4′-Dichlorophenyl	3.14	155 ± 20	−0.635	0.92(1)	66
						−1.170	0.28	98
9	OCH_3	H	4′-Chlorophenyl	2.58	210 ± 10	−0.727	1(1)	70
						−1.518	0.40	140
11	OCH_3	H	3′-Trifluoromethyl-4′-chlorophenyl	3.32	50 ± 39	−0.611	0.37(0.72)	75
						−1.317	0.50	182
12	H	OCH_3	4′-Chlorophenyl	2.57	148 ± 31	−0.729	0.85(0.95)	70
						−1.416	0.45	115
13	OCH_3	H	3′,4′-Dichlorophenyl	3.18	100 ± 7	−0.634	0.80(1)	68
						−1.315	0.44	136
17	OCH_3	H	Phenyl	2.00	184 ± 53	−0.760	0.30(0.50)	100
						−1.518	0.15	148
18	OCH_3	H	4′-Tolyl	2.25	52 ± 48	−0.704	1(1)	74
						−1.588	0.35	176
19	H	CF_3	4′-Chlorophenyl	3.26	186 ± 15	−0.504	0.96(1)	76
						−1.276	0.55	140
20	OCH_3	H	4′-Methoxy-naphthalen-2-yl	3.03	135 ± 45	−0.620	0.32(0.7)	80
						−1.326	0.30	195
21	OCH_3	H	4′-Chlorobiphenyl-4-yl	4.06	630 ± 248	−0.726	1(1)	74
						−1.440	Irreversible	
22	OCH_3	H	4′-Methoxyphenyl	2.04	40 ± 30	−0.782	1(1)	72
						−1.650	0.44	166
24	OCH_3	H	4′-Phenoxyphenyl	3.51	193 ± 55	−0.750	0.96(1)	68
						−1.497	0.51	184
25	OCH_3	H	4′-Trifluoromethoxyphenyl	2.90	20 ± 0	−0.720	0.92(0.96)	68
						−1.590	0.85	172
26	OCH_3	H	4′-Dimethylaminophenyl	2.30	~ 3	−0.882	0.60(0.80)	82
						−1.570	Irreversible	
27	OCH_3	H	4′-Isopropoxyphenyl	2.81	17 ± 2	−0.816	0.89(1)	74
						−1.590	0.31	252
29	OCH_3	H	4′-Methoxy-3′-tolyl	2.25	44 ± 4	−0.836	0.75(1)	94
						−1.625	0.20	170
30	OCH_3	H	4′-Aminophenyl	1.58	24 ± 19	−0.876	0.83(1)	68
						−1.511	0.42	169
35	H	OCF_3	4′-Methoxyphenyl	2.88	113 ± 50	−0.681	0.95(1)	68
						−1.487	0.37	142
37	OCH_3	H	4′-Trifluoromethylphenyl	2.74	43 ± 23	−0.643	0.92(0.96)	68
						−1.387	0.45	200
39	OCH_3	H	3′-Chlorophenyl	2.60	21 ± 1	−0.698	0.92(1)	68
						−1.470	0.47	180
40	OCH_3	H	3′-Methyl-4′-tolyl	2.48	37 ± 28	−0.802	0.92(1)	74
						−1.574	0.44	226
42	OCH_3	H	4′-Tert-butylphenyl	3.03	204 ± 14	−0.791	1(1)	68
						−1.615	0.42	202
47	OCH_3	H	2′-Thiophen-3-yl	1.93	270 ± 10	−0.785	0.91(1)	70
						−1.573	0.34	232
48	H	NO_2	4′-Methoxyphenyl	1.97	345 ± 81	−0.477	0.81(1)	70
						−0.932	0.90	68
						−1.704	0.45	108
49	H	H	i-Butyl	1.61	~4,500[b]	−0.842	0.86(1)	64
						−1.549	0.60	192
50	H	H	n-Propyl	1.41	12,160 ± 500	−0.838	0.77(0.90)	80
						−1.457	0.41	194
56	H	H	4′-Nitrophenyl	1.96	56 ± 4	−0.518	0.90(1)	65
						−0.987	0.68	172
						−1.544	0.59	191
57	H	OPh	4′-Phenoxyphenyl	4.79	665 ± 135	−0.780	0.35(0.67)	74
						−1.425	0.40	138
60	H	H	4′-Ethylphenyl	2.81	2,750 ± 150	−0.726	0.67(1)	67
						−1.360	0.39	140
61	H	H	4′-Trifluoromethylphenyl	2.71	1,170 ± 80	−0.652	1(1)	72
						−1.434	0.30	132
67	H	$(CO)CH_3$	4′-Methoxyphenyl		584 ± 27	−0.603	0.79(1)	72
						−1.264	0.90	130
						−1.932	0.80	158

Table 1 (*continued*)

Compounds	R^1	R^3	R^2	LogP$^a_{calc}$ (VCCLAB)	IC_{50}/nM FcB1 strain^b	$E_{1/2}$/V$^{(c)}$ (vs. SCE)	Rlp^d	ΔEp/mV^e
68	OCH_3	H	4′-Dimethylamino-3′-tolyl	4.0 ± 0.8	40 ± 30	0.815 1.012	0.98(1) 0.39	69 125

[a] LogP calculated with VCCLAB (http://www.vcclablaboratory.org/lab/alogps/start.html).
[b] The drug concentration giving a 50% decrease in parasite growth.
[c] The highest tested concentration.
[d] The electrochemical data are gathered at 0.1 V/s; for irreversible systems $E_{1/2}$ was obtained under a higher potential scan rate when the backward peak appeared.
[e] $E_{1/2} = (Ep_{backward} + Ep_{forward})/2$.
[f] Rlp = $|Ip_{backward}/Ip_{forward}|$; in parentheses, value when the potential scan is restricted to the first system.
[g] $\Delta Ep = |Ep_{backward} - Ep_{forward}|$.

rise to pharmacomodulation studies to develop new hits and leads. The conjugated system between the nitrone group and the ketonic function of the indolone moiety was found to be essential for optimal *in vitro* antiplasmodial activity.

In parallel, we explored some ADMET properties (Absorption, Distribution, Metabolism, Excretion and Toxicity) with the binding affinity of these compounds to human serum albumin [15] and cell based metabolism studies. In this context, we showed that indolone-*N*-oxides undergo a bioreductive transformation in red blood cells [16]. In previous studies we evaluated the spin trapping properties of these compounds and demonstrated their ability to trap hetero- and carbon-centered radicals [17]. Major differences in antiplasmodial activities as well as in spin trapping properties studied by EPR spectroscopy were observed between aryl and alkyl substituted indolones at R^1. It can be noted that indolone-*N*-oxides are built around three reducible functions, the *N*-oxide moiety, the highly electrophilic carbon in the nitrone moiety and the ketone function. At this point we considered it necessary to study the redox properties of indolone-*N*-oxides to establish the relationship between the electrochemical behavior and the biological activity and to continue with the study of the structures required to obtain more potent *in vivo* antimalarial activity.

We report here the redox behavior of 37 indolone-*N*-oxides using cyclic and stationary voltammetry, and EPR spectroelectrochemical measurements. The relationships between the substituents, redox potentials, lipophilicity and antiplasmodial activities were examined.

2. Experimental

2.1. Reagents

Acetonitrile (ACN, Fisher) and tetrabutylammonium perchlorate (TBAP, Fluka) were obtained commercially at the highest purity available and used without further purification. The methods for the synthesis of indolone-*N*-oxides have been previously reported [12]. The two new compounds (**67**, **68**) were obtained from Idealp-Pharma (Villeurbanne, France).

2.2. Voltammetric measurements

Electrochemical experiments were carried out at 25 °C in acetonitrile containing TBAP 0.1 mol L^{-1} using a Voltalab 80 PGZ 402 (Radiometer) with a conventional cell with three electrodes: reference electrode, a double junction calomel electrode (SCE); counter electrode, a platinum electrode sheet (5 × 5 mm); working electrode, a platinum disk (0.5 mm diameter) for cyclic voltammetry and a rotating disk electrode (EDI, Tacussel) for stationary conditions. All solutions were deoxygenated by argon bubbling through the solution for 15 min and maintaining a blanket of the inert gas over the solution during the experiment. Voltamperograms were recorded for a compound concentration equal to 10^{-3} mol L^{-1} (ferrocene or indolone-*N*-oxides) and a potential scan rate ranging from 0.02 V/s up to 25 V/s depending on experiments. The electrochemical data shown in Table 1 were recorded at a potential scan speed of 0.1 V/s.

2.3. EPR spectroelectrochemical analysis

For spectroelectrochemical measurements, the EPR spectrometer was coupled to a potentiostat-galvanostat (EG&G Princeton Applied Research-Model 362). A PHN 81 (Tacussel) voltammeter was used to control the applied potential. A flat quartz cell adapted to electrochemical measurements (Bruker, Wissembourg, France) was used for analysis. The electrochemical reduction was performed using a three-electrode set-up: the working and counter-electrode were platinum and the reference electrode was a silver wire. The applied potential was chosen to be on the diffusion plateau of the first reduction wave obtained under stationary conditions: $E_{applied} = E_{1/2} - 0.2$ V. The electrolysis potential was applied for 5 min to the solution containing the compound in acetonitrile/TBAP and the EPR spectrum was immediately recorded as a function of time. EPR spectra were obtained at X-band at room temperature on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). WINEPR and SIMFONIA software (Bruker, Wissembourg, France) were used for EPR data processing and spectrum computer simulation. Typical scanning parameters were scan rate, 1.2 G/s; scan number, 1; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 20 mW; sweep width, 100 G; sweep time, 83.88 s; time constant, 40.96 ms; center field, 3 480 G; receiver gain 5×10^4.

2.4. Biological properties

Log P and antiplasmodial *in vitro* activities of the compounds studied have been previously reported [12] and are given in Table 1 (same numbering as in Ref. [12]). The IC_{50} value is defined as the concentration inhibiting the growth of the parasite *Pf* FcB1 reference strain by 50% when cultured in red blood cells.

3. Results and discussion

3.1. Electrochemical behavior

In the electroactivity domain of CH_3CN/TBAP, the cyclic voltammograms of the compounds showed oxidation and reduction processes at a platinum electrode. As this paper deals with the reductive properties of the indolone-*N*-oxides, only the cathodic behavior is described. Table 1 reports the electrochemical data obtained at a potential scan speed of 0.1 V/s: $E_{1/2} = (Ep_{backward} + Ep_{forward})/2$; $Rlp = |Ip_{backward}/Ip_{forward}|$ and $\Delta Ep = |Ep_{backward} - Ep_{forward}|$.

Fig. 1 shows typical voltammograms obtained for the series. The voltammogram is characterized by two electron transfers. The first electron transfer (-0.882 (**26**) < $E_{1/2} < -0.477$ (**48**) vs. SCE) is perfectly reversible (Rlp around 1) independently of the scan rate when the potential scan is restricted to this system. As expected the

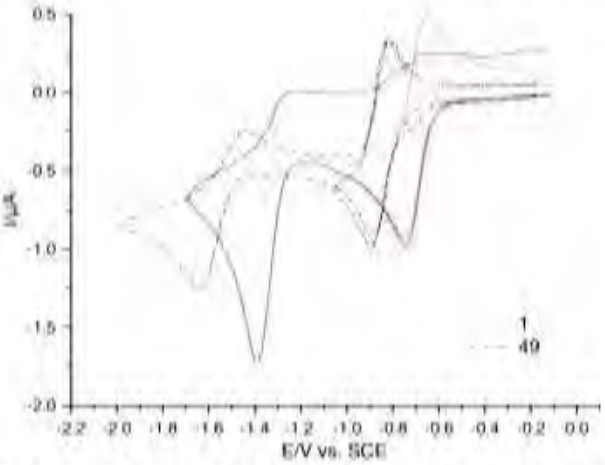


Fig. 1. Electrochemical properties of indolone-*N*-oxides. Cyclic voltammograms of **1** and **49**. (1 mmol L^{-1}) at a platinum electrode in ACN–TBAP (0.1 mol L^{-1}); potential scan rate 0.1 V/s.

potentials $E_{1/2}$ depend on the nature of the substituents. The ΔEp values are in agreement with the quasi-reversible definition of an electron transfer. The peak currents are linearly related to the square root of the potential scan rate as for a diffusion controlled process. The second electron transfer (−1.704 (**48**) < $E_{1/2}$ < −1.264 (**4, 67**) vs. SCE) is more complicated: the backward peak current is lower than the forward peak current (Rlp< 1). The peak currents are of the same order of magnitude as those of the first electron transfer, which means that the same number of electrons is exchanged. When the potential scan rate was increased, the electrochemical system became reversible; a simple EC scheme (electron transfer followed by a chemical reaction) could account for these observations. As above, the peak currents are linearly related to the square root of the potential scan rate as for a diffusion controlled process and the potentials $E_{1/2}$ depend on the nature of the substituents.

Considering the basic structure of the indolone-*N*-oxides (Scheme 1), two reducible functions are present, the *N*-oxide and the ketone, which may correspond to the two electron transfers observed by cyclic voltammetry. By contrast, compounds **48**, **56**, and **67**, that have a third reducible function in their structure such as NO_2 at R^2 (**48**), NO_2 at R^1 (**56**) and a ketone at R^2 (**67**), gave a third redox signal (Table 1).

By comparison with the oxidation peak current of ferrocene recorded under the same conditions, monoelectronic transfers are expected for the reductions of the indolone-*N*-oxide compounds. Moreover, as explained below, EPR spectroelectrochemical experiments demonstrated that the first reduction step gives rise to the appearance of a nitroxide radical-anion consistent with a monoelectronic transfer. These results confirm that the first reduction step can be attributed to the reduction of the *N*-oxide to a nitroxide radical-anion (Scheme 2). The second electron transfer is then attributed to the ketone function. The irreversibility of this reduction is indicative of high chemical reactivity of the intermediate, the radical-anion C°—O⁻.

The ketone reduction potential depends on the delocalization [18] whereas the nature of the products formed depends on the medium: pinacol, through a monoelectron transfer, or alcohol through a bielectron transfer [18,19] that requires protonation. Pinacol originates from the dimerization and then protonation of the radical-anion. The reversibility of the second electrochemical system depends on the stability of the radical-anion C°—O⁻: the peak ratio Rlp is lower than unity (Table 1) but when the potential scan rate was increased (around 5 V/s) a quasi-reversible system was observed (data not shown). In dry acetonitrile, the one electron reduction yields the C°—O⁻ radical-anion, which gives pinacol after dimerization.

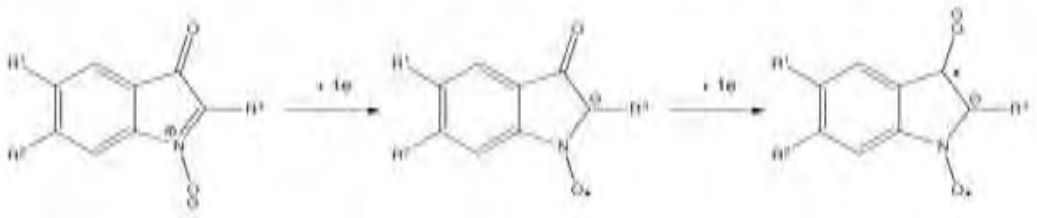
Voltammograms were recorded with increasing quantities of water (Fig. 2). Addition of small quantities of water has a slight effect on the first reduction process whereas it considerably affects the second one. The potential of the first redox process (*N*-oxide reduction) is slightly displaced towards less negative values when increasing the percentage of water (around 120 mV on the figure). Moreover, the system remains reversible, in particular when the potential scan is restricted to this system. The anodic displacement of the reduction potential can be explained by the protonation of the nitroxide radical-anion according to the potential-pH diagram theory, the water being the proton source. This protonation is confirmed by the EPR spectroelectrochemical analysis (cf. 3.2).

On the contrary, the potential of the second electron transfer (ketone reduction) is strongly displaced towards less negative values when the percentage of water is increased (around 500 mV). The forward peak current also increases showing that the system moves from a one electron transfer to a bielectronic transfer, leading to the production of alcohol [19,20].

For compounds **48**, **56**, and **67** that have a third reducible function in their structure, the signals are consistent with those reported in the literature. The acetophenone moiety in **67** is reduced around −2 V [20–22]. In the case of the NO_2 derivatives, their reductions are monoelectronic [23,24] in aprotic solvents. The potential of NO_2 reduction for **48** is then attributed at −0.932 V and for **56** at −0.987 V. The C—O reduction potentials are in the same order as the reported values of other compounds (Table 1). However, as part of our study, only the data corresponding to the first reduction step were correlated with anti-malarial properties.

3.2. EPR spectroelectrochemical analysis

To confirm the radical nature of the species formed after the first one-electron step reduction and to study its stability, electrochemistry was coupled with electron paramagnetic resonance (EPR) spectroscopy. An illustration of the EPR spectrum obtained and its evolution with time is shown in Fig. 3 for compound **1** after 5 min of electrolysis in acetonitrile containing TBAP 0.1 mol L^{-1} (potential on the plateau of the diffusion wave due to the first electrochemical system: $E_{applied} = E_{1/2} - 0.2$ V). The spectrum recorded 4 min after the end of electrolysis (Fig. 3a) consists in a triplet characterized, as demonstrated by simulation, by the hyperfine splitting constants



Scheme 2. Reduction of indole *N*-oxide.

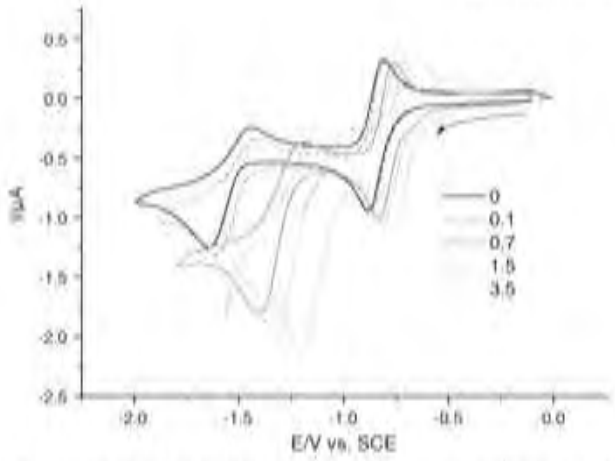


Fig. 2. Electrochemical properties of indolone-*N*-oxides. Cyclic voltammograms of **49** (1 mmol L^{-1}) at a platinum electrode in ACN-TBAP (0.1 mol L^{-1}) with increasing amount of water, potential scan rate 0.1 V/s.

$a_N = 6.27$ G, $a_H^1 = 1$ G (protons on carbons 4 and 7 for compound 1). This nitroxide radical-anion detected is relatively stable (about 10 min). This stability is consistent with an aprotic medium and the highly conjugated character of the indolone-*N*-oxide structure allowing the delocalization of the unpaired electron. The high stability of the spin adducts derived from indolone-*N*-oxides has already been demonstrated in previous studies dealing with spin trapping properties of these molecules [17]. This triplet evolves with time to a signal (Fig. 3c) with hyperfine splitting constants $a_N = 9.5$ G and $a_H^1 = 2.3$ G indicating that the carbon of the nitroxide anionic form has been protonated (Scheme 3). A protonation on the nitroxide function would give a hydroxylamine form which is EPR silent. Before the protonation at carbon 2, the unpaired electron is strongly delocalized on the whole structure which is nearly planar and it couples with the nuclear spins of the nitrogen and proton at C4 and C7 (for compound 1). X-ray crystallography of a similar compound (2-phenylisatogen) shows that the isatogen ring and the phenyl ring are almost fully co-planar [25,26]. After protonation at C2, the planarity of the molecule is decreased thus reducing the electronic delocalization and resulting in a higher electronic density at the NO bond. The unpaired electron then couples with the nuclear spins of the nitrogen and

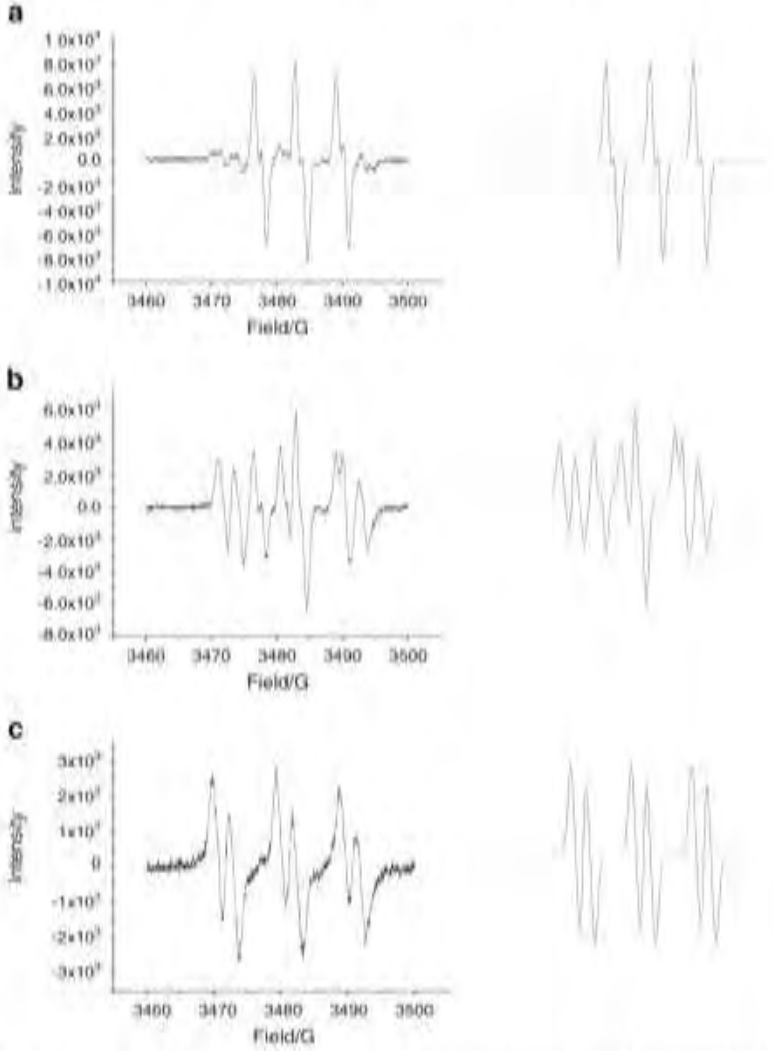
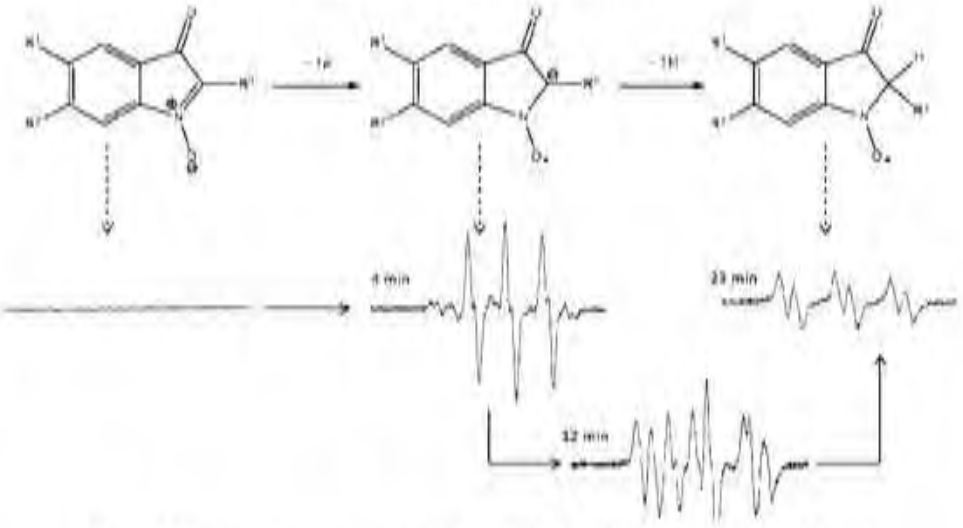


Fig. 3. EPR spectra of the reduced form of indolone-*N*-oxide: EPR spectra and the corresponding simulated spectra recorded for compound **1** (1 mmol L^{-1}) a) 8 min, b) 12 min and c) 23 min after the end of the 5 min electrolysis at −0.9 V vs. SCE in ACN containing TBAP (0.1 mol L^{-1}).



Scheme 3. Relationship between the reduced forms of indolone *N*-oxides and the corresponding EPR spectra.

proton at C2. From this structural change resulting from the protonation at C2, the higher electronic density on the NO bond leads to a higher hyperfine splitting constant, a_N, shifting from 6.27 G to 9.5 G. These observations are in agreement with Scheme 3. For compound **1** a mixture of the two species (nitroxide radical-anion and its protonated form) is observed over about 8 min. As demonstrated by the simulation, the spectrum recorded 12 min after the end of electrolysis is the superimposition, in the same proportion, of the spectra of the two species (Fig. 3b). The coupling between the electrochemistry and EPR spectroscopy leads to a better understanding of each step of the reduction/protonation process compared with that reported for some isatogens [27].

3.3. Relationship between reduction potentials and substituents on indolone-N-oxides

All the compounds studied gave at least two electrochemical systems: the first one presented a half-wave potential ranging from −0.882 to −0.477 V vs. SCE while for the second one the limits are in the domain [−1.650, −1.264] V vs. SCE. These values are highly dependent on the three substituents, R^1, R^2 and R^3. Studies reported for other *N*-oxide derivatives, such as phosphorylated nitrones [28], phenyl-butyl nitrone derivatives [29] or quinoxaline-*N*, *N*′-dioxides [11], showed such a relationship. In our series, the presence of electron-donating groups induces an important shift of the first reduction wave. For example compounds **49** or **50**, (R^1 = alkyl) have the highest reduction potential value. With the introduction of electron-withdrawing substituents (Cl, CF_3, O−CF_3, NO_2) on the phenyl ring (R^1 = aryl) (compounds **1**, **8**, **9**, **11**, **12**, **13**, **19**, **25**, **37**, **39**, **56** and **61**) or on the indolone moiety at R^3 (compounds **19**, **35**, **48**), the products are more readily reduced. For example, the substitution of the alkyl chain (R^1) (compound **49** or **50**) by a 3,4-dichlorophenyl (compound **8**) or by a 4-trifluoromethylphenyl (compound **61**) group resulted in a positive shift equal to 23 mV for the first cathodic wave. This shift was only 9 mV when R^1 was a 4-ethylphenyl group (compound **60**). By comparing the redox potentials of compounds **9**, **37** and **25** or **56** and **61** the withdrawing effect of substituents appears clear: $NO_2 > CF_3 > Cl$. Similarly, by comparing compounds **37** and **25**, one can note that the replacement of CF_3 by O-CF_3 induces, as expected, a negative shift in the reduction potential. A smaller shift is recorded with the introduction of an electron-donating group (−O−R or −Ph−O−R) as R^1 or R^3 (compounds **2**, **4**, **5**, **7**, **18**, **20**, **22**, **24**, **27** **29** and **57**). By comparing compounds **9** and **12** it appears that the position of the methoxy group (R^1 or R^2) did not affect the reduction potential.

These effects are consistent with the facilitation of reduction by lowering the electron density at C2 which is confirmed by the Hammett plot (Fig. 4), considering the σ_R values for substituents in the 4-position of the phenyl ring [27,30]. As predicted by Hammett, the number and position of electron-withdrawing groups influence the reduction potential.

Note that the most cathodic potentials were obtained in the case of amine substituents (compounds **26** and **30**). The addition of a

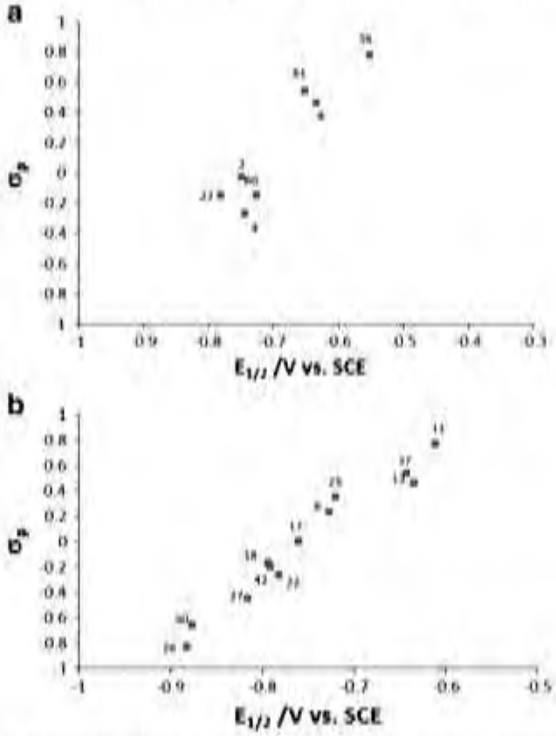


Fig. 4. Relationship between reduction potentials and Hammet constants of indolone *N*-oxides. Plot of $E_{1/2}$ against Hammet substituent constants σ_R for compounds having (a) o- and (b) OMe at R^1 [31].

methoxy group at R^1 does not modify the reduction potential as seen by comparing compounds **2** with **24** and **37** with **61**.

3.4. Relationship between the electrochemical behavior and antiplasmodial activity

The indolone-*N*-oxides gave rise to two reduction steps in ACN/TBAP. The first reduction of the *N*-oxide moiety is reversible and involves the formation of a stable nitroxide radical-anion as confirmed by EPR spectroscopy. This relatively stable radical could be the starting point for the redox events deleterious to the parasite. In this case, the ease of the first reduction of the *N*-oxide moiety would play an important role in the antiplasmodial activity. Indeed, further experiments carried out on the series have demonstrated that the antiplasmodial properties of these indolone-*N*-oxides were controlled by a bioreductive transformation in red blood cells [16].

Many parameters including solubility and log P, absorption, diffusion and metabolism, as well as the cellular targets affect the biological activities of compounds. It is clear that a global analysis of each parameter versus the biological activity studied may not produce a clear relationship. The effects of these parameters may be additive or subtractive, rendering any correlation difficult to observe on a large series. No simple relationship was found between the reduction potential and the antiplasmodial activity. For example, one can note that compounds with aliphatic substitutions (**49**, **50** and **60**) are hardly reduced and poorly active against *Plasmodium*. On the contrary, compounds having amine groups (**26**, **30** and **68**) also have very cathodic reduction potentials whereas they are among the most biologically active compounds of the series. The amine groups at R^3 on these compounds give cationic protonated forms at the pH of the cell model tested (parasitized red blood cells), which are determinant for the absorption mechanism of the cell. In the case of these compounds (**26**, **30** and **68**), the absorption surpasses the electron withdrawing effects of substituents compared with other compounds. Interestingly, we previously showed that antiplasmodial activities were more strongly affected by the substitutions at R^1 than by substitutions at the phenyl group of the indolone moiety (R^1 and R^2). Consequently, the relationship between activity and the ease of reduction have been studied for molecules with $R^1 = -OCH_3$ and $R^2 = H$, compounds distinguished by electron-withdrawing or -donating substituents on the phenyl group at R^3. The corresponding graphs are shown in Fig. 5. In the case of electron-withdrawing substituents (Fig. 5a), the antiplasmodial activity increases when the compounds are more readily reduced. These results are consistent with an antiplasmodial property controlled by a bioreductive transformation in red blood cells [16] and in agreement with mechanisms of action that we recently reported [14].

On the other hand, for electron-donating substituents (Fig. 5b), a large variation in activity is observed, whereas the potential is almost constant due to their weak influence. In this second case, variation in lipophilicity could explain such disparate IC_{50} values. Indeed, in this group, the less potent compounds **20**, **24** and **42** are characterized by the highest partition coefficient ($logP > 3$), reaching 3.63 for **42**.

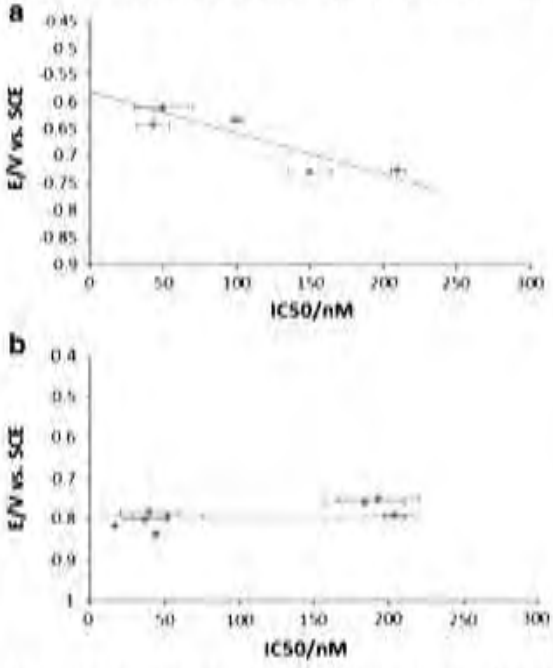


Fig. 5. Relationship between antiplasmodial activity and reduction potential. Plot of $E_{1/2}$ against IC_{50} for a) compounds having the methoxy group (R^1) and electron-withdrawing substituents on the phenyl group at R^3 (**9**, **11**, **12**, **13**, **37**); b) compounds having the methoxy group (R^1) and electron-donating substituents at R^3 (**17**, **18**, **22**, **24**, **27**, **29**, **40**, **42**).

4. Conclusion

The thirty seven indolone-*N*-oxides studied in aprotic solvents, showed two reduction steps located around -0.68 ± 0.2 V and -1.45 ± 0.2 V vs. SCE. The first reduction step was reversible for all the compounds studied under the conditions of the study and ascribed to the reduction of the —C=N—O double bond, whereas the second irreversible reduction step was ascribed to the carbonyl reduction. EPR spectroscopy analysis confirmed that this first reduction gives rise to the formation of a relatively stable radical, which could be the starting point for the redox events deleterious to the parasite. A relationship between electrochemical behavior and indolone-*N*-oxide structure can be established for compounds having electron-withdrawing substituents. The insertion of an electron-withdrawing group on the *N*-oxide ring results in a less negative reduction potential making the bio-reduction easier and thus the compound more active against *Plasmodium*. These results will orientate the selection of substituents for the design of new molecules with better pharmacological activities.

Acknowledgements

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3. Conclusion

Les potentiels d'oxydo-réduction d'une série d'indolone-*N*-oxyde (37 représentants), en milieu aprotique ont été étudiés par voltammétrie cyclique et spectroélectrochimie RPE. La réduction de cette série se caractérise par deux étapes de transfert d'électron situées à environ - 0,68 ± 0,2 V et -1,45 ± 0,2 V. La réduction du groupe -C = N-O avec le transfert du premier électron, est totalement réversible. La seconde étape de réduction correspond à la réduction du groupe carbonyle qui est quasi-réversible en raison de la formation du pinacol après dimérisation.

L'étude indique que les composés ont une plus grande activité antipaludique quand ils sont plus facilement réductibles en raison de deux évidences i) une relation entre le comportement électrochimique et les structures chimiques des composés INODs établies pour des groupements électro-attracteurs sur le carbone alpha de la fonction nitrone qui facilite la réduction, ii) ces composés sont bio-réductibles dans les globules rouges.

Par couplage électrochimie RPE, la formation d'un radical cation stable formé au cours de la première réduction est confirmée. La protonation du radical se produit sur le carbone en position alpha de la fonction nitrone plutôt que sur le groupe NO. Ce radical intermédiaire est obtenu avec un potentiel compatible avec l'environnement intracellulaire. Ce radical pourrait déclencher un signal redox activant plusieurs signaux d'oxydo-réduction en cascade et la génération d'un stress oxydant fatal au globule rouge parasité

3. Conclusion

The redox potential of thirty seven indolone-*N*-oxides in aprotic solvent was studied using cyclic voltammetry and EPR spectroelectrochemistry. The reduction of this series is characterized by two electron transfer steps located around - 0.68 ± 0.2 V and -1.45 ± 0.2 V. The reduction of the –C=N–O double bond is the first electron transfer which is totally reversible. The second reduction step corresponds to the reduction of the carbonyl group that is quasi-reversible due to the formation of pinacol after dimerization.

The study established a relationship between the electrochemical behaviour and the chemical structures of INODs for compounds having electron-withdrawing substituents. These derivatives have less negative reduction potential, resulting in easier bio-reduction. In addition, the previous study showed that INODs are bio-reducible in infected RBCs. Therefore, when the compound is more easily reducible, the antiplasmodial activity is higher.

By coupling electrochemistry to EPR, the formation of stable radical cation derived from the first reduction is confirmed. The protonation of the radical occurs on the carbon in alpha position to the nitrone function and not on the NO group. This radical intermediate is obtained at a potential compatible with an intracellular environment. This radical activates several redox signals in cascade to generate, finally a fatal oxidative stress to parasitized RBC.

III. PRO-OXIDANT PROPERTIES OF INDOLONE-*N*-OXIDE DERIVATIVES IN RELATION TO THEIR ANTIMALARIAL PROPERTIES

1. Introduction

Les relations entre le comportement redox et l'activité antpaludique des indolone-*N*-oxydes ont été étudiées dans le chapitre précédent (page 42). Leurs potentiels redox sont proches de ceux de la quinine (Bunney et al, 1970). Les travaux antérieurs dans le laboratoire avaient démontré la bio-réductibilité des INODs, enzyme et thiol-dépendante au sein des globules rouges. Etant donné la quantité importante de thiols dans le globule rouge, en particulier le glutathion (GSH), l'intéraction des INODs avec ces thiols pourrait jouer un rôle essentiel dans la biotransformation des INODs dans les globules rouges. Le but de cette étude a été d'étudier les premiers stades de l'interaction biochimique des INODs avec certains composants erythrocytaires (GSH, L-cystéine) en comparaison avec la chloroquine et l'artémisinine, dans des modèles biomimétiques. Le composé **1** (INOD-1) a été choisi pour cette étude en raison de ses puissantes propriétés antipaludiques et *in vitro* et *in vivo* sa faible toxicité.

Ces trois composés (INOD, chloroquine, artémisinine) modifient le métabolisme redox du parasite *P. falciparum* à l'intérieur des globules rouges. La chloroquine interfère avec le processus de biocrystallization de l'hémoglobine évitant la formation d'hémozoïne (Zhang et al, 1999). L'artémisinine ou ses dérivés génèrent des intermédiaires radicalaires qui crée un stress oxydant et exerce ainsi une activité parasicide (Bray et al, 2005). Dans ce chapitre, nous avons voulu répondre à la question suivante: est-ce que le mode d'action des INODs est identique ou différent de ceux de la chloroquine ou l'artémisinine? Pour comparer l'activité des INODs avec la chloroquine et l'artémisinine, leur interaction avec l'hème et leur capacité à générer ou à piéger des radicaux ont été étudiées.

1. Introduction

The relationship between redox behavior and antimalarial activity of indolone-*N*-oxides was reported in aprotic solvent in the previous chapter (page 42). Their redox potentials are near to those of quinine (Bunney *et al*, 1970). The previous work in the laboratory had demonstrated the bio-reducibility of INODs, enzyme and thiol dependent, inside the RBCs. Since there is high abundant amount of thiol compounds especially glutathione (GSH) in red blood cells (RBCs), the interaction of INODs and thiol compounds could play a pivotal role in the biotransformation of INODs within RBCs. The aim of this study was to explore the early stages of the biochemical interaction of INODs with some RBCs components (GSH and L-cysteine) in comparison to chloroquine and artemisinin, in biomimetic model. Compound **1** (6-(4-chlorophenyl)-7H-[4,5-f]indol-7-one-5-oxide) was selected for this study due to its potent antimalarial properties *in vitro* and *in vivo* and low toxicity.

These three compounds (INODs, chloroquine and artemisinin) change the redox metabolism of parasite *P. falciparum* inside RBCs. Chloroquine interferes with the haemoglobin biocrystallization process to prevent hemozoin formation (Zhang *et al*, 1999). Artemisinin or artemisinin derivatives generate radical intermediates which creat oxidative stress and exert parasicide activity (Bray *et al*, 2005). In this chapter we wanted to answer the following question: Is the mode of action of INODs the same as chloroquine or as artemisinin or none of them? To compare the activity of INDOs with chloroquine and artemisinin, their interaction with heme and their capacity to generate and/or trap radicals should be investigated.

2. Electron spin resonance and cyclic voltammetry studies of indolone-*N*-oxide derivatives in relation to their antimalarial properties

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Pro-oxidant properties of indolone-*N*-oxides in relation to their antimalarial properties

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Indolone-*N*-oxides (INODs) are bioreducible and possess remarkable anti-malarial activities in the low nanomolar range *in vitro* against different *Plasmodium falciparum* (P.f.) strains and also *in vivo*. INODs have an original mechanism of action: they damage the host cell membrane without affecting non-parasitized erythrocytes. These molecules produce a redox signal which activates SYK kinases and induces a hyperphosphorylation of AE1 (band 3, erythrocyte membrane protein). The present work aimed to understand the early stages of the biochemical interactions of these compounds with some erythrocyte components from which the redox signal could originate. The interactions were studied in a biomimetic model and compared with those of chloroquine and artemisinin. The results showed that INODs i) do not enter the coordination sphere of the metal in the heme iron complex as does chloroquine; ii) do not generate iron-dependent radicals as does artemisinin; iii) generate stable free radical adducts after reduction at one electron; iv) cannot trap free radicals after reduction. These results confirm that the bioactivity of INODs does not lie in their spin-trapping properties but rather in their pro-oxidant character. This property may be the initiator of the redox signal which activates SYK kinases.

Introduction

Molecular and proteic redox systems play an important role in the control of cellular homeostasis and antioxidant defences. Some drugs containing a redox pharmacophore (quinone and quinoid compounds, *N*-oxide, nitro and thiol derivatives, endoperoxides) may generate an oxidative stress in the cell that can be fatal for the hosted microbes and for the cells. We recently reported that indolone-*N*-oxides, which are bioreducible, posess remarkable anti-malarial activities in the low nanomolar range *in vitro* against different *Plasmodium falciparum (P.f.)* strains and are also active *in vivo*.[1,2] Moreover, these compounds are only cytotoxic at very high doses (micromolar range) thus giving a very interesting selectivity index. Early studies showed that INODs have redox potentials near to those of 1,4-quinones and therefore may exert their biological action by oxidizing essential biomolecules.[3] In addition, they containing a nitrone function that may react with the glutathiyl radical formed within the cells upon oxidation of glutathione.[4] To explore the mechanism of action of INODs, we screened for changes in INOD-treated *P. falciparum*-infected red blood cells (RBCs) using a comprehensive proteomic approach. INODs have an original mechanism of action: they damage the host cell membrane, without affecting non-parasitized erythrocytes, with the consequent RBC membrane vesiculation and destabilization responsible for parasite death. The mechanism leading to the selective destabilization of the membrane of parasitized erythrocytes involves the activation of a stress responsive phosphorylation pathway which finally induces the uncoupling of membrane-cytoskeleton interactions. Marked hyperphosphorylation of AE1 (band 3) appears to be the hallmark of the process.[5] We have also studied the kinetics of penetration and biotransformation of these molecules in the erythrocytes. The compounds penetrate very rapidly, accumulate and are rapidly bio-transformed in the RBC cytosol by a thiol-dependent reduction possibly *via* an enzymatic pathway.[6] Because INODs contain the *N*-oxide functional group, this bioreductive transformation was expected, as it had previously been described for other *N*-oxides derivatives.[7] We recently reported the examination of the antimalarial properties of these compounds in relation to their redox properties using cyclic voltammetry coupled to

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EPR spectroscopy.[8] Given the redox events occurring in Plasmodium infected RBCs, this bioreductive transformation may be pivotal for the parasites' redox balance and for the antiplasmodial activity. Altogether these studies showed that these compounds target the redox metabolism of the infected host cell.

In a previous study[2] we investigated the *in vitro* antimalarial properties of compound **1** (6-(4-chlorophenyl)-7H-[1,3]dioxolo[4,5-f]indol-7-one-5-oxide) against fresh clinical isolates of *P. falciparum*. This study was carried out to (i) compare the activity of compound **1** with that of chloroquine and dihydroartemisinin to assess the potential for cross-resistance, (ii) investigate drug interactions of indolone-*N*-oxides with standard antimalarials and (iii) determine the stage-dependent activity of indolone-*N*-oxides. Compound **1** was equipotent against chloroquine-susceptible and chloroquine-resistant isolates. There was no correlation between responses to chloroquine and compound **1** (r¼ 0.015; P>0.05), but the *in vitro* responses of compound **1** and dihydroartemisinin were significantly and positively correlated (r¼ 0.444; P<0.05). INODs, as well as artesunate, inhibited parasite maturation at the ring stage.

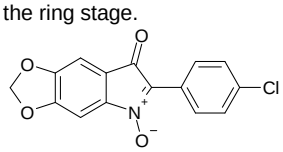


Figure 1. Structure of compound **1**.

The antimalarials, chloroquine (CQ) and artemisinin (ART), also target the redox metabolism of *P. falciparum.* The former belongs to the quinolines that interfere with haemoglobin biocrystallization[9] preventing hemozoin formation, while artemisinin-like derivatives create oxidative stress by generating radical intermediates[10]. Considering i) the mechanisms of action of artemisinin and chloroquine; ii) the redox properties of INODs; iii) and the interactions observed between these molecules in fresh clinical isolates, the present study aimed to understand the early stages of the biochemical interactions of the INODs with some RBC components and to compare them with the actions of chloroquine and artemisinin, in biomimetics models. As the lead compound of the INOD series, compound **1** was selected for these studies (Figure 1). EPR and electrochemical experiments were designed to study i) the bioreductive properties of compound **1** in relation to those of ART and CQ: ii) the interaction with non-protein thiols (L-cysteine and glutathione); iii) the capacity to interact with heme; iv) the capacity to generate and/or trap radicals.

Results and Discussion

Electrochemical behaviour of compound 1 and interaction with thiol compounds

The use of electrochemical methods to obtain relevant information about drugs containing a redox pharmacophore is particularly important to predict their biotransformation in cells. The main structural feature of INODs is the redox heterocyclic core (redox pharmacophore) in which the nitrone moiety ($C=N^{+}–O^{-}$) is conjugated to the ketone function giving to the molecule the capabilities to undergo several oxidation-reduction reactions.

The electrochemical behaviour of this kind of compounds has been previously reported in non-aqueous media [8, 11]. In this work, the reducible behaviour of INODs was studied in presence of water using cyclic voltammetry. Due to their low aqueous solubility, INODs were studied in DMSO/water 80/20 (*v/v*). This solvent mixture was selected after testing different solvent systems (ex. ACN/H_2O; DMF/H_2O). The system selected (DMSO/H_2O) was the best compromise to simultaneously solubilize INOD compounds and L-Cys (or GSH) where other systems had failed to do so. Figure 2 represents the voltammograms obtained for compound **1**. The first cycle shows the reduction of the electroactive compound **1** around – 0.5 V; on the backward scan, two anodic peaks are observed around – 0.4 V and – 0.1 V. In the second cycle and forward scan, a cathodic peak around -0.2 V appeared and is coupled with the anodic peak around – 0.1 V. It appears that the electrochemical system of compound **1** can be translated by two electron transfers: $E_{1/2}$ = – 0.43 V and $E_{1/2}$ = – 0.15 V. The cathodic peak currents of the first cycle are linearly related to the square root of the potential scan rate as for a diffusion controlled process. By comparison with the oxidation peak current of ferrocene recorded under the same conditions, mono-electronic transfers are expected for the reductions of INODs. The electron transfer recorded during the first reduction process corresponds, as demonstrated in non-aprotic solvent [8], to the reduction of the *N*-oxide to a nitroxide radical-anion (Scheme 1). It can be noted that, by comparison with the voltammogram recorded in non-aqueous medium, the nitroxide function is more easily reduced in presence of water, due to the fact that electrochemical oxido-reduction requires higher energy when increasing the content of the organic phase in the solvent supporting electrolyte.

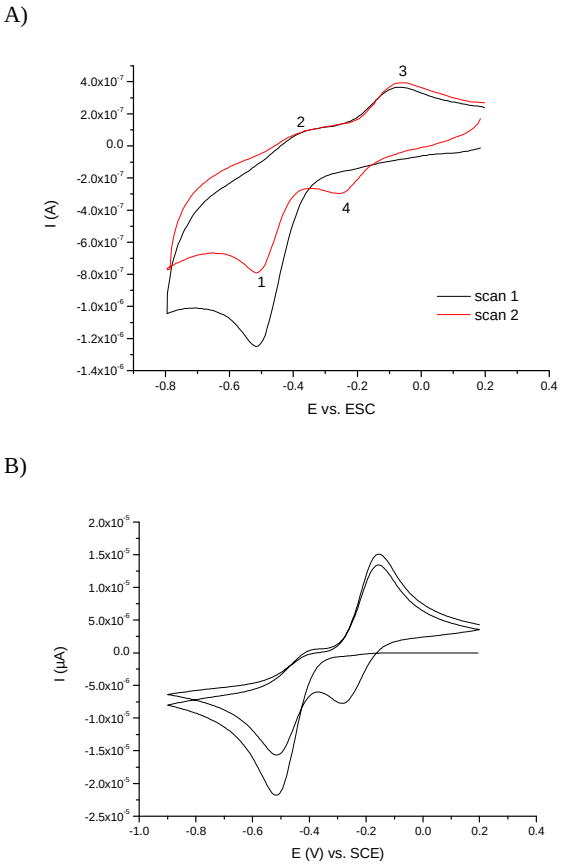
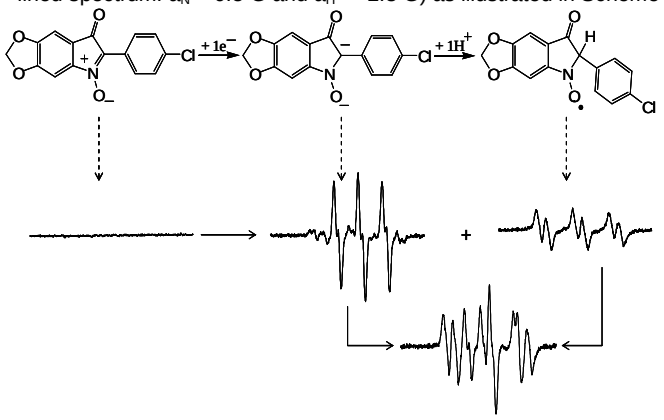


Figure 2. A) cyclic voltammograms at a glassy carbon electrode (1 mm diameter) in DMSO/water (80/20 v/v) of compound **1** (10 µM), potential scan speed 0.1 V/s; B) the corresponding simulated voltammograms (DigiElch 6.F (Gamry)).

EPR spectro-electrochemical experiments were carried out in order to characterize the reduction products. The in-situ electrolysis ($E_{applied}$ = - 0.9 V vs. Ag/AgCl) was monitored by recording the EPR spectra. The EPR spectrum obtained at the beginning of the electrolysis (Figure 3A) consists of a multi-lined pattern characteristic of the superposition of two species, the nitroxide radical-anion (three lined spectrum: a_N = 6.27 G, a_H^β =1 G) and its protonated form (six lined spectrum: a_N = 9.5 G and a_H^β = 2.3 G) as illustrated in Scheme 1



Scheme 1. Relationship between the reduced forms of compound **1** and the corresponding EPR spectra.

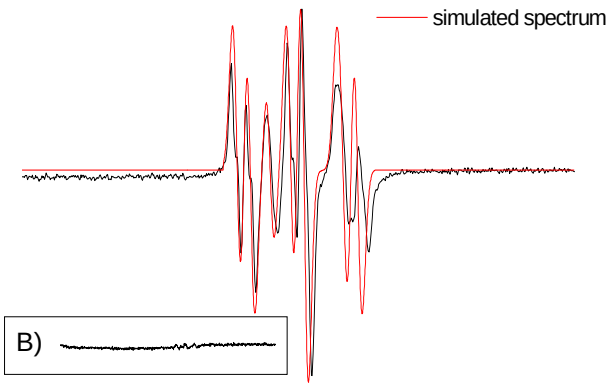
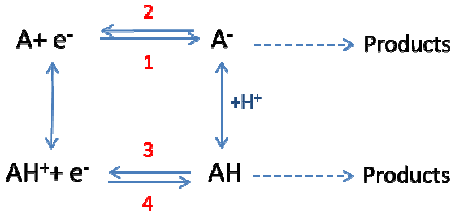


Figure 3. EPR spectra of the reduced form of compound **1**: EPR spectra and the corresponding simulated spectra recorded during the electrolysis at -0.9 V vs. Ag in DMSO/water (80/20, v/v) containing TBAP (0.1 mol.L^{-1}) in the absence (A), and in presence (B) of L-cysteine (100 mmol.L^{-1}).

These results confirmed the expected monoelectronic transfer and the formation of two reduction products responsible, on the voltammogram, for the two oxidation peaks recorded (2 and 3) on the reverse scan and for the appearance of a second reduction wave (4) during the second scan. The reductive pathway of compound **1** (A) is presented in Scheme 2.



Scheme 2. Reductive pathway of compound **1.**

The reduction of compound **1** (A) gives the radical-anion A$^-$ that is detected by EPR spectroscopy which is rapidly protonated into AH. On the backward scan, the oxidation peak of A$^-$ is small (Ep_2 ~ - 0.4 V) compared to that of the protonated AH (Ep_3 ~ - 0.06 V). On the forward scan of the second cycle, the reduction of AH$^+$ is observed (Ep_4 = - 0.25 V). Figure 4 shows the normalized voltammograms ($I/v^{1/2}$ versus E) of the second cycle as a function of the potential scan rate. Due to the ohmic drop coming from the DMSO, the voltammograms shifted when the potential scan speed was increased. From Figure 4, it can be seen that the oxidation peak of A$^-$ (Ep ~ - 0.4 V) did not increase with the potential scan rate, indicating a fast protonation. On the contrary, the normalized currents (anodic oxidation of AH or cathodic reduction of AH$^+$) increased with the potential scan speed because decomposition of AH is restricted and AH$^+$ is a weak acid.

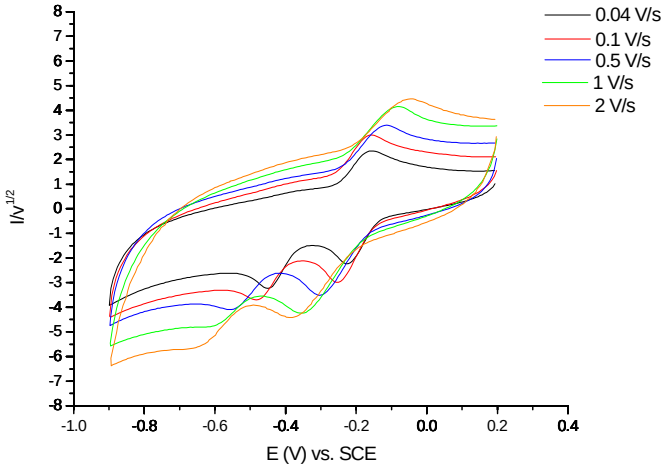


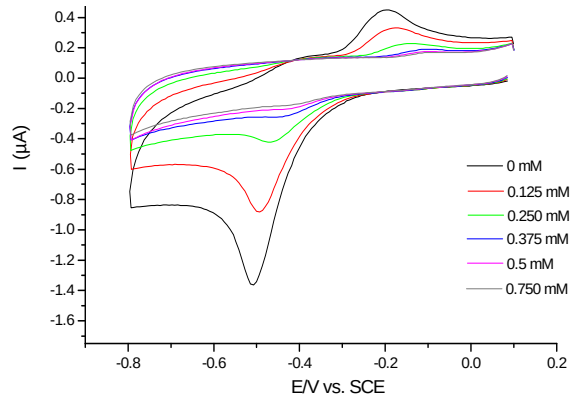
Figure 4. Normalized cyclic voltammograms at a glassy carbon electrode (1 mm diameter) in DMSO/water (80/20, v/v) of compound **1** at different potential scan rates v; the current is normalized by $I/v^{1/2}$.

Simulations with DigiElch 6.F (Gamry) (Figure 2B) confirmed the shape of the voltammograms according the scheme proposed in Scheme 2. The instability of the radicals has been taken into account: the kinetic constant, k, (0.1 s^{-1}) for the radical-anion A$^-$ is five hundred times greater than that of AH. In the case of the protonation, the kinetic constant k_H (pseudo first order rate 1 s^{-1}) for the radical-anion A$^-$ is a thousand times greater than that of A. The radical-anion A$^-$ is nearly a strong base which explains the weak peak current (Ep ~ - 0.4 V) on the reverse scan. The difference in the acidity properties is in agreement with the potential shift of the redox couples A/A$^-$ and AH$^+$/AH as in Pourbaix's diagrams.

Previous studies have demonstrated that the antiplasmodial property of the INODs was controlled by a bioreductive transformation in RBCs [6], with the compounds being immediately reduced when entering the RBC by a pathway that is thiol- and enzyme-dependent. Some thiol reagents, like mercaptoethanol [12], cysteine or *N*-acetylcysteine mimic the reactivity of thiol-containing enzymes, such as topoisomerase [12,13]. To study the interaction of the compound with such thiol-containing enzymes, the electrochemical behaviour of compound **1** was studied in presence of L-Cys. As shown in Figure 5A, increasing the concentration of L-Cys resulted in a decrease in the first reduction wave proportional to the concentration added, whereas the oxidation wave remained unchanged. Above 0.75 mM L-Cys, the two reduction waves had almost totally disappeared. The decrease in peak intensity was also accompanied by a peak potential shift to the more positive region. The decline of peak potential and intensity associated to a change of colour of the solution indicated

that there was a chemical reaction between compound **1** and L-Cys. This result is confirmed by the disappearance of the EPR signal after reduction in presence of L-cysteine (Figure 3B). The effect of GSH, the most abundant RBC non-protein thiol, is shown in Figure 5B. In this latter case, there was a greater decrease in the intensity of the reduction waves with a concomitant displacement to low potential.

A)



B)

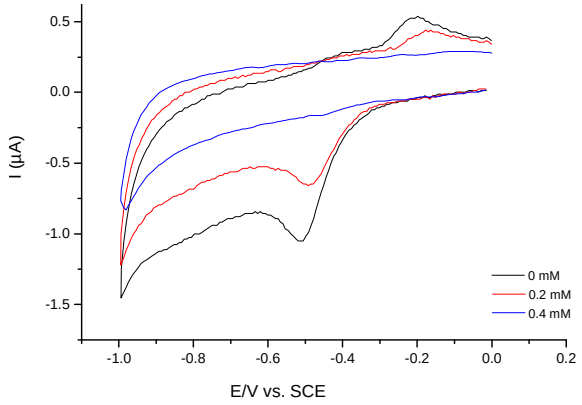


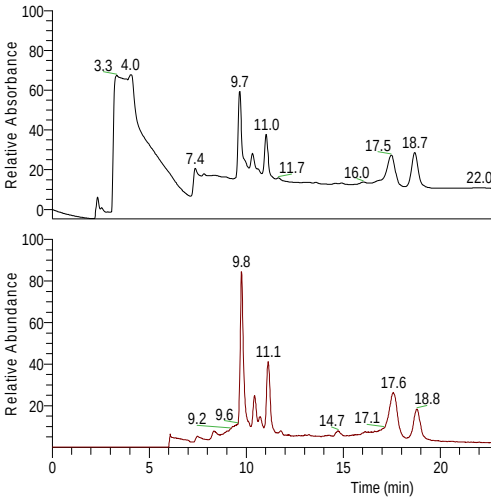
Figure 5. Cyclic voltammograms of compound **1** (0.25 mM) at a glassy carbon electrode in presence of various concentration of (**A**) L-cysteine and (**B**) GSH recorded in DMSO/phosphate buffer (80/20, v/v), pH = 7.

The same behaviour has been described for quinone compounds [13], where 1,2- and 1,4-Michael-type adducts are formed by the addition of the thiol group to the quinone ring. The pseudo-quinone structure of the INOD could explain the similar reactivity towards L-Cys. It was also reported that L-Cys can directly interact with artemisinin (without iron mediation) to form a binary adduct, enhancing artemisinin stability with subsequent negative shift of the reduction potential from - 0.64 V to - 1.03 V [14]. The decrease in the peak intensity recorded in our case, with the subsequent potential shift, may be due to a reaction of INOD with thiols.

Chemical analysis of the interaction between compound 1 and L-Cys

Chromatographic analysis of the products obtained from the chemical interaction between compound 1 and L-Cys in solution is shown in Figure 6. The reaction between compound 1 and L-Cys leads to the reduction of the nitrone function moiety (C=NO) and the formation of three major products (P1, P2, and P3) as shown in Figure 6. As the parent compound 1 (t_r = 18.7 min) is chlorinated, therefore the follow-up of its products (P1, P2, and P3) is favoured by mass detection. These three major products had different UV-Vis spectra with masses of 303, 303, and 319 amu for P1, P2, and P3, respectively. This suggests that two reduced (dihydrogenated) isomers (303 amu) and a hydrated form (319 amu) are produced. Of particular interest is the product P1, eluted at t_r = 9.7 min, with the reduced nitrone moiety (C(OH)–NH). It corresponds to the same compound isolated from human erythrocytes when compound 1 was incubated inside these cells [6]. The second product, P2, (t_r = 11.0 min) may correspond to another reduced form (C(H)–NOH) whereas the third product, P3, (t_r = 17.5 min) may correspond to (C(OH)–NOH). These three reduced forms are in agreement with the reduction and hydration reactions known for the nitrone moiety [15].

A)



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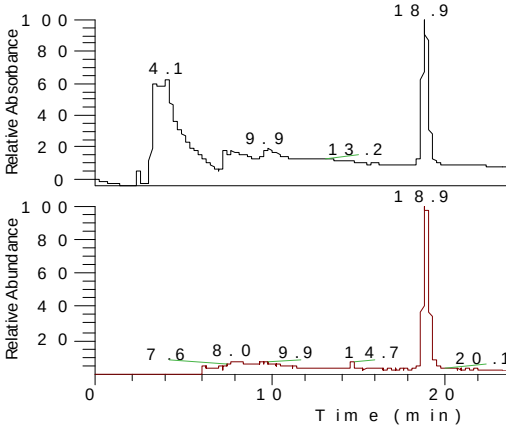
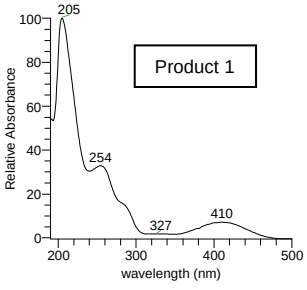
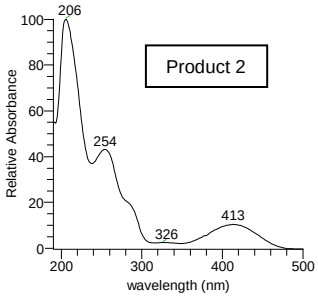


C)



D)



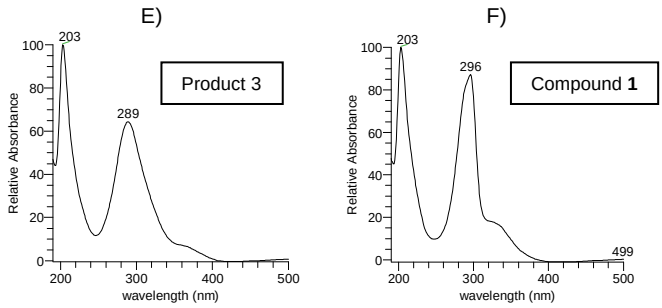


Figure 6. LC-(-)APCI-MS analysis of A) the interaction between compound **1** (0.25 mM) and L-Cys (2.5 mM), the top chromatogram was obtained from a photodiode array detector (total scan) and the bottom chromatogram obtained from (-)APCI-MS (total ion current); B) the same as (A) but without L-Cys; C), D), E), and F) represent the corresponding UV-Vis spectra of each compound.

Reactivity of compound 1 towards Fe (II)-heme and Fe(III)-hemin.

In infected RBC, the parasite responsible for malaria digests the hemoglobin to use the aminoacids to its advantage. In the course of this hemoglobin digestion, four equivalents of [Fe(II)-heme] are released and oxidized into hematin which is detoxified by the parasite into the malaria pigment or hemozoin, a highly insoluble microcrystalline form of [Fe(III)(protoporphyrin-IX)]. One mechanism proposed for the quinoline-type antimalarial drugs, such as chloroquine (CQ), quinacrine, quinine, and mefloquine is that they exert their action by disrupting the formation of the hemozoin pigment, thus eliciting toxicity to the parasite from the build-up of free heme [15,16]. Studies suggested that the interaction of these drugs with ferriprotoporphyrin-IX occurred through coordination of the amino-quinoline group of the drug to the iron center [17, 18]. Therefore it appeared interesting to study the interaction of compound 1 with iron heme model complexes in solution using EPR spectroscopy, to compare with artemisinin and chloroquine. The paramagnetic monomeric hemin complex, [Fe(III)-hemin] presents, at 107K (liquid nitrogen flow), a single EPR asymmetrical broad line at g = 5.656 characteristic of a ferric high-spin complex (S=5/2) [19] (Figure 7a). Adding compound 1, ART, CQ or quinine (Q) to the medium containing the hemin complex, did not change the EPR spectra (Figure 7b, 7c, 7d and 7e). In a second step, hydroxylamine ($NH_2OH.HCl$) was added to the hemin complex solution (Figure 7f). A large decrease of the EPR signal intensity was observed under these conditions indicating a loss of the paramagnetic properties of the sample. The lack of any new EPR lines on the spectra and this strong decrease in intensity are in favour of the formation of the hematin μ-oxo-dimer (μ[Fe(III)PPIX]$_2$O) from hemin [21, 22]. This dimer does not yield an EPR signal due to an antiferromagnetic coupling between the two S = 5/2 ferric ions. This spin state has been proposed from susceptibility measurements and Mössbauer studies [21, 22]. When compound 1 or ART are added to the mixture under these conditions, the same weak intensity line is observed (Figure 7g and 7h, respectively) showing that the EPR silent species (μ-oxo dimer proposed) is not modified in its ligand coordination and spin state. It is completely different when CQ is added to the mixture since there are no traces of high spin iron(III) at g = 5.656 but a strong and unique signal appears at g = 2.026 (Figure 7i). The same result was obtained with quinine (Figure 7j). Antimalarial drugs such as chloroquine and quinine are known to exert their antimalarial activity by binding to hematin in its μ-oxo-dimer form, avoiding the formation of hemozoin [23]. Interaction of the hematin μ-oxo dimer with CQ and Q could modify the redox and spin state of the metal ion. The line observed at g = 2.026 could then correspond, as described in literature [24, 25], to an oxidized form of the μ-oxo form dimer (μ-[PPIXFe(III)-O-Fe(IV)PPIX(L)$_x$]) (L = CQ or Q) which is paramagnetic. Whatever the EPR silent species formed by adding hydroxylamine (μ-oxo dimer hypothesized), only chloroquine and quinine are able to change the iron coordination and spin state of this species showing that they enter the coordination sphere of the metal while compound 1 and artemisinin cannot. These results show that the mechanism of action of these indolone-*N*-oxides does not lie in their ability to interact with an iron centre to prevent the biocrystallization of hemozoin.

Interaction of INOD with radicals derived from artemisinin and iron

Our results on fresh clinical isolates showed a slight synergistic action between artemisinin and INODs [2]. The key pharmacophore of INODs is the indolone-*N*-oxide core (conjugated nitrone function) which may trap radicals [26] and the key pharmacophore of artemisinin is the endoperoxide bridge [27] which may produce radical species with iron(II) [16,18]. Iron(II) salts reductively activate the peroxide bond of artemisinin leading to the formation of a pair of oxyl radical intermediates that rapidly rearrange *via* either a 1,5 H-shift or β-scission to produce the more stable carbon-centred radicals [28]. These alkyl radicals can be readily formed *in vivo* by the reaction of artemisinin with iron(II)-heme [16,29,30], the most abundant source of iron in Plasmodial-infected erythrocytes. It is proposed that these reactive C-radicals interact with cellular components such as heme and parasite proteins resulting in the death of the parasite. Because of these opposite properties between INOD and artemisinin, the possibility that compound **1** or its reduced form, may or may not trap the radicals generated by artemisinin in the presence of iron(II), has been studied using EPR to understand the synergistic effect recorded in our case. The EPR spectrum obtained from the mixture compound **1**/artemisinin/iron(II) is presented in Figure 8A. The EPR spectrum consists of a six-lined spectrum characterized by the hyperfine splitting constants: a_N = 9.57 G; a_H = 2.50 G. In the absence of iron(II) and/or artemisinin, no EPR spectrum was recorded. The results confirm that the INOD compound can efficiently trap the "iron-mediated" artemisinin radicals, whereas INOD did not generate any radicals in the presence of Fe(II), under our experimental conditions (Figure 8B). On the contrary, after reduction of compound **1,** no trapped radicals were observed on the EPR spectrum (Figure 8C). This result is in contradiction with the synergic effect recorded for the two drugs and demonstrates that the mechanism of action of compound **1** do not lie on its radical trapping properties.

Conclusion

These results show that the compound **1** i) does not enter the coordination sphere of the metal in the iron complex as does chloroquine; ii) cannot trap free radicals after reduction; iii) generates stable free radical adducts after reduction at one electron. These results are in good agreement with the fact that no correlation was observed between INODs and CQ responses on fresh clinical isolates of *P. falciparum*[2] while *in vitro* the responses of compound **1** and dihydroartemisinin were significantly and positively correlated. The results suggest different mechanisms of action or different molecular targets for these three antimalarial drug classes. These hypotheses are reinforced by the fact that INODs are equipotent against both types of chloroquine resistant and sensitive strains of *P. falciparum*[1,2]

INODs lose radical trapping properties after reduction which happens *via* a rapid intracellular erythrocytic thiol-dependent reduction as reported previously.[6] These results confirm that the bioactivity of INODS compounds does not lie in their spin-trapping properties but rather in their pro-oxidant character. This property may initiate the redox signal that activates SYK kinases and induces a hyperphosphorylation of AE1 (band 3) and could be connected to the pro-oxidant effects of both derivatives but with different targets.[5]

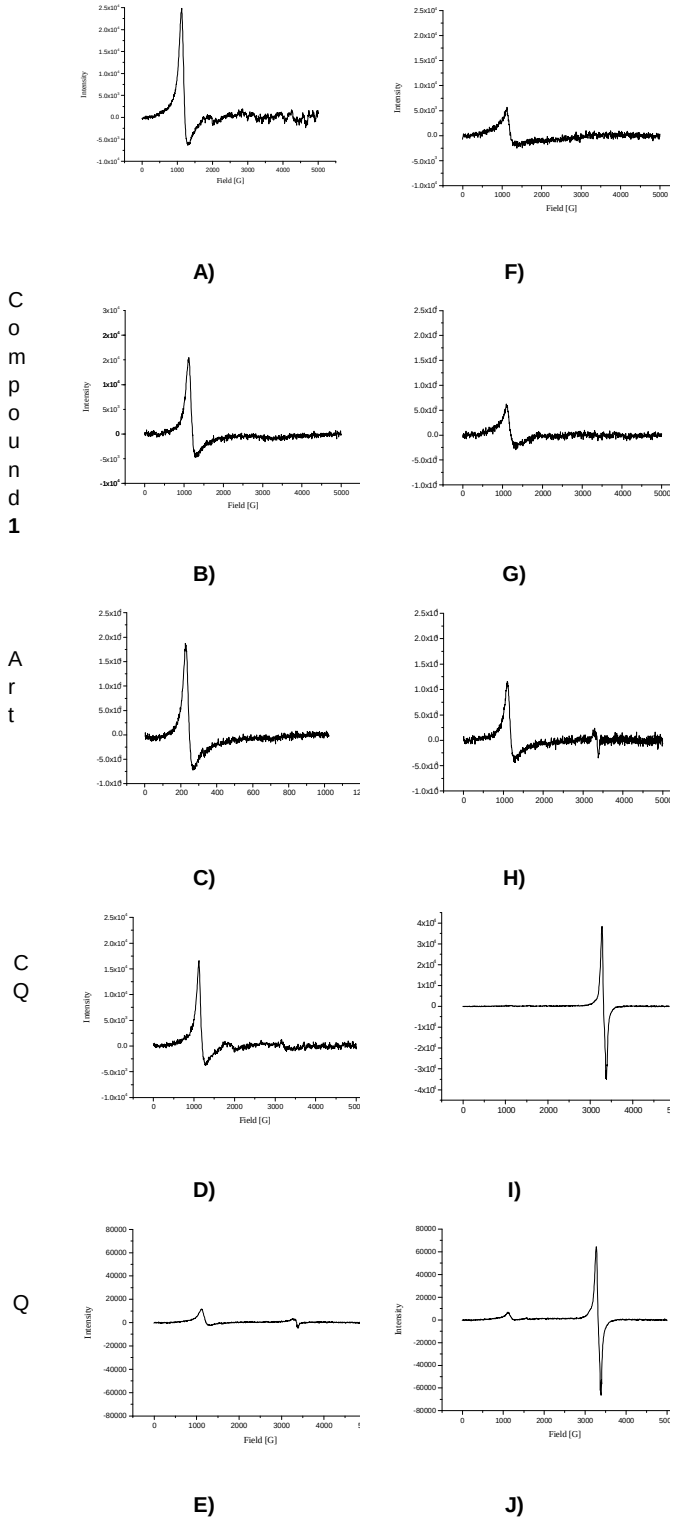


Figure 7. EPR spectra recorded in frozen DMSO at 107K with **A**) hemin (0.75 mM), **B**) hemin/compound **1** (0.75/3 mM)**, C**) hemin/artemisinin (0.75/3 mM), **D**) hemin/chloroquine (0.75/3 mM), **E**) hemin/quinine (0.75/3 mM),**F**) hemin/hydroxylamine (0.75/7.5 mM), **G)** hemin/hydroxylamine/compound **1** (0.75/7.5/3 mM), **H**) hemin/hydroxylamine/artemisinin (0.75/7.5/3 mM), and **I**) hemin/hydroxylamine/chloroquine (0.75/7.5/3 mM), J) hemin/hydroxylamine/quinine (0.75/7.5/3 mM).

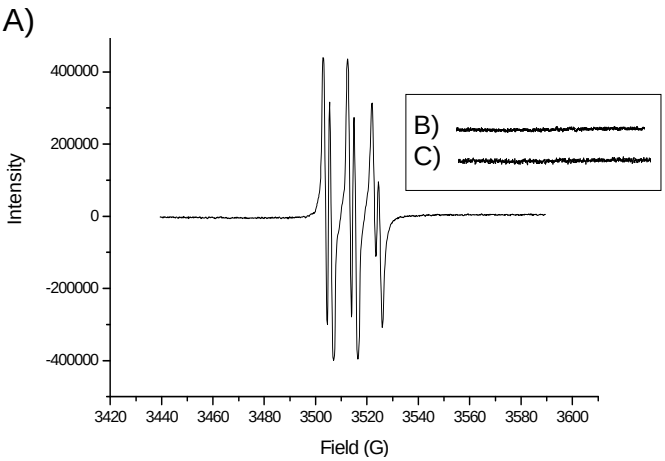


Figure 8. EPR spectra recorded after 10 min incubation in DMSO/water (90/10, v/v) of a mixture containing A) artemisinin 2.5 mM, Fe^{2+} ($(NH_4)_2Fe(SO_4)_2.6H_2O$) 0.25 mM and compound **1** (0.25 mM); B) Compound **1** and Fe^{2+}; C) compound **1**/ Fe^{2+}/ART/L-Cys

Experimental Section

Chemicals

6-(4-chlorophenyl)-7H-[1,3]dioxolo[4,5-f]indol-7-one-5-oxide (compound **1**) was synthesized in our laboratory as previously reported[1]. Ferrous ammonium sulphate, hemine chloride, hydroxylamine hydrochloride ($NH_2OH.HCl$), sodium acetate, sodium dihydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4) and ferrocene were purchased from Prolabo (VWR, France); dimethylsulfoxide (DMSO), HCl 37%, NaOH, tris-HCl, hydrogen peroxide (H_2O_2), L-cysteine (L-Cys), anhydrous acetonitrile (ACN), ferrocene, chloroquine diphosphate salt, artemisinin and glutathione (GSH) were purchased from Sigma-Aldrich (St. Quentin, France); ethylenediaminetetraacetic acid (EDTA) and tetrabutyl ammonium perchlorate (TBAP) were purchased from Fluka, RPMI from Cambrex (Verviers, Belgium).

Electrochemical analysis

Electrochemical experiments were carried out at 25 °C (thermal bath) in DMSO/Tris-HCl buffer (0.1 M) (80/20, v/v), using a Voltalab 80 PGZ 402 (Radiometer) with a conventional three-electrode system including an Ag/AgCl electrode or an SCE as the reference electrode, a platinum electrode (5x5 mm) as the counter electrode and a glassy carbon disk (1 and 3 mm diameter) as the working electrode. All solutions were deoxygenated by passing a gentle, constant stream of pre-purified argon through the solution for 10 min and maintaining a blanket of the inert gas over the solution during the experiment. The glassy carbon electrode was cleaned after each run by electrochemical cleaning to avoid aggressive changes. The electrochemical cleaning process was done in acetate buffer pH 4.5 by polarization for 10 min at - 500 mV then at 2000 mV vs. The reference electrode (the electrochemical cleaning process was done according to the Princeton Co protocol). Between experiments, glassy carbon was rubbed on the polishing pad with blue diamond slurry

particles (Waters, USA), then washed with methanol and distilled water.

Chemical analysis of the interaction between compound 1 and L-Cys

LC and LC-MS analysis were carried out with an LC-PDA-MS^n system (Thermo electron Corporation and Spectra system (SS)) including an automatic injector with an oven (SS-AS3000), a degasser (SS-SCM1000), and a quaternary pump (SS-P1000 XR) coupled to a photodiode array detector PDA (SS-UV6000LP), and an ion trap mass spectrometer (Finnigan LCQ Deca XP Max). Nitrogen was used as a nebulizing and drying gas. Data acquisition was carried out using Finnigan Xcalibur software (version 1.4). The atmospheric pressure chemical ionization (APCI) source was used in the negative ion mode. Mass scans were done in the range *m/z* 50 – 650. The chromatographic separation was done on an analytical column Luna® C-18 (5 *µ*m, 250 mm × 4.6 mm) using a C-18 pre-column (5 µm, 4.6 mm x 3 mm) (Phenomenex, France). The separation was done in gradient mode using solvent A (water) and solvent B (CH_3OH). The gradient program was the following: at *t* = 0-3 min; solvents (95% A / 5% B, v/v); at *t* = 4-20 min, solvents (15% A / 85% B, v/v); at *t* = 22 min, solvents (95% A / 5% B, v/v); the column regeneration time was 10 min and the mobile phase flow rate was 1 ml/min. The analysis was performed at room temperature (RT), with 20 µl of sample injected. Compound **1** was dissolved in DMSO and L-Cys in phosphate buffer and they were mixed to give a final concentration of 0.25 and 2.5 mM for compound **1** and L-Cys, respectively.

Electron paramagnetic resonance (EPR) experiments

EPR spectra were obtained at X-band on a Brüker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). EPR data processing and spectrum computer simulation were performed using WINEPR and SIMFONIA software (Bruker, Wissembourg, France).

a. Interaction with heme

Compound **1**, artemisinin and chloroquine were each incubated under an argon atmosphere with hemin or heme obtained by reducing hemin with $NH_2OH.HCl$. The analyzed samples were prepared in DMSO by mixing hemin/compound at the stoichiometric ratio 1/4 (0.75/3 mM) or hemin/$NH_2OH.HCl$/compound at the stoichiometric ratio 1/10/4 (0.75/7.5/3 mM). The solutions were stirred at room temperature for 5 h before recording the EPR spectra. The recording was carried out at 107°K using a liquid nitrogen flow in a quartz tube (inner diameter: 4 mm) containing 250 µL analysed solution. Typical scanning parameters were: scan rate, 0.6 G/s; scan number, 1; modulation amplitude, 5.10-3 G; modulation frequency, 100 kHz, microwave power, 20.2 mW; time constant, 40.96 ms, sweep width, 5000 G.

b. EPR spectroelectrochemical analysis

For spectro-electrochemical measurements, the EPR spectrometer was coupled to a potentiostat-galvanostat (EG&G Princeton Applied Research-Model 362). A flat quartz cell adapted to electrochemical measurements (Bruker, Wissembourg, France) was used for analysis. The electrochemical reduction was carried out using a three-electrode set-up: the working and counter-electrode were platinum and the reference electrode was a silver wire. The applied potential was chosen to be on the diffusion plateau of the first reduction wave obtained under stationary conditions: $E_{applied}$ = – 0.9 V. The electrolysis potential was applied for 5 min to the solution containing the compound in acetonitrile/TPAB and the EPR spectrum was immediately recorded as a function of time. Typical scanning parameters were: scan rate, 1.2 G/s; scan number, 1; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 20 mW; sweep width, 105 G; sweep time, 83.88 s; time constant, 40.96 ms; receiver gain 5 $x10^4$.

c. Interaction of INOD with radicals derived from artemisinin and iron

The compound was dissolved in DMSO to obtain a 3 mM stock solution. EPR spectra were recorded in DMSO/water 90/10, v/v with the final concentrations: artemisinin 2.5 mM, $Fe^{2+}((NH_4)_2Fe(SO_4)_2.6H_2O)$ 0.25 mM and compound **1** 0.25 mM. The final solution was vortexed for 15 s and then transferred into the quart flat cell (FZK160-5x0.3; Magnettech). Typical scanning parameters were: scan rate 0.6 G/s, scan number 1, amplitude modulation 1 mG, modulation frequency 100 kHz, microwave power 20 mW, field center 2500 G, spectrum width 5000 G, gain 80, frequency 9.86 10^9 Hz, sweep width 190 G, sweep time 41.94 ms, time constant 20.48 ms, receiver gain 2.10^5, 10 scan.

Table of Abbreviations

INODs : indolone-*N*-oxide derivatives
P. f. : *Plasmodium falciparum*
DMSO : dimethylsulfoxide
CQ: chloroquine
ART: artemisinin
EPR: electron paramagnetic resonance
L-Cys: L-Cysteine
RBC: red blood cell

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Keywords: Pro-oxidant drugs · indolone-*N*-oxide · antimalarials · cyclic voltammetry · electron spin resonance (EPR)

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3. Conclusion

Les études sur les interactions biochimiques des INODs avec certains composants des globules rouges montrent que ces composés i) n'entrent pas dans la sphère de coordination du métal dans le complexe fer-héme contrairement à la chloroquine; ii) ne génèrent pas d'espèces radicalaires fer-dépendantes comme le fait l'artémisinine; iii) génèrent des intermédiaires radicalaires après réduction à un électron dans un milieu polaire, qui est réversible, iv) ne peuvent pas piéger les radicaux libres après la réduction. Ainsi les propriétés antipaludiques des INODs sont associées à leur caractère pro-oxydant (bio-reductibilité) et non à un caractère de piégeur de spin ou d'interaction avec l'hème. Ce caractère pro-oxydant pourrait déclencher une voie de signalisation redox via l'activation de SYK-kinases et l'hyperphosphorylation de la protéine bande 3, AE1, qui aboutit à la destruction du globule rouge et du parasite.

Ce résultat est en bon accord avec l'étude précédente sur les isolats frais humains de *P. falciparum* (Tahar *et al*, 2011) qui ne montrent aucune corrélation entre les INODs et la chloroquine ($r^{1/4}$ 0.015, P 0.05) et une corrélation positive et significative entre les INODs et la dihydroartemisinine ($r^{1/4}$ 0.444, P 0.05). Ces résultats suggèrent que les INODs présentent un mode d'action et des cibles moléculaires differents de ceux des antipaludiques actuels. Cette hypothèse est en accord avec le fait que lesINODs sont actifs sur les deux types de souches de *Plasmodium falciparum*, résistantes et sensibles à la chloroquine

3. Conclusion

The investigation of the early stages of the biochemical interactions of the INODs with some RBC components show that these compounds i) are not included in the coordination sphere of the metal in the iron-heme complex unlike chloroquine; ii) do not generate iron-dependent free radical species such as does artemisinin; iii) generate radical intermediates after one-electron reduction in polar medium which is reversible; iv) cannot trap free radicals upon reduction. The antimalarial properties of INODs are associated with the pro-oxidant characters rather than the spin strap character or interaction with heme. This pro-oxidant character could trigger a redox signal pathway via SYK activation and hyperphosphorylation of band 3 protein (AE1) which induces an oxidative stress fatal to the RBC and the parasite.

This finding is in good agreement with the previous study on human fresh isolated of *P. falciparum* (Tahar *et al*, 2011) that shows no correlation between INODs and chloroquine ($r^{1/4}$ 0.015, P 0.05) and significant and positive correlation between INODs and dihydroartemisinine ($r^{1/4}$ 0.444, P 0.05). These results suggest that INODs exhibit different modes of action and molecule targets from these current antimalarials. This hypothesis emphasizes the fact that INODs are active toward both strains of *Plasmodium falciparum,* resistant and sensitive to chloroquine.

PART B.

REDOX PROPERTIES OF *Crinum latifolium* EXTRACTS IN RELATION TO THEIR ANTICANCER PROPERTIES

I. BIBLIOGRAPHY

1. Prostate cancer

a. Description

Cancer is a general term used for a group of diseases where abnormal cell growth is observed. The abnormal cells then invade surrounding tissue and spread to different organs in the human body, resulting in death. This process is called metastase. Cancer is a serious public health problem which caused 7.6 millions of death (around 13% of all death) in 2008 including lung, stomach, liver, colorectal, breast, cervical cancer, prostate cancer (WHO, 2013). It can be recalled that prostate cancer is considered as one of the most prevalent cancer in elder males (90% in men ages 70 to 90) (Dunn *et al*, 2011). Treatments including surgery, radioactive therapy, hormone therapy, chemotherapy may induce adverse effects which often reduce the quality of life. Moreover, these treatments are high-priced especially for people in developing countries.

b. Anatomy

Prostate gland is located below urinary bladder and in front of rectum. It protects and nourishes the sperms by producing semen. The gland starts to develop in fetus and continues to grow until the baby becomes adult. Its size is about walnut size in young men. The gland slowly grows all the life due to male hormone which could cause much larger size in elder men (Porche, 2011).

c. Risk factors

While the exact causes of prostate cancer stay as mysterious as all other cancers do, we do know some risk factors involved in prostate cancer. Aging is the first risk factor which should be concerned since prostate cancer is found in 90% of men from 70 to 90 and it is rarely found in men younger than 65 (Dunn *et al*, 2011). Genetic is an important factor in prostate cancer incidence. If family history has been reported any prostate cancer cases, the men have higher risk to get this malignancy (Dunn *et al*, 2011). Moreover, race is also a common risk factor. While the highest prostate cancer ratio was obtained in the African-American men ($\geq$ 250/100000), the lowest incidence was reported in Asian/Pacific Islanders and American Indian/Native Alaskan men ($\leq$ 100/100000) (Dunn *et al*, 2011). In addition, the habit and the living environment could be important factors. The highest rate of prostate cancer is observed in the region of Americas, following by Europe and the lowest rate is in Southeast Asia

(WHO, 2013). More than 30 % of cancer death could be reduced by modifying the way of life style such as limiting tobacco, alcohol use and overweight and promoting physical activity, healthy diet (WHO, 2013).

Tobacco contains cadmium which may lead to prostate cancer and other cancers in men (Drasch *et al*, 2005). Cadmium destroys antioxidant system in our body. Therefore, there is an enhancement of free radical, resulting in cellular damage and aging process (Drasch *et al*, 2005). Moreover, it interacts with p53 protein, a tumor suppressor protein which creates favorable conditions for cancer cells growth (Drasch *et al*, 2005). Cadmium also encourages the growth of human prostate epithelial cells at low levels (10 – 9 to 10 – 7 M) and facilitates malignant transformation (Drasch *et al*, 2005). Finally, excessive amount of cadmium in body may cause severe prostate cancer in smokers.

As we know, heavy alcohol consumption could lead to depression and liver damage. Not surprisingly, heavy alcohol drinking is related to prostate cancer risk (Fillmore *et al*, 2009).

Doing physical activity certainly reduces the risk of most major cancer sites (colon, breast, endometrium, lung, prostate, ovarian, gastric, rectal, pancreatic, bladder, testicular, kidney and haematological cancers) (Friedenreich *et al*, 2010). It also improves circulation, controls body weight and positively affects the immune system (Friedenreich *et al*, 2010). It is recommended to do slight physical exercise for 30-60 min 5 times a week or vigorous exercise for 30 min 3 times a week in order to reduce the development of chronic diseases, including cancer.

Healthy diet is essential for daily life since it provides right nutrient, energy as well as prevents the body from diseases including coronary and cerebrovascular diseases, various cancers, type 2 diabetes mellitus, hypertension, liver disease and asthma (Knight, 2011), (Calle *et al*, 2003). Obviously, people should concern about the way of living so that we could prevent many types of diseases and have healthy happy life.

d. Diagnostic

Since prostate cancer is a slow-growing cancer, annual screening with prostate specific antigen (PSA) test, digital rectal examination (DRE) and prostatic biopsy is suggested for men age from 50 to 70.

Prostate specific antigen is a protein produced by normal prostate tissue. In case of prostate cancer, this protein escapes from prostate then enters into the circulation (Papsidero *et al*, 1980). The rise of prostate specific antigen in serum is not only caused by cancer but also

caused by prostatitis or benign prostatic hypertrophy. Misuse of test screening could lead to over-diagnosis and over-treatment (Haythorn *et al*, 2011).

Prostatic biopsy will be done when there are abnormal results in PSA and DRE tests. 8 to 16 different samples taken in the peripheral zone are required. At the same time, grading is used for determining how aggressive cancer progression is. The grades range from 1 to 5 (Dunn *et al*, 2011). The higher grade of cancer cells is, the more abnormal cells are, the more likely cancer spreads.

e. Treatment

There are different treatment options for prostate cancer including active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, biologic therapy. Depending on diagnosis results, type of treatments will be selected.

Table 1. Treatment options in prostate cancer.

Treatments	Types/Drugs	Procedure	Disadvantage
Active surveillance (at early stage)		No treatment until cancer is more aggressive	
Surgery	-Radical prostatectomy -Laparoscopic radical prostatectomy -Robot-assisted laparoscopic radical prostatectomy, -Cryotherapy	Removal of the entire prostate gland and seminal vesicles	
Radiation therapy	-External beam radiation -Brachytherapy	Supply high power energy to the prostate without damaging surrounding tissues	Urinary urgency, dysuria, diarrhea, proctitis, erectile dysfunction
Hormone therapy	-Luteinizing hormone-releasing hormone agonists: leuprolide, goserelin, and buserelin. -Antiandrogens : flutamide and nilutamide.	Reduction of male hormone, resulting in stop cancer cell growth	Hyperlipidemia, insulin resistance, cardiovascular disease, anemia, osteoporosis, sexual dysfunction and cognitive deficits
Chemotherapy	Docetaxel, prednisone, mitoxantrone	Drugs adminstration to stop the division of cancer cells	Myelosuppression, hypersensitivity, gastrointestinal upset, peripheral neuropathy
Biologic therapy	Cabazetaxel, abirateron, sipuleucel-T	Encourage immune system to fight cancer	Neutropenia, gastrointestinal disturbance, renal insufficiency

2. Functions of macrophages in immune system

Macrophages (MΦ) are professional phagocytes which are able to express a receptor on their surface and detect abnormal foreigners, death cells and toxics in body. MΦ play an important role in innate immune system including immune surveillance, tissue homoeostasis and wound healing. MΦ precursors are monocytes which originate from haematopoietic stem cells, in the bone marrow. Monocytes circulate in blood, bone marrow and spleen. Spleen serves as storage of immature monocytes since monocytes do not proliferate in healthy state. If inflammation occurs in the body, monocytes migrate to the inflammatory site and differentiate into dendritic cells (DC) and MΦ. Mature MΦ are also present in many tissues such as bone (osteoclasts), lungs (alveolar macrophages), interstitial connective tissue (histiocytes), liver (Kupffer cells), brain (microglia) where they detect and remove dead cells, pathogens and toxic materials (Murray *et al*, 2011). In stressed tissue (acute and chronic inflammation), the proliferation and recruitment of monocytes and MΦ fates are controlled by different cytokines and factors. There are four different activated forms of macrophages: classical activated macrophages (M1 macrophages), alternatively activated macrophages (M2 macrophages), tumor-associated macrophages (TAMs) and myeloid-derivated suppressor cells (MDSCs). While M1 is activated by interferon-γ (IFN- γ), bacterial lipopolysaccharide (LPS), tumor necrosis factor α (TNF α), resulting in reactive oxygen species (ROS) and nitric oxide (NO) production, M2 activation is triggered by interleukin 4/interleukin 13(IL4/IL13)-stimulated macrophages, IL10-induced macrophages and immune complex-triggered macrophages (Wang *et al*, 2010). After activation, M1 macrophages are able to produce inflammatory cytokines, ROS to fight against various types of bacteria, protozoa, virus and tumor cells (Biswas *et al*, 2008). M2 phenotype expresses anti-inflammatory function as well as immunosuppression and wound healing regulation (Biswas *et al*, 2008). TAMs are present in malignant tumors where they are responsible for anti-tumor suppression (Biswas *et al*, 2008). Myeloid-derived suppressor cells (MDSCs) are precursors of certain TAMs that are involved in protecting the host from excessive immune stimulation, autoimmune responses, limiting the activation of T cells. They are believed to inhibit antitumor immunity and to promote tumor expansion (Bronte *et al*, 2010) (Quatromoni *et al*, 2012). Depending on the requirement of immunology system, MΦ can shift from one functional phenotype to another (Murray *et al*, 2011).

Table 2. Phenotype of macrophages.

Phenotypes	Activated by	Function
M1	IFN- γ, LPS, TNF α	antibacteria, anti-protozoa, anti-virus, anti-tumor
M2	IL4/IL13, IL10, immune complex-triggered MΦ	Anti-inflammatory function, immunosupression , wound healing regulation
TAMs	Growth factor, chemokines	Tumor progression: limiting excessive immune stimulation, autoimmune responses and T cells activation
MDSCs	Endotoxin, CD8+ T cell-induced acute entrocolitis	Tumor progression: limiting excessive immune stimulation, autoimmune responses and T cells activation

3. Function of macrophages in cancer

Cancer progression is a multi-steps process including in situ cancer, invasion and metastasis (Steeg, 2002). At initial step, normal cell growth is disrupted and new blood vessels are formed to supply nutrient for the transformation from normal cells to abnormal cells (Yoshida *et al*, 2000). Then, the degradation of basement membrane allows for invasion into adjacent tissues. Tumor cells continue to spread to the secondary sites via blood or lymphatic stream (Yoshida *et al*, 2000). Among these steps, metastasis is a major cause of cancer death which involves many factors such as increased expression of metastasis-promoting genes or decreased expression of metastasis-suppressor genes (Shin *et al*, 2006). In summary, there are eight capabilities which are essential for tumor progress: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replication potential, evasion the immune system, incitation of mutations to arise in a *bona fide* tissue stem cell, sustained angiogenesis, tissue invasion and metastasis (Jaiswal *et al*, 2010).

Solid tumors consist of tumor cells, extra cellular matrix (ECM) and inflammatory cells (tumor-associated macrophages (TAM), dendritic cells, lymphocytes) (Balkwill *et al*, 2001). It has been shown that chronic inflammation is associated with some cancers, examples given in Table 3. TAM is one of the macrophages phenotype which is attracted to the tumor site by chemokines (Balkwill *et al*, 2001). TAM enhances proliferation and metastasis of tumor cells as well as tumor resistance to chemotherapy (de Palma *et al*, 2011).

Table 3. Inflammation and cancer risk.

Inflammatory condition	Malignancy
Schistosomiasis	Bladder
Papillomavirus	Cervical
Prostatitis	prostate cancer
H. pylori induced gastritis	Gastric
Inflammatory bowel disease	Colorectal
Hepatits virus (B and C)	Hepatocellular

Importantly, there is low oxygen in the environment of tumor solid that ranges from normoxia (2-9% O_2), to mild hypoxia (2-0.02% O_2), and to severe hypoxia (< 0.02% O_2). It is unfavorable for ROS activity since their formation requires oxygen. It could explain how tumor cells often resist to antitumor drugs, especially ROS-generating agents (Daweale *et al*, 2010).

Although the mechanism of invasion and progression of tumor is known, the major cause of cancer is still a big question mark for science. Hopefully, this question will be answered in near future, that is a foundation for the development of new and effective antitumor strategies.

4. Reactive Oxygen Species (ROS)

While tumor cells are able to progress in the body, how can immune system fight against the tumor cells? When a mice has lack of the immune development genes such as recombination activation gene 2 ($RAG2^{-/-}$), interferon gamma (IFN $\gamma^{-/-}$), different types of tumors spontaneously develop (Jaiswal *et al*, 2010). Therefore, the immune system is essential for tumor surveillance.

Immune cells fight microbes and tumor cells by two ways:

1. Phagocytosis-mediated lysosomal degradation, production of antimicrobial peptides (defensins, lactoferrins, proteases, cathepsins, reactive oxygen species (ROS)) (Lam *et al*, 2010)
2. Production of tumor-growth inhibitory cytokines (TNF, TRAIL, IL-12, Il-18) (Jakóbisiak *et al*, 2003).

One of the most important fighting ways of immune system is ROS production. In general, the ROS consist of radical species (O_2^-, $HO_2^\bullet$, $HO^\bullet$, $NO^\bullet$, $ONOO^\bullet$) and non-radical species (H_2O_2, HOCl). They are not only considered highly toxic for living mammalian cells but also highly effective in immune defense strategy. Not surprisingly, their functions are still

debatable. ROS are generated by various pathways in the body, for instance nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase (XO), the mitochondrial electron transport chain, peroxisomes, and the endoplasmic reticulum. ROS regulate protein phosphorylation cascade, transcription factors activity and gene expression which result in a modification of signal transduction pathway such as in cell growth, differentiation, survival, inflammation and the immune response (Dewaele *et al*, 2010)

Although the superoxide anion is toxic for cells but it is rapidly removed by superoxide dismutases (SOD) under physiological condition ($K = 2x10^9\ M^{-1}s^{-1}$) (Estevez *et al*, 2002). It could be converted to more active oxidizing agents ($ONOO^-$, H_2O_2, $^{\bullet}OH$) due to its instability. After conversion of superoxide to H_2O_2, Fenton reaction rarely happens since its reaction rate is slow in comparison with other possible reactions by catalase and glutathione (Estevez *et al*, 2002).

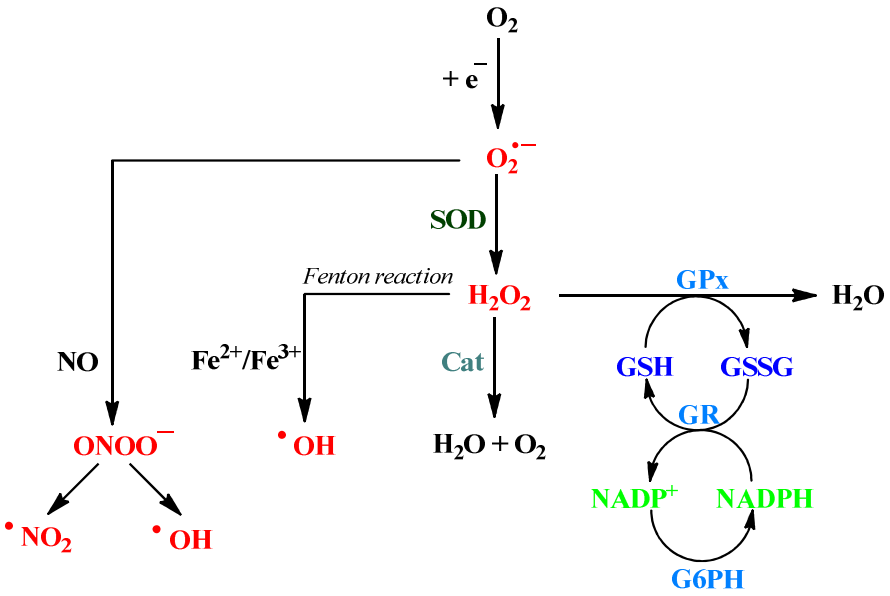


Figure 1. ROS formation and detoxification.

Nitric oxide (NO) has opposite effects in different biological system (Estevez *et al*, 2002). It can be used as a cellular messenger in vascular and nervous system or as a toxic for intracellular pathogen and tumor in nonspecific host defense. Its actions depend on the oxidative state of the cells (Estevez *et al*, 2002). It is stable and not toxic for living cells by itself under physiological conditions (Estevez *et al*, 2002). Under pathophysiological conditions, it can react with superoxide to form peroxynitrite ($ONOO^-$), a potent oxidant which is able to induce apoptosis in various cell types (Estevez *et al*, 2002). $ONOO^-$ can easily decompose into nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radical ($HO^{\bullet}$) under physiological conditions (Figure 2) (Pacher *et al*, 2007). NO is formed by the conversion of

arginine to citrulline, catalyzed by nitric oxide synthases (NOS). NOS is present in brain (neuronal NOS- nNOS or NOS1), in macrophages (inducible NOS-iNOS or NOS2) and in plasma membrane (endothelial NOS-eNOS or NOS3) (Pacher *et al*, 2007). NO rapidly diffuses through cell membrane to blood stream after its formation, followed by reaction with oxyhemoglobin to produce nitrate. Specially, activated NOS2 by proinflammatory cytokines results in NO production which diffuses through the membrane and reacts with superoxide at the membrane surface, producing peroxynitrite (K = 6 to $10x10^9$ $M^{-1}s^{-1}$) (Pacher *et al*, 2007) (Estevez *et al*, 2002) (Figure 2).

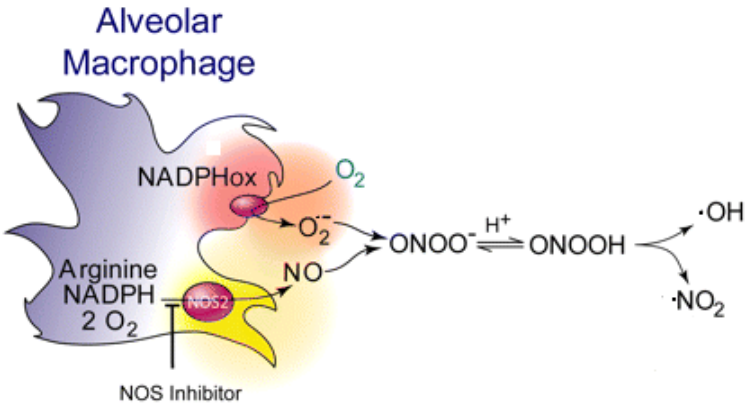


Figure 2. Production of peroxynitrite (Pacher *et al*, 2007).

In order to maintain redox-homeostasis, there are two systems to neutralize the ROS: i) enzymatic systems including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (Cat), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD); ii) non-enzymatic systems including glutathione (GSH) and antioxidant agents (vitamin C, vitamin E) (M. Dewaele, 2010). SOD, Cat and GPx play important roles in removing superoxide or H_2O_2, resulting in the formation of water and oxygen (Figure 1). GPx requires the cofactor GSH which is reduced to glutathione disulfide (GSSH). Then, GR regenerates GSH from oxidized glutathione, GSSG by oxidizing one mole nicotinamide adenine dinucleotide phosphate (NAPDH). Finally, NAPDH is regenerated by G6PD (Figure 1).

Cancer cells produce hypoxic and endogenous oxidative environment which is sensitive to the highly toxic exogenous ROS-generating agents. Therefore, antitumor strategies using ROS-generating agents can directly act toward tumor cells by activating immune cells producing ROS or by inhibiting the antioxidant mechanism. They are called pro-oxidant agents.

Among the phagocytes, neutrophils and macrophages are important producers of ROS via NOX2 NADPH that is an enzymatic system responsible for respiratory burst. This enzyme is located in the membrane of macrophages and neutrophils. It is composed of Rho guanosine

triphosphatase, gp91-phox, p22phox, p40phox, p47phox, p67phox. Phosphorylation of p47phox results in the conformation change of these subunits. Electrons are then transported from cytoplasmic NADPH to extracellular medium where they interact with free molecular oxygen to produce superoxide anions (Figure 3) (de Oliveira-Junior *et al*, 2011).

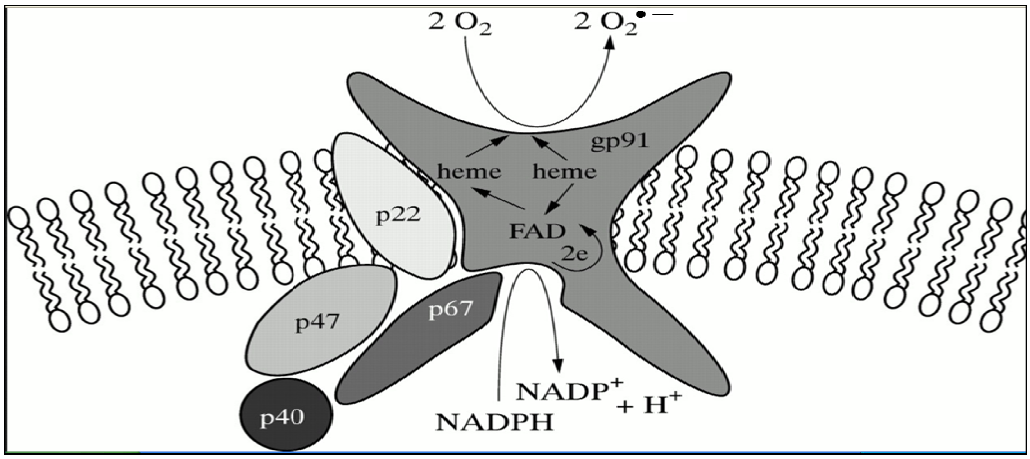


Figure 3. NADPH oxidase system (van Heerebeek *et al*, 2002).

As mentioned above, low level of ROS is important in cellular regulation since it regulates various protein kinases and transcription factors (Chen *et al*, 2009). As a result, ROS are active key compounds in redox signaling, microbicidal activity, immune modulation, adaptive immune activation, chemoattraction and antitumor activity. Interestingly, ROS are not only involved in tumor-suppression mechanism but also in tumor-development mechanism (Dewaele *et al*, 2010), (Chen *et al*, 2009). These two effects depend on the level and species of ROS (Chen *et al*, 2009). For example, H_2O_2 modifies cardiolipin, a lipid in mitochondria membrane, followed by leaking of cytochrom *c* and results in activation of apoptosis (Chen *et al*, 2009). In contrast, small amount of superoxide without association with the H_2O_2 formation inhibits the apoptosis pathway and up-regulates Bcl-2, an anti-apoptotic regulator in leukemic tumor cells (Chen *et al*, 2009). Furthermore, autophage induced by ROS could play tumor suppressive role in early stage of cancer or tumor progressive role in advance stage (Dewaele *et al*, 2010). Cancer could modulate autophage to remove their opponents and create a favorable environment for their survival and invasion (Dewaele *et al*, 2010). Since the antitumor activity of ROS is due to their source, types, amount and cancer stage, it is important to get early diagnosis and proper treatment.

5. Botanic and ethnobotanic aspects

Crinum latifolium is a plant used in Vietnamese herbal medicine. Leaves can be used fresh or dried (Loi, 2000). In ancient time it was used for longevity. Nowadays, it is believed as a good remedy for benign prostate enlargement, uterine fibroids, ovarian cysts and tumor (Loi, 2000). Since the effects and the mechanism of action of *Crinum latifolium* are still not understood, scientists study the biological properties of the extracts and natural compounds from the plant. Besides the botanical name, there are common names for this plant given in Table 4.

Table 4. Botanical and common name of *Crinum latifolium*:

Family	Botanical name	Common name
Amaryllidaceae	*Crinum latifolium*	English name: milk lily, wine lily. Vietnamese name: Royal virgin female

Crinum latifolium is an herbaceous, lily-like perennial plant. It often has 6 to 18 huge showy flowers on a fairly stout stem which is developed from tunicated bulbs. A bulb looks like a big onion, 10-15 cm in diameter which produces neck or a pseudo-stem made up of the sheathing bases of the old leaves. From one bulb, it might grow many bulbs which can be separated and planted easily. The leaves are linear to sword-shaped, sheathing at the base with reddish purple, arranged in a rosette or rarely in two opposite rows. Leaf veins are parallel, the upper surface forms a groove along the leaf, the main vein is strong visible on the lower surface. The inflorescences arrange in umbel. Flower's lobes are white, often tinged with red, lanceolate to oblong-lanceolate (Loi, 2000).

The plant loves the sun and water but needs a good drainage. They require some spaces between each other. Propagation by division is a major method as well as by seed but it takes a long time from germination to flower. Since most Crinum plants have lovely lily-like flowers with sweet fragrance, these are used as landscape plant.

In the world, the plant is distributed in America, Australia, Southern Asia and Africa. In Viet Nam, *Crinum latifolium* is cultivated in the North, in the Middle and in the South.

A

B

Figure 4. *Crinum latifolium*. A) The mother bulb in the center and its 'calves', B) Flower (Photo by Prof. Vo thi Bach Hue, 2009)

6. Substances isolated from *Crinum latifolium*.

Some compounds have been isolated from *Crinum latifolium* in the past few years. Most of them are alkaloids and a few non-alkaloid compounds (sugars, flavonoids).

Three types of alkaloids have been found in *Crinum latifolium*: crinane (5,10b-ethanophenanthridine), lycorine (pyrrolophenanthridine) type and cherylline types. The alkaloids isolated so far are listed in:

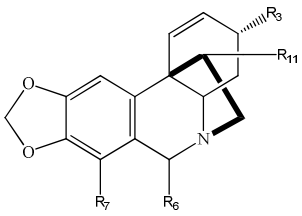
- Crinane type: these alkaloids are mainly found in bulbs (Table 5 and Figure 5). Some representatives have significant biological activities (Tram *et al*, 2002).

- Cherylline type: the representative alkaloids are cherylline and latifine which are found from leaves of this plant (Table 6 and Figure 6). They are biogenetic isomers. Their skeletons are base on 4-phenyltetrahydroisoquinoline. Their mass spectra are similar. They could be metabolites of *N*,*O*-dimethylnorbelladine (Kobayashi *et al*, 1984).

- Lycorine type: these alkaloids are extracted mainly from fruits and bulbs (Table 7 and Figure 7). Under stress conditions (incision injury, insect attack), these alkaloids are hydrolyzed and oxidized to participate in protective and repair mechanism of the plant. During the isolation, to prevent the hydrolysis and oxidation of these alkaloids, pre-treatment with anaesthetic (e.g lidocaine) is necessary (Tram *et al*, 2002).

Table 5. Crinan type, alkaloids isolated from *Crinum latifolium.*

Alkaloids	Their metabolites	References
Buphanidrine (**1**)	Oxidized products: undulatine, 6-hydroxy undulatine, ambelline, 6-hydroxybuphanidrine (**2**), (higher concentration after flowering).	Tram *et al*, 2002
6-hydroxybuphanidrine (**2**)		Tram *et al*, 2002
Powelline (**3**)	Oxidized products: 6-hydroxypowelline, crinamidine, 6-hydroxycrinamidine (higher concentration at flowering). Product of 3-*O*-methylation: buphanidrine (higher concentration at flowering).	Tram *et al*, 2002
6-hydroxypowelline (**4**)	Product of 3-*O*-methylation: 6-hydroxybuphanidrine (higher concentration at flowering).	Tram *et al*, 2002
Ambelline (**5**)		Ghosal *et al*, 1983
11-*O*-Acetylambelline (**6**)		Ghosal *et al*, 1985
3α-1.2-Didehydro-crinan-3-ol (**7**)		Tram *et al*, 2002
Crinine (**8**)		S. Kobayashi, 1983
Undulatine (**9**)		Tram *et al*, 2002
Hydroxyundulatine (**10**)		Tram *et al*, 2002
Crinamidine (**11**)	Product of 3-*O*-methylation: Undulatine (**9**) (higher concentration at flowering).	Tram *et al*, 2002
6-Hydroxycrinamidine (**12**)	Product of 3-*O*-methylation: 6-Hydroxyundulatine (**10**), (higher concentration at flowering).	Tram *et al*, 2002
1,2-β-epoxyambelline (**13**)		Ghosal *et al*, 1984
11-*O*-Acetyl-1,2-β-epoxyambelline (**14**)		Ghosal *et al*, 1985
Epoxy-3,7-dimethoxycrinane-11-one (**15**)		Tram et al, 1999
Crinamine (**16**)		Kobayashi *et al*, 1983
11-O-methyl-crinamine (**17**)		Kobayashi *et al*, 1983
Hamayne (**18**)		Kobayashi *et al*, 1983
Diacetylhamayne (**19**)		
3-*O*-Acetylhamayne (**20**)		
Dihydro-oxo-demethoxy haemanthamine (**21**)	Isolation from leaves, GC-MS detection	Tram *et al*, 2002
Crinane-3α-ol (**22**)	Isolation from leaves, GC-MS detection	Tram *et al*, 2002
Augustamine (**23**)	Isolation from leaves, GC-MS detection	Tram *et al*, 2002
Oxoassoanine (**24**)	Isolation from leaves, GC-MS detection	Tram *et al*, 2002
Crinafoline (**25**)		Ghosal *et al*, 1986
Crinafolidine (**26**)		Ghosal *et al*, 1986



R_3= OMe, R_6=H, R_7= OMe, R_{11}= H: Buphanidrine (**1**)

R_3= OMe, R_6=α-OH, R_7= OMe, R_{11}= H: 6-hydroxybuphanidrine (**2**)

R_3= OH, R_6=H, R_7= OMe, R_{11}= H: Powelline (**3**)

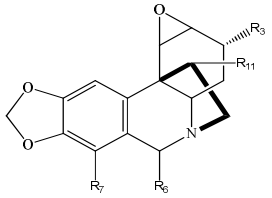
R_3= OH, R_6= OH, R_7= OMe, R_{11}= H: 6-hydroxypowelline (**4**)

R_3= OMe, R_6=H, R_7= OMe, R_{11}= OH: Ambelline (**5**)

R_3= OMe, R_6=H, R_7= OMe, R_{11}= OAc: 11-*O*-Acetylambelline (**6**)

R_3= α-OH, R_6=H, R_7= H, R_{11}= H: 3α-1.2-Didehydro-crinan-3-ol (**7**)

R_3= OH, R_6=H, R_7= H, R_{11}= H: Crinine (**8**)



R_3= OMe, R_6=H, R_7= OMe, R_{11}= H: Undulatine (**9**)

R_3= OMe, R_6=OH, R_7= OMe, R_{11}= H: 6-Hydroxyundulatine (**10**)

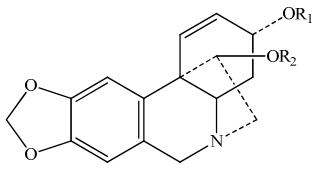
R_3= OH, R_6=H, R_7= OMe, R_{11}= H: Crinamidine (**11**)

R_3= OH, R_6=OH, R_7= OMe, R_{11}= H: 6-hydroxycrinamidine (**12**)

R_3= OMe, R_6=H, R_7= OMe, R_{11}= OH: 1,2-β-epoxyambelline (**13**)

R_3= OMe, R_6=H, R_7= OMe, R_{11}= OH: 11-*O*-Acetyl-1,2-β-epoxyambelline (**14**)

R_3= OMe, R_6=H, R_7= OMe, R_{11}= O: epoxy-3,7-dimethoxycrinane-11-one (**15**)



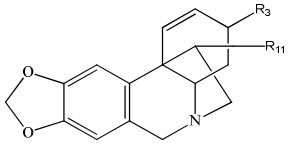
R_1=Me, R_2=H: Crinamine (**16**)

R_1=, R_2=Me: 11-O-methyl-crinamine (**17**)

R_1= R_2=H: Hamayne (**18**)

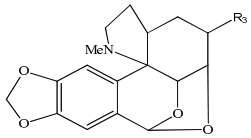
R_1=R_2=Ac: Diacetylhamayne (**19**)

R_1=Ac, R_2=H: 3-O-Acetyl-hamayne (**20**)

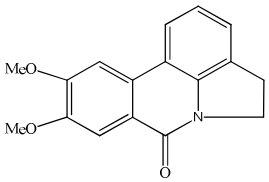


R_3=H, R_{11}=O: Dihydro-oxo-demethoxy haemanthamine (2**1**)

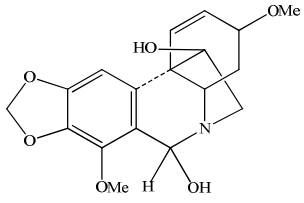
R_3=α-OH, R_{11}=H: Crinane-3α-ol (**22**)



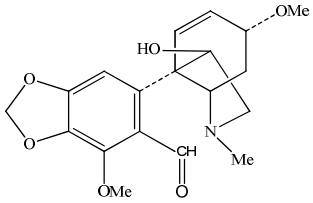
Augustamine (**23**)



Oxoassoanine (**24**)



Crinafoline (**25**)



Crinafolidin (**26**)

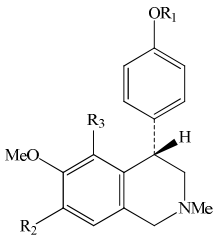
Compounds **6**, **13** and **14** have been reported as immunomodulator (Ghosal *et al*, 1984, 1985).

Compounds **25** and **26** have been reported as antitumor (Ghosal *et al*, 1986).

Figure 5. Chemical structure of the Crinan type alkaloids isolated from *Crinum latifolium*

Table 6. Cherylline type alkaloids isolated from *Crinum latifolium.*

Alkaloids	References
Latifine (S) (**27**)	Kobayashi *et al*, 1983, 1984
Cherylline (S) (**28**)	
O, O-dimethylcherylline (**29**)	Kobayashi *et al*, 1983



R_1=R_2=H, R_3= OH: Latifine (**27**)

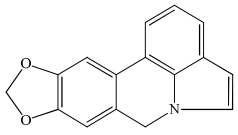
R_1=R_3=H, R_2= OH: Cherylline (**28**)

R_1=Me, R_2= OMe, R_3=H: O, O-dimethylcherylline (**29**)

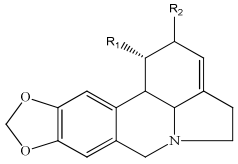
Figure 6. Chemical structures of Cherylline type alkaloids isolated from *Crinum latifolium*.

Table 7. Alkaloids, lycorine type isolated from *Crinum latifolium.*

Alkaloids	Their metabolites	References
Lycorine (**31**)		Ghosal *et al*, 1983
Hippadine (**39**)	Its concentration is maximum during the pre- and post-flowering stage.	
Pratorinine (**38**)	Its concentration is maximum during the pre- and post-flowering stage.	
Pratorimine (**35**)	Its concentration is maximum during the pre- and post-flowering stage.	
O-acetyl-pratorimine (**36**)		
Pratosine (**37**)	Its concentration is maximum during the pre- and post-flowering stage.	
4.5-Dehydroanhydrolycorine (**30**)		Tram *et al*, 2002
2-Epilycorine (**32**)	2-Epilycorine produces 2-epipancrassidine via 3,3a-epoxide	Ghosal *et al*, 1989
2-Epipancrassidine (**33**)	It is metabolic product of 2-Epilycorine	
Hippeastrine (**34**)		Jeffs *et al*, 1984

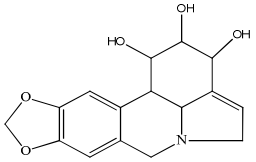


4.5-Dehydroanhydrolycorine (**30**)

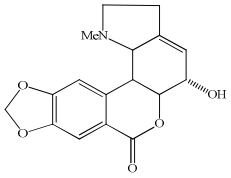


R_1= OH, R_2= Lycorine (**31**)

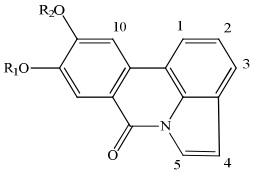
R_1= OH, R_2= 2-Epilycorine (**32**)



2-Epipancrassidine (**33**)



Hippeastrine (**34**)



R_1 = Me, R_2 = H : Pratorimine (**35**)

R_1 = Me, R_2 = Ac: O-acetyl-pratorimine (**36**)

R_1 = R_2 = Me : Pratosine (**37**)

R_1 = H, R_2 = Me : Pratorinine (**38**)

R_1 + R_2 = CH_2 :Hippadine (**39**)

Figure 7. Chemical structure of lycorine type alkaloids isolated from *Crinum latifolium*.

Table 8. Sugars isolated from *Crinum latifolium*

Compounds	Notes	References
Glucan A	Glucan A is composed of 12 glucose unites, a water soluble neutral oligosaccharide	Tram *et al*, 2002
Glucan B	Glucan B is composed of approximately 110 glucose residues, mainly composed of α-1 → 4 linked D-glucopyranose moieties with branches linked at position 6	

7. Traditional use of *Crinum latifolium* in Viet Nam

In Vietnam, the plant is called "Royal virgin female" because it was used for treatment of gynecological diseases of virgin women who were selected for the emperor *but the emperor did not care about them* (Loi, 2000). Since 1989-1990 people believe that leaves of *Crinum latifolium* have good effect for prostatitis, adenoma, benign prostate enlarge, uterine fibroids, ovarian cysts and tumor (Loi, 2000). *Crinum latifolium* has reputation for antioxidant activity, a cellular immunity effective T-lymphocyte activator. It is used for hypoxia, infection, chronic inflammation, detoxification, regeneration of tissue, hormone balancing, supportive prostate and ovaries (Loi, 2000).

In India, the leaf juice is used for ear-ache. The leaves are smeared with castor oil and then warmed which is a good remedy for whitlows and inflammation of toes and fingers. The bulb juice is used for emetic and vomiting in poisoning. It can be used for children, then it should be paid attention in over dose. The bulbs, after roasting laid on the skin to ease rheumatic pain (Loi, 2000).

Crinum latifolium is widely used as traditional medicine not only in Vietnam and India but also in the world, for treatments of benign prostate hypertrophy (BPH) and prostate cancer.

8. Biological activities

Different biological activities of extracts of *Crinum latifolium* have been reported and given in Table 9.

Table 9. Biological activities of *Crinum latifolium.*

Compounds/Uses	Biological activity	References
Combination of Glucan A and Phosphatidyl-lycorine: *Mast cell stabilizing effects, for treatment of allergic disorders*	Combination of glucan A and phosphatidyl-lycorine significantly protect against Tween 80-induced degranulation of mast cells in the presence of antigen *in vitro* and protect against compound 48/80-induced degranulation of mast cells *in vivo*.	Ghosal *et al*, 1988
Cold and hot extract of *Crinum latifolium: Antitumor activity*	*In vitro*, hot/cold plant extract stimulates human T-lymphocyte activation especially cell-mediated immune response of CD4+T lymphocytes (T-helper cell). 20% co-stimulation on T-helper cells was evaluated when human peripheral blood mononuclear cells (PBMCs) was cultivated in the presence of plant cold/hot water extracts. There was no evidence for a specific CD8+T-lymphocytes reaction when PBMCs were cultivated in the presence of plant cold/hot water extract.	Tram *et al*, 1999
Crinafoline and Crinafolidine: *Anti-tumor effect*	Crinafoline, crinafolidine and crinafoline methochloride, at different concentrations, produce significant reduction in the growth of S-180 ascites tumor cells in *vivo*.	Ghosal *et al*, 1986
11-*O*-Acetylambelline and 11-*O*-Acetyl-1,2-β-epoxyambelline: *Potential immunoregulant*	11-*O*-Acetylambelline and 11-*O*-Acetyl-1,2-β-epoxyambelline inhibit tumor growth and rise of macrophage and spleen weight.	Ghosal *et al*, 1985
Crinamine and Haemanthamine: *Apoptosis-inducing activity*	While crinamine and haemanthamine induce apoptosis (85% to 90%) at 25 µM after 48 h in 5123tc cancer cells, they do not induce apoptosis in a non-cancerous human embryonic kidney (HEK 293t) cell-line when treated at similar concentration. The structure requirements for the selective apoptosis induction of Amaryllidaceae alkaloids of the crinane-type are the alpha-C2 bridge and the free hydroxyl group at C-11.	McNulty *et al*, 2006
Aqueous extracts of Crinum latifolium*: Immunomodulatory properties in human peripheral blood mononuclear.*	Blockage of neopterin production induced by the mitogens or IFN-γ suggests that water soluble components from *Crinum latifolium* may play an important effect on the human immune system.	Zevetkova *et al*, 2001
Alcoholic extract of Crinum latifolium*: Anti-acetylcholinesterase*	A strong anti-AChE activity was observed, 83.44%, at concentration 0.5 mg/mL. This is a strong anti-AChE activity. The control was Galanthamine whose % inhibitor activity was 86.67%, at concentration 0.1 mg/mL.	Khoa *et al*, 2011

9. Conclusion

Crinum latifolium is extensively used in traditional medicine in Viet Nam and in other parts of the world particularly in Asia. A few reported studies show that the extracts of *Crinum latifolium* may induce cell apoptosis, tumor growth reduction and stimulation of the immune system which are encouraging to further contribute to these studies. In consequence, different properties will be studied in this work:

1. Redox activity
2. Cytotoxic activities of the extracts against cancer cells
3. Capacity of the extracts to modify the phenotype of macropahges against cancer cells.
4. Immunostimulating capacities on different models.

II. EXTRACTS OF *Crinum latifolium* LEAVES ENHANCE ANTITUMOR CAPACITIES OF MACROPHAGES

1. Introduction

La médecine traditionnelle asiatique est considérée comme celle utilisant le plus de plantes pour le traitement de maladies depuis très longtemps. *Crinum latifolium*, appartenant à la famille Amarylliaceae, est l'une des plantes de la médecine populaire asiatique utilisée dans les thérapies anti-inflammatoire, anti-tumorale, antivirale, antibactérienne et antifongique (Fennell et al, 2001), (Loi et al, 2000). Dans les aspects anticancéreux, l'extrait aqueux des feuilles de *Crinum latifoium* est utilisé pour le traitement du cancer de la prostate. Le traitement du cancer de la prostate à l'aide d'extraits de feuilles chaudes de *C. latifolium* a été fondé sur les connaissances traditionnelles, sans connaître les mécanismes d'action. Dans des études récentes, il a été rapporté des propriétés immunostimulantes et anti-inflammatoires de l'extrait aqueux de feuilles de *Crinum latifolium* telles que la stimulation de la prolifération des cellules T, la suppression de la formation mitogène, la formation de l'interféron γ-induite et indoleamine 2,3-dioxygénase activité (Tram et al, 1999), (Zvetkova et al, 2001), (Jenny et al, 2011). Cependant, la voie biologique impliquant une activité anticancéreuse n'est toujours pas élucidée. Par ailleurs, il serait intéressant de savoir si les propriétés anti-cancéreuses sont dues à un composé pur ou à une activité plus complexe de l'ensemble des composés présents dans les extraits provenant de *Crinum latifolium*.

Dans ce contexte, nous avons évalué les activités d'oxydo-réduction et antitumorale de trois extraits, une fraction alcaloïde et un composé pur, 6-hydroxycrinamidine obtenus de *Crinum latifolium* par des méthodes physico-chimiques et biologiques. En particuliér, les effets des extraits de *C. latifolium* sur la polarisation des macrophages et des espèces réactives de l'oxygène (ROS) produites par les macrophages ont été étudiés dans des modèles *in vitro*. Les résultats préliminaires permettent d'expliquer en partie, les activités antitumorales des différents extraits de *Crinum latifolium*.

1. Introduction

Asian folk medicine is believed as the richest medicinal plant source used for treatment diseases for a long time. *Crinum latifolium*, belonging to Amarylliaceae family, is one of the plants in Asian folk medicine used in therapy against anti-inflammatory, antitumoral and antiviral, antibacterial and antifungal effects (Fennell *et al*, 2001), (Loi *et al*, 2000). In anticancer aspects, aqueous extract of *Crinum latifoium* leaves has good effect in treatment of prostate cancer. The treatment of prostate cancer by using hot leaves extracts from *C.latifolium* has been based on traditional knowledge, without knowing the mechanism of action. In recent studies, there were some evidences about immunostimulatory and anti-inflammatory properties of aqueous extract of *Crinum latifolium* leaves such as stimulation of T-cells proliferation, suppression of mitogen formation, interferon-γ-induced formation and indoleamine 2,3-dioxygenase activity (Tram *et al*, 1999), (Zvetkova *et al*, 2001), (Jenny *et al*, 2011). However, biological pathway involving anti-cancer activity is still not elucidated. Moreover, it would be interesting to know whether the anti-cancer properties require only action of a pure compound or a complex activity of compounds in extracts derived from *Crinum latifolium*.

In this context, we assessed redox and antitumor activities of *Crinum latifolium* including three different extracts, one alkaloid fraction and one pure compound, 6-hydroxycrinamidine, by physicochemical and biological methods. Specially, the effect of *C. latifolium* extracts on macrophages polarization and reactive oxygen species (ROS) produced by polarized macrophages were investigated *in vitro* assays which have not been reported so far in literature. The results allow to explain in part the antitumor actions of different extracts from *Crinum latifolium*.

2. Extracts of *Crinum latifolium* inhibit the cell viability of mouse lymphoma cell line EL4 and induce potent activation of antitumor activity of macrophages *in vitro*

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Extracts of *Crinum latifolium* inhibit the cell viability of mouse lymphoma cell line EL4 and induce potent activation of antitumour activity of macrophages *in vitro*

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Ethnopharmacological relevance: Crinum latifolium (CL) leave extracts have been traditionally used in Vietnam and are now used all over the world for the treatment of prostate cancer. However, the precise cellular mechanisms of the action of CL extracts remain unclear.

Aim of the study: to examine the effect of CL extracts on the anti-tumor effect of peritoneal macrophages also in relation with their capacity to scavenge reactive oxygen species (ROS).

Materials and methods: The effects of extracts (aqueous, flavonoid, alkaloid) and fraction (alkaloid) were studied i) on murine peritoneal macrophages (MTT assay) and on lymphoma EL4-luc2 (luciferine assay) for cytotoxicity, ii) on macrophage polarization (ROS production assays and gene expression by PCR), and iii) on tumoricidal functions of murine peritoneal macrophages (lymphoma cytotoxicity by co-culture with syngeneic macrophages).The results were analyzed in parallel with those obtained with the DPPH and the bleaching beta-carotene assays giving basic redox properties of CL extracts. One pure alkaloid, 6-hydroxycrinamidine, isolated from CL, was introduced as a control for comparison reason.

Results: The total flavonoid extract exerted the strongest antioxidant activity and a powerful inhibitory activity on cancer cells. Alkaloid extracts showed inhibitory activity on the proliferation of lymphoma cells either by direct act on tumour cells or by activation of tumoricidal functions of syngeneic macrophages. The aqueous extract induced mRNA expression tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin 6 (IL-6) indicating differentiation of macrophages into pro-inflammatory M1 polarized macrophages. The flavonoid fraction (F) and alkaloid extracts induced expression of the formyl peptide receptor (FPR) on the surface of the polarized macrophages which could lead to the activation of macrophages toward M1 phenotype. Aqueous and flavonoid extracts enhanced NADPH quinoneoxido-reductase 1 (NQO1) mRNA expression in polarized macrophages which could play an important role in cancer chemoprevention. All the studied samples were not toxic for normal living cells and the pure alkaloid tested, 6-hydroxycrinamidine, presents no activity in the models investigated.

Conclusions: Our results indicate that CL extracts and alkaloid fraction (but not pure 6-hydroxycrinamidine) inhibit the proliferation of lymphoma cells in multiple pathways. Our results are in accordance with traditional usage and encourage further studies and *in vivo* assays.

Abbreviation: **CL**, *Crinum latifolium*; **ROS**, reactive oxygen species; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PCR**, *polymerase chain reaction,* **DPPH***,* 2,2-diphenyl-1-picrylhydrazyl, **M1**, classically activated macrophages, **PBMC**, peripheral blood mononuclear cells.

Introduction

Crinum latifolium (CL), belonging to the family of Amarylliaceae, is a lily-like flowers plant that grows throughout the tropics and warm temperature regions of the world (Fennell et al., 2001). The plant is distributed in America, Australia, Southern Asia, and the majority in Africa.

Herbal preparations of CL have been used therapeutically for over a thousand years in Asian folk medicine for their anti-inflammatory, anti-tumour and anti-microbial effects (Fennell et al., 2001; Loi, 2000).

In the case of the traditional use against cancer, especially in Vietnamese and Chinese medicine, hot aqueous extracts of CL are consumed as a good remedy for prostate cancer, with successful cases being reported (Nhu, 2002). It can be noted that prostate cancer is considered as one of the most common cancers in older

males (90% of men aged 70 to 90) (Dunn et al., 2011). Treatments including surgery, radiotherapy, hormone therapy, and chemotherapy may induce adverse effects that often reduce the quality of life. Moreover these treatments are expensive especially for people in developing countries. Herbs are not expensive and have been used for a long time in Asia as cures for many diseases (Loi, 2000).

Recent studies have produced some evidence for immunostimulatory and anti-inflammatory effects of aqueous extracts of CL leaves that could explain their antitumour effects (ref Tram et al., 1999; Zvetkova et al., 2001; Jenny et al., 2011). In the case of immunostimulation, the ability of CL extracts to activate and stimulate T-cell proliferation *in vitro* and *in vivo* has been demonstrated (Tram et al., 1999). In addition, hot and cold aqueous Yes it is extracts suppressed mitogen and interferon (IFN)-γ-induced formation of neopterin in human peripheral blood mononuclear cells (PBMC) (Zvetkova et al., 2001). Extracts of CL also suppressed indoleamine 2,3-dioxygenase (IDO) activity in stimulated PBMC with phytohaemagglutinin (PHA), which corresponds to the down-regulation of the formation of IFN-γ in T-cells, tryptophan degradation and neopterin production in macrophages (Jenny et al., 2011) that are responsible for anti-proliferative activity. These results are starting points to understand the mechanism of the anti-tumour activities of CL. Macrophages are the dominant population of leukocytes found in the tumor microenvironment. These cells are not a homogeneous population; they are composed of several distinct pro-and anti-tumour subpopulations with overlapping functions depending on a variety of external factors in the tumour microenvironment and signals from lymphocyte subsets (Biswas and Mantovani, 2010). Thus, these cells may represent potential targets for anti-cancer therapy (Mantovani et al., 2009).

In addition, many pure compounds have been isolated from CL extracts in the past few years. Most of them are alkaloids and though there are a few non-alkaloid compounds (sugars, flavonoids). Three types of alkaloids have been found in CL extracts: crinane (5,10b-ethanophenanthridine), lycorine (pyrrolophenanthridine) and cherylline types (Tram et al., 2002). Interestingly, some pure compounds such as 11-*O*-acetylambelline and 11-*O*-acetyl-1,2-β-epoxyambelline have been shown to be immunoregulators, while crinafoline and crinafolidine possess anti-tumour activity on sarcoma 180 ascites tumour cells in mice(Ghosal et al., 1985; Ghosal et al., 1986).

To assess the potential anti-tumour properties of CL, we carried out biochemical and biological studies on three extracts of CL (aqueous extract (**AqEx**), total alkaloid extract obtained by Supercritical Fluid Extraction (SFE) (**AkEx**) and total flavonoid extract (**FlEx**)) and on an alkaloid fraction (**FrF**) obtained from the total chloroform alkaloid extract. The major pure compound, 6-hydroxycrinamidine (**6HC**), was isolated from the alkaloid extract to compare its activities with the three extracts and with the alkaloid fraction. In the present study we report the biochemical and biological properties of these extracts which could be responsible for their anti-tumour activities, namely their redox activities, cytotoxicity, capacity to induce activation and differentiation of macrophages.

Experimental Section

2.1. Plant material

Whole leaves of CL were collected in Binh Dinh province, Vietnam, in October 2010. The leaves was identified and authenticated by Prof. Vo Thi Bach Hue, Analytical Department, Medicine and Pharmacy University of Ho Chi Minh city, Vietnam. After collection, the leaves were cleaned and shade-dried in a cool place and then ground into a homogeneous powder.

2.2. Preparation of botanical extracts

The leaf powder was extracted by four different procedures. The materials were deposited at the Analytical Department, Medicine and Pharmacy University of Ho Chi Minh City, Vietnam. Their voucher numbers were as follows: aqueous extract (CL011010-1, **AqEx**), total chloroform alkaloid extract (CL011010-2), fraction F from CL011010-2 (CL011010-2-F, **FrF**), total alkaloid extract by SFE (CL011010-3, **AkEx**), total flavonoid extract (CL011010-4, **FlEx**).

The aqueous extract (**AqEx**) was prepared as follows It is a product in market: 100 g of powered leaves was boiled in distilled water (1:10, w:v). The water extract was filtered through paper (Munktell No 2, USA) and was further heated to 60 – 70 ^{o}C to concentrate the solution. The yield of water extract was 5%. Finally, 0.14 g sodium benzoate was added to 100g aqueous extract for preservation.

To obtain the total chloroform alkaloid extract (CL011010-2), 100 g of powdered leaves was suspended in 1 L of 1‰ HCl in 96% ethanol, pH 4. The acidic ethanolic solution was filtered through paper (Munktell No 2, USA) and then made alkaline (pH 9) with ammonia solution. The basic ethanolic solution was successfully extracted with $CHCl_3$. After evaporating under reduced pressure, 3 g of product were collected (yield: 0.3%).

Total alkaloids (**AkEx**) were extracted by using Supercritical Fluid Extraction (SFE). With CO_2 100 g of leaf powder was passed through a supercritical fluid extraction system using 96% ethanol as modifier. The extraction was carried out under supercritical temperature (31 ^{o}C) and pressure at (72 bar). The SFE extract was acidified to pH 3-4 and filtered through paper (Munktell No 2, USA). The acidic solution was then basified with ammonia solution to pH 10 and extracted with chloroform to yield the basic chloroform extract. The resulting extract was concentrated under reduced pressure to give 1 g of the total alkaloid residue (yield: 0.1%).

The total flavonoid extract (**FlEx**) was prepared as follows: 100 g of powdered leaves was mixed with 1 L 70% ethanol and was then filtered through paper (Munktell No 2, USA). The collected solvent was further extracted with ethylacetate. The final extract was evaporated under reduced pressure to give 4 g of total flavonoid residue (yield: 0.4%).

2.3. Fractionation and isolation

Fractionation of the CL total chloroform alkaloid extract was achieved as follows. The total crude alkaloid extract (CL011010-2) was dissolved in $CHCl_3$ (South Basic Chemicals Company, China) and subjected to vacuum liquid chromatography with $CHCl_3$:MeOH (South Basic Chemicals Company, China) as the mobile phase and the amount of methanol increased gradually from 0 to 100%. Fractions (150 ml) were collected and verified by thin layer chromatography (TLC) (silica gel $60F_{254}$, Merck, Germany) with the solvent system $CHCl_3$–MeOH–NH_4OH 25% (6:1:0.05) (South Basic Chemicals Company, China). Among the eight main fractions collected (A, B, C, D, E, F, G, H), the isolation of the active compound was obtained

from fraction F (**FrF**) using column chromatography (MeOH/ethylacetate = 50/50) (Fisher Chemical, UK). The purity of the substance was checked by TLC (silica gel $60F_{254}$, Carlo Erba Reagents, France) with the solvent mixture MeOH/ethylacetate = 50/50 (R_f = 0.63). Finally, 15.3 mg of pure compound (white crystals) was obtained by crystallization.

2.4. Identification of the pure compound, 6-hydroxycrinamidine

NMR spectra (^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC and ADEQUATE) were recorded on a Bruker AVANCE 500 MHz spectrophotometer equipped with a 5mm TCI ^{1}H, ^{13}C, ^{31}P cryoprobe. The sample was dissolved in dimethyl sulfoxide (DMSO)-d_6 (Aldrich, USA).

The pure compound (**6HC**) was investigated by using an LC-MS (Thermo-Finnigan, France) equipped with a C18-column (5µm, 4.6 x 250 mm, Luna, Phenomenex, France). The column temperature was set at 25 °C and the flow rate was 0.7 mL/min with the mobile phase A (acetonitrile, VWR), B (distilled water), C (0.1% formic acid, Sigma). The gradient elution was started at 10% A for 4 min, increased linearly to 90% at the 25th minute for 3 min and then returned rapidly to the initial volume. Phase C always remained at 10%. The injected volume was 10 µL.

2.5. Redox assays

2.5.1. DPPH assay

The free radical scavenging activity was evaluated using the stable 2,2-diphenyl-2-picrylhydrazyl nitrogen-centred free radical (DPPH). DPPH in ethanol (300 µM, 500 µL) was added to 1 mL of the test compounds at different concentrations in ethanol. Each mixture, tested in triplicate, was then mixed thoroughly and the absorbance recorded at 530 nm every minute for 30 min, using a spectrophotometer (Specord 205, Analytik Jena, Germany). The decrease in DPPH absorbance was monitored. DPPH solution (300 µM, 500 µL) in the respective solvent (1 mL) served as the blank. Trolox in ethanol (20 mg/L) was used as the reference. The radical scavenging activity of the samples (antioxidant activity) was expressed in terms of IC_{50} (concentration in mg/L required for a 50 % decrease in DPPH absorbance).

2.5.2. β-carotene-bleaching assay

The antioxidant activity of the extracts was determined according to Karadeniz et al. (2005) with the following modifications: a solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 1 mL of chloroform. 500 µL of this solution was then transferred into a round-bottom rotary flask containing 20 mg of linoleic acid and 200 mg of Tween 20. After removing the chloroform using a nitrogen stream, 50 mL of aerated distilled water was added to the flask with manual shaking. Aliquots of 5 mL of this emulsion were transferred into tubes containing 200 µL of extracts or α- tocopherol (50 mg/L, positive control), which was used as positive control. Each measurement was done in triplicate. The blank consisted of 0.2 mL of the respective solvent without the extract. Zero time absorbance was recorded at 470 nm as quickly as possible. The samples were then subjected to thermal autoxidation at 50 °C in a temperature controlled 8-cell holder. Subsequent absorbance readings were recorded at regular time intervals until the colour of the β-carotene in the blank sample had disappeared (80 min). The extent of inhibition of β-carotene bleaching is related to the concentration of antioxidant compounds. All samples were assessed in triplicate. Antioxidant activity (AA) was calculated as the percentage inhibition of β-carotene bleaching relative to the blank using the following equation: AA (%) = [1 - (Ai - At)/ (A'i- A't)] x 100 (Ai = absorbance of the sample at t = 0; At = absorbance after incubation (80 min) at 50 °C; A'i= absorbance of the blank at t = 0; A't = absorbance of the blank after incubation (25 min) at 50 °C

2.6. Macrophages preparation

2.6.1. Animals

All the mice were bred and used under protocols approved by the Conseil Scientifique du Centre de Formation et de Recherche Expérimentale Médico Chirurgical and the ethic boards of the Midi-Pyrénées (France) ethics committee for animal experiments (Experimentation permit number 31-067, approval n° 3155503). All the C57B1/6 mice (Janvier, France) used for the *in vitro* macrophages studies were 8-12 week old males.

2.6.2. Preparation of mouse resident peritoneal macrophages

After euthanasia with CO_2, resident peritoneal cells were harvested by washing the peritoneal cavity of the C57BI/6 mice (Janvier, France) with 5 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, France). The collected cells were centrifuged at 1400 rpm for 10 minutes and the cell pellet was suspended in Macrophage-SFM (Serum-Free Medium) (Gibco Life Technologies, France) supplemented with glutamine (Gibco Life Technologies, France). Cells were allowed to adhere in 48- or 96-well culture plates (1.5×10^5 cells/well) for 2 hours at 37 °C and under 5% CO_2 atmosphere. Non-adherent cells were then removed by washing with phosphate-buffered saline (PBS, Gibco, Life Technologies, France) and the remaining adherent cells were cultured and stimulated like those described below.

2.6.3. Human monocytes preparation

Mononuclear cells were obtained from the buffy coats from healthy blood donors (Etablissement Français du Sang, Toulouse, France) by a standard Ficoll-Hypaque gradient method as previously described (Bureau et al., 2001). Human monocytes were isolated from mononuclear cells by adherence to plastic for 2 hours in special macrophage serum-free medium (Bibco, Life Technologies, France) with L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by gentle washing with Hank's Balanced Salt Solution (HBSS, Bibco, Life Technologies, France). The number of adherent cells was standardized and the remaining adherent cells (85% monocytes) (Bureau et al., 2001) were incubated in Macrophage-SFM (Gibco, Life Technologies, France).

2.7. Sample preparation

The samples studied were dissolved in 0.1% DMSO to obtain an initial concentration of 25 mg/mL. In the experiments, the final concentration of the test samples was 25 µg/mL.

2.8. MTT assay

Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfo-phenyl)-2*H*-tetrazolium) (Sigma-Aldrich, France) assay. After 2 hours of adhesion of mice peritoneal macrophages according to the experimental protocol, the different types of extracts from CL were plated in a total volume of

200 µL in 96-well plates (Becton Dickinson, France). The wells containing only mice peritoneal macrophages were used as control groups. Following 24 hours of incubation at 37 °C, 0.02 mL MTT (final concentration 0.5 mg/mL) was added in each well and the plates were incubated for 2 hours, and then 100 µL of DMSO was added to dissolve the blue formazan crystals. The absorbance was measured by spectrophotometry at 545 nm (EnVisionMultilabel Plate Reader, Perkin Elmer, France).

2.9. Cytotoxicity on lymphoma, EL4-luc2

EL4-luc2 cells, a syngeneic murine lymphoma cell line from C57BL/6 mouse expressing the firefly luciferase gene (luc2) (Caliper Life Science) were used as a tool to detect drug efficacy *in vitro* on tumour cell proliferation and the activation of macrophage tumoricidal activity. The CL extracts (Final concentration 25 µg/mL. There is only one concentration in order to to avoid repeat the same sentence about concentration, then I made remark about concentration in section 2.6.) were incubated with EL4-luc2 lymphoma cells (3×10^4 cells/well) for 24 and 48 hours and cell viability was evaluated by an MTT assay.

2.10. Assay for ROS production

To assess ROS production, human monocytes (1.5×10^5 cells/well) were placed in a 96-well white microplate and the ROS production was measured by chemiluminescence in the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol, Sigma, France) using a thermostatically (37 °C) controlled EnVisionMultilabel Reader (PerkinElmer, France). The generation of chemiluminescence was continuously monitored for 90 minutes after incubation of the cells with luminol (60 µM) under basal conditions and in the presence of either studied samples 25 µg/mL and/or 12-*O*-tetradecanoylphorbol 13-acetate (PMA) (100 nM/well), a specific activator of Protein Kinase C (PKC); zymosan (50 ng /well), from the cell wall of *Saccharomyces cerevisiae* or the chemotactic peptide, *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (1µM/well) using the method reported by Bureau *et al.* (2001).

2.11. Determination of inhibition of proliferative response via macrophages activity

To test the anti-tumour reactivity of macrophages, we seeded 1.5×10^5 mouse peritoneal macrophages per well in a 96-well plate and co-cultured them with 3×10^4 cells of the EL4-luc2 cell line for 24, 48 and 72 hours. An *in vitro* bioluminescence assay with firefly D-luciferin (150 µg/mL) (Caliper Life Science) was carried out using a thermostatically (37 °C) controlled Envision Multilabel Reader (PerkinElmer, France).

2.12. Quantification of mRNA by reverse transcription and real-time PCR

Total RNA obtained from peritoneal macrophages was prepared with RNeasyMini Kit columns (Qiagen) using the manufacturer's protocols. Synthesis of cDNAfor reverse transcription-quantitative PCR (RT-qPCR) was performed from 1 mg of total RNA with QuantiTectH Reverse Transcription (Qiagen) according to the manufacturer's recommendations and primed with hexamers. Quantitative real-time PCR was performed on a LightCycler480 system (Roche Diagnostics) using LightCycler SYBR Green I Master (Roche Diagnostics). Ten microliters of reaction mixture was incubated; the amplifications were performed for 50 cycles (10 s at 95°C and 60 s at 60°C). The primers (at a final concentration of 10 mM) were designed with the software Primer 3 and listed in Table 1.

Table 1. Primers sequences used in quantitative PCR experiments.

Gene	Sequences
GAPDH	sense5'AAC TT GGC ATT GTG GAA GG3' antisense 5'ACA CAT TGG GGG TAG GAA CA3'
IL-1β	sense5' CAA CCA ACA AGT GAT ATT CTC CAT G3' antisense 5' GAT CCA CAC TCT CCA GCT GCA3'
TNF-α	sense 5'AGG CTG TGC ATT GCA CCT CA3' antisense 5'GGG ACA GTG ACC TGC ACT GT3'
IL-6	sense5' GAG GAT ACC ACT CCC AAC AGA CC3' antisense 5' AAG TGC ATC ATC GTT GTT CAT ACA3'
Mannose receptor	sense 5'ATG CCA AGT GGG AAA ATC TG3' antisense 5'TGT AGC AGT GGC CTG CAT AG3'
CD36	sense 5'GCA GAA TCA AGG GAG AGC AC3' antisense 5'GAG CAA CTG GTG GAT GGT TT3'
CD11b	sense 5' GAC TCA GTG AGC CCC ATC AT3' antisense 5' AGA TCG TCT TGG CAG ATG CT3'
Dectin-1	sense 5'CAT CGT CTC ACC GTA TTA ATG CAT3' antisense 5'CCC AGA ACC ATG GCC CTT3'
TGFβ	sense 5'AGG GGC CTC TAA GAG CAG TC3' antisense 5'AGG TTG GCA TTC CAC TTC AC3'
NQO-1	sense 5'TTC TCT GGC CGA TTC AGA GT3' antisense 5'GGC TGC TTG GAG CAA AAT AG3'
HO-1	sense 5'CCA GAG TGT TCA TTC GAG CA3 antisense 5'CAC GCA TAT ACC CGC TAC CT3'
Nrf2	sense 5'CTC GCT GGA AAA AGA AGT GG-3' antisense 5'CCG TCC AGG AGT TCA GAG AG3'

2.13. Statistical analysis

The results from the three independent experiments were presented as mean ± S.E. Differences between group averages were analysed by ANOVA, followed by a Bonferroni Dunnett test. Differences of in *p*-value of less than 0.05 were considered statistically significant.

Results

3.1. Identification of the pure 6-hydroxycrinamidine.

The LC-MS chromatogram of a pure compound isolated from *C. latifolium* had an R_t at 6.24 min with a molecular mass, *m/z* 334.13 $[M+H]^+$. The pure compound, $C_{17}H_{19}NO_6$ has been identified as 6-hydroxycrinamidine (Table 2 and Fig. 1) in previous reports of alkaloids isolated from herbal medicines (Hue et al., 1997; Machocho et al., 1999).

Table 2. ^{1}H NMR (500 MHz), COSY (500 MHz) and 2D NMR data of 6-hydroxycrinamidine

C/H Position	H Posistion	^{1}H	COSY (H→ H#)	HMBC (H→ C#)	^{13}C	ADEQUATE (C→ C#)
1	-	3.8 (m)	2	2, 4a, 10a, 10b	53.2 (s)	2, 10b
2	-	3.1 (s)	1,3	3, 4	54.5 (s)	1, 3
3	-	4.2 (s)	2, 4α, 4β	1, 4a	64.2 (d)	2, 4
4	4α	1.3 (m)	3, 4β, 4a	4a, 10b	29.4 (d)	4a, 3
	4β	1.3 (m)	3, 4α, 4a	4a, 10b		
4a	-	3.5 (dd ; 3.50, 3.52)	4α, 4β, 4a	1, 4, 6, 10a, 11	56.7 (d)	3, 4
6	-	4.9 (d)	-	4a, 6a, 7, 8, 10a, 12	84.9 (d)	6a
6a	-	-	-		121.8 (d)	-
7	-	-	-		143.1 (s)	-
8	-	-	-		134.9 (s)	-
9	-	-	-		149.0 (s)	-
10	-	6.9 (s)	-	6a, 8, 9, 10b	97.7 (s)	9, 10a
10a	-	-	-	-	140.2 (d)	-
10b	-	-	-	-	41.8 (s)	-
11	11 endo	1.5(m)	11exo, 12endo, 12exo	4a, 10a, 10b	36.4 (s)	10b, 12
	11 exo	2.1 (m)	11endo, 12endo,12exo	C-1, C-10a, C-10b, C-12		
12	12 endo	2.5 (m)	11endo,11exo, 12exo	4a, 6, 11	46.2 (s)	11
	12 exo	2.9 (m)	11endo, 11exo,12endo	4a, 6		
OCH2O	-	5.9 (d)	-	8, 9	101.2 (s)	-
7-OMe	-	3.9 (m)	-	7	59.9 (s)	-

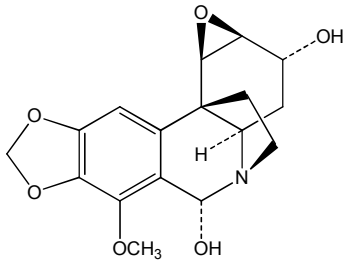


Fig.1. 6-hydroxycrinamidine

3.2. Redox properties

In both DPPH and β-carotene bleaching assays, the total flavonoid extract had the highest antioxidant activity with IC_{50} values of 107.36 mg/L and 1010.2 mg/L, respectively. The aqueous extract presented weaker IC_{50} values, 496.9 mg/L (DPPH assay) and 1513.3 mg/L (bleaching beta-carotene assay), respectively. Other samples had weak or no activity. These results are given in Table 3.

3.3. Effect of CL extracts on cell viability

3.3.1. Macrophages

The MTT assay is a well-known assay to assess the function of mitochondria in living cells. The cell viability is established by determining the reduction of tetrazolium salts into formazan crystals. There were no significant changes observed in macrophages incubated with CL samples 25 µg/mL in comparison with the control (macrophages alone): the viability of macrophages was not affected in the presence of the samples studied, showing that CL extracts were not toxic to immune cells (Fig. 2).

3.3.2. Tumour cells (EL4-luc2)

The flavonoid and alkaloid CL extracts at 25 µg/mL were directly toxic to the EL4-luc2 tumour cells, with the flavonoid being the more toxic. Indeed, the level of lymphoma proliferation was always below 2500 luminescence units in the presence of the flavonoid extract whereas the initial number of lymphoma cells added to each well was 30 000 corresponding to ~ 3385 luminescence units. The results showed that the flavonoid extract inhibited the proliferation of tumour cells (Figs. 3).

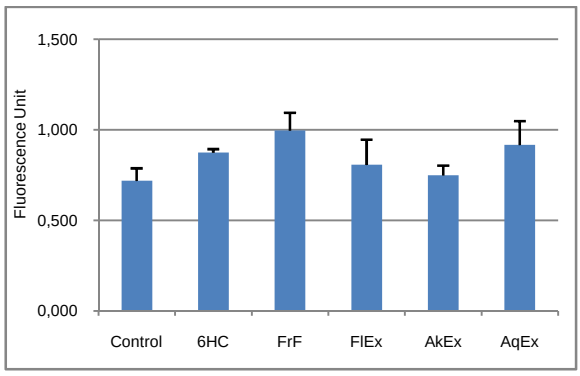


Fig. 2. The effect of CL extracts on the viability of macrophages.Control: macrophages (without test samples), 6HC: 6-hydroxycrinamidine, FrF: fraction F, FlEx: flavonoid extract, AkEx:

alkaloid extract, AqEx: aqueous extract. Data represented as mean ± SEM of three independent experiments.

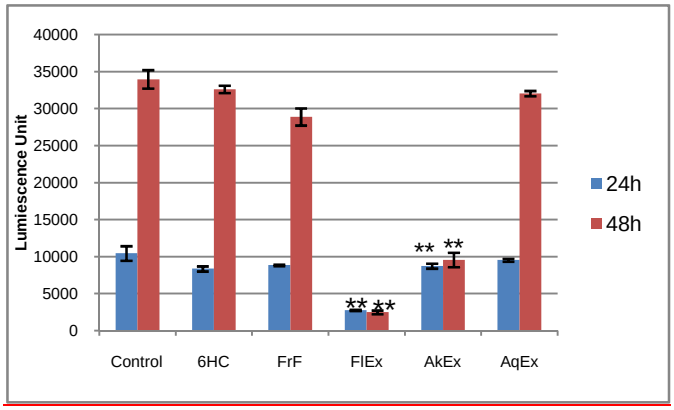


Fig. 3. The effect of CL extracts on the proliferation of EL4-luc2 lymphoma . at 24 h and 48 h of culture. Control: EL4-luc2 cells without CL extracts, 6HC: 6-hydroxycrinamidine, FrF: fraction F, FlEx: flavonoid extract, AkEx: alkaloid extract, AqEx: aqueous extract. Data are represented as mean ± SEM of three independent experiments. Statistical significance versus control: ** $P < 0.001$

Table 3. Antioxidant properties of the CL extracts

Plant extract	DPPH assay IC_{50}(mg/L)	β-carotene bleaching assay IC_{50}(mg/L)
Flavonoidextract(CL011010-4)	107.4± 9.5	1010.2 ± 95.4
Aqueousextract(CL011010-1)	496.9 ± 37.04	1513.3 ± 229.6
Alkaloidextract (CL011010-3)	1163.7 ± 23.1	No activity
Fraction F(CL011010-2-F)	No activity	No activity
6-hydroxycrinamidine	No activity	No activity
Trolox	10.03 ± 0.9	-
Vitamin E	-	6.6± 0.5

3.4. Study of ROS production by monocytes in the presence of CL extracts

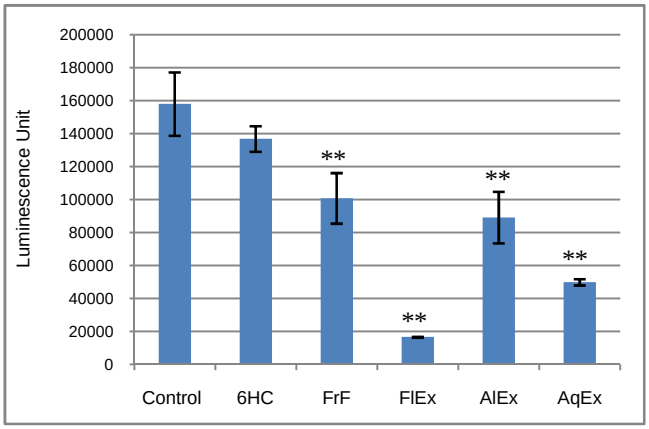
Figure 4A shows the effect of *C. latifolium* extracts on basal ROS production by human monocytes. Basal ROS production was suppressed by unstimulated human monocytes incubated with fraction F, flavonoid, alkaloid and aqueous extracts for 90 minutes (Fig. 4A). Monocytes cultured for 90 min with CL extracts were then stimulated by 100 nM PMA. The ROS production stimulated by PMA was inhibited in monocytes incubated with flavonoid, alkaloid and aqueous extracts, and was slightly induced in monocytes incubated with 6HC (Fig. 4B).

3.5. Induction of macrophages polarization by CL extracts

3.5.1. Production of ROS

This part of the study investigated whether polarization for 24 hours with different *C. latifolium* extracts affected the ability of macrophages to produce ROS in response to PMA, zymosan and fMLP. ROS production at the basal level and in response to fMLP was increased when the macrophages were differentiated with fraction F, flavonoid and alkaloid extracts. Moreover, opsonized zymosan proliferation was strongly inhibited by macrophages treated with alkaloid and aqueous extracts, while PMA proliferation was slightly inhibited by macrophages treated with the same extracts (alkaloid and aqueous). Macrophages treated with fraction F and 6HC showed increased ROS production when stimulated by PMA. Finally, 6HC induced ROS production in macrophages exposed to opsonized zymosan (Fig. 5).

A)



B)

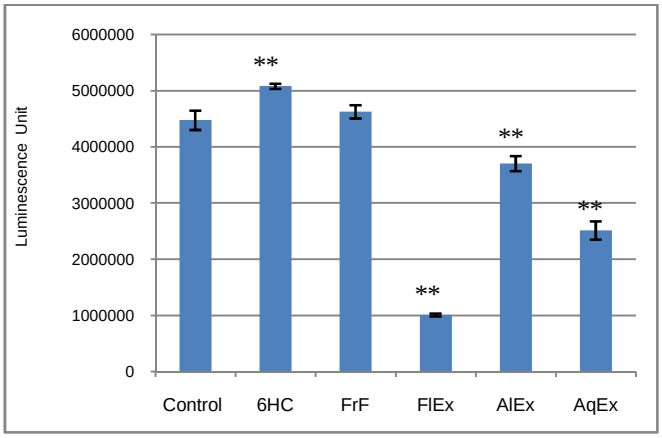


Fig. 4. The effect of CL extracts on ROS production in human monocytes. CL extracts induced a respiratory burst in monocytes as measured by chemiluminescence. Total chemiluminescence emission (area under the curve expressed in chemiluminescence index) was measured over 90 min. (A), monocytes cultured for 90 min with CL extracts, corresponding control: unstimulated human monocytes without CL extracts (B), after 90 min of treatment with CL extracts, the ROS production in monocytes was stimulated by PMA (100nM), corresponding control: human monocyte stimulated by PMA without CL extracts, 6HC: 6-hydroxycrinamidine, FrF: fraction F, FlEx: flavonoid extract, AkEx: alkaloid extract, AqEx: aqueous extract. The data are the means ± SEM of three independent experiments. ** p < 0.001 indicates a significant difference compared with the corresponding control groups without CL extracts.

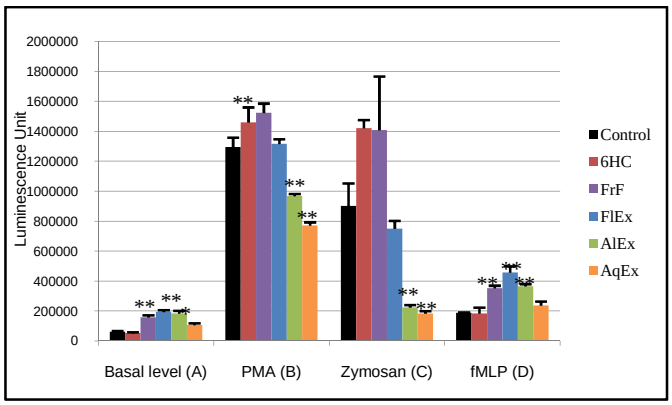


Fig. 5. Effect of CL extracts to modulate the capacity of human monocytes after 24 hours of treatment to produce ROS after stimulation by PMA, zymosan or fMLP. The respiratory burst of monocytes was measured by chemiluminescence. Total chemiluminescence emission (area under the curve expressed in chemiluminescence index) was measured over 90 min. (A) represents the ROS production by unstimulated monocytes, cultured 24 h with CL extracts. After 24 h of culture with CL extracts, the monocytes were stimulated by PMA (B), zymosan (C) or fMLP (D) in the absence of extracts. The data are the means ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.001$ indicate a significant difference compared with the untreated macrophages by CL extracts (control) without stimulator (A) or with various stimulators (B, C, D). 6HC: 6-hydroxycrinamidine, FrF: fraction F, FlEx: flavonoid extract, AkEx: alkaloid extract, AqEx: aqueous extract.

3.5.2. Evaluation of mRNA level

To define the polarization of peritoneal macrophages by aqueous and flavonoid extracts of CL, the expression of markers of the M1 and M2 macrophage activation states was assessed by RT-PCR (Figs 6 and 7). We observed that after treatment for 24 hours with an aqueous extract of CL the peritoneal macrophages from mice expressed the mRNA encoding the gene for IL1-β, TNFα and IL-6, which are established markers of classical M1 macrophage polarization (Fig. 6). By contrast, aqueous and flavonoid extracts of CL had no significant effect on the expression of the mRNA of the mannose receptor, CD36, Dectin-1, and TGFα, which are markers of alternative M2 macrophage activation (Fig. 7).

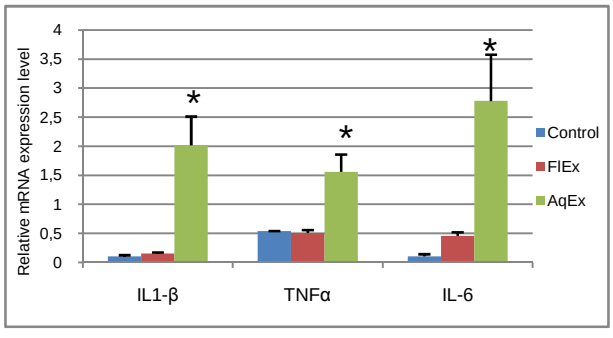


Fig. 6. Effects of aqueous and flavonoid extracts of CL on IL1-β, TNFα, and IL-6 mRNA expression in peritoneal macrophages. The mRNA expression in mice peritoneal macrophages pretreated or not (control) over 24 h with aqueous (AqEx) and flavonoid (FlEx) extracts of CL was evaluated by RT-PCR. The data are the means ± SEM of three separate experiments. * $p < 0.05$ indicates a significant difference compared with the untreated macrophages by CL extracts (control).

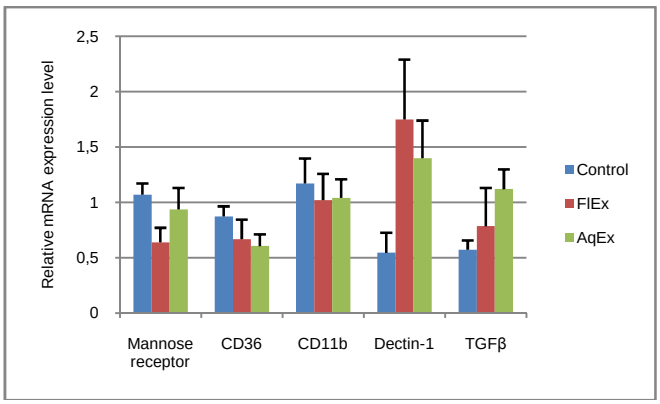


Fig. 7. Effects of aqueous and flavonoid extracts of CL on mannose receptor, CD36, Dectin-1 and TGF mRNA expression in peritoneal macrophages.The mRNA expression in mice peritoneal macrophages pretreated or not (control) over 24 h with aqueous (AqEx) and flavonoid (FlEx) extracts of CL was evaluated by RT-PCR. The data are the means ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.001$ indicate a significant difference compared with the untreated macrophages by CLextracts (control). The data are the means ± SEM of three separate experiments.

The molecular activities of flavonoids include activation of the nuclear factor-erythroid 2-related factor 2 (Nrf2). Several studies (Fahey et al., 2002; Pae et al., 2007; Nakamura et al., 2004) have shown the importance of this transcription factor in regulating the ARE-dependent transcription of heme oxygenase-1 (HO-1) and NAD(P)H: quinoneoxidoreductase (NQO1) genes. Thus, we determined whether aqueous and flavonoid extracts of CL augmented mRNA expression of HO-1, NQO1 and Nrf2 in resident murine peritoneal macrophages. While treatment of macrophages with the flavonoid extract for 24 hours resulted in a 2-fold increase in NQO-1, the aqueous extract induced a 3-fold increase in the same gene expression (Fig. 8). The expression of HO-1 and Nrf2 mRNA were not significantly increased (Fig. 8).

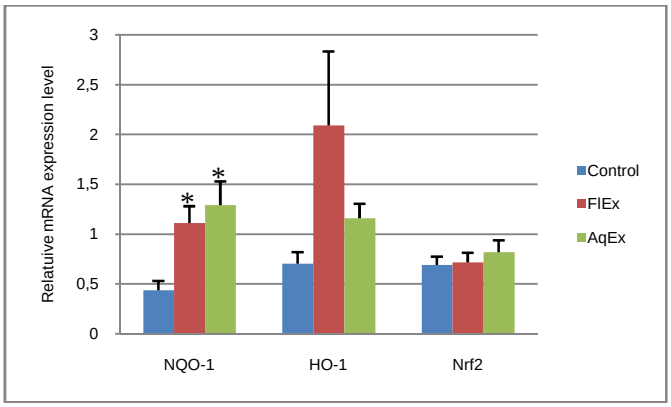


Fig. 8. Effects of aqueous and flavonoid extracts of CL on heme oxygenase-1 (HO-1), NAD(P)H: quinoneoxidoreductase (NQO1) and Nrf2 mRNA expression in peritoneal macrophages. The mRNA expression in mice peritoneal macrophages pretreated or not (control) over 24 h with aqueous (AqEx) and flavonoid (FlEx) extracts of *C. latifolium* was evaluated by RT-PCR. The data are the means ± SEM of three separate experiments. * $p < 0.05$ indicates a significant difference compared with the untreated macrophages by CL extracts (control).

3.6. Proliferative responses of EL4-luc2 lymphoma via macrophages activity

Figure 9 shows that the macrophages polarized after 24 hours of culture with the alkaloid and aqueous extracts of CL inhibited the proliferation of EL4-luc2 tumour cells. This inhibitory effect on lymphoma proliferation was very significant after 72 hours of EL4-luc2 culture. These results indicate that macrophages polarized by the alkaloid or aqueous extracts of CL have acquired a phenotype that can reduce tumour growth. Other extracts did not have any significant effect.

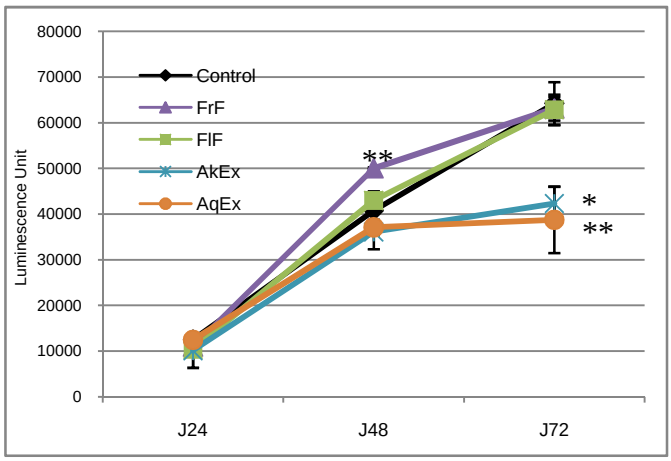


Fig 9.Effects of extracts of *C. latifolium* on antitumor activity of murine peritoneal macrophages.The proliferation of EL4-luc2 in coculture with macrophages was evaluated during 72h. The ratio EL4/macrophages was $3x10^4$/ $15x10^4$ at J0. The number of EL4-luc cells was evaluated by bioluminescence after 24, 48 or 72 h of coculture with macrophages. Macrophages was pretreated or not (control) during 24h with Fraction F (FrF), Flavonoid extract (FlEx), Alkaloid extract (AkEx) and Aqueous extract (AqEx) of *C. latifolium*. The data are the means ± SEM of three separate experiments. * p< 0.05 (**p<0,01) indicates a significant difference compared with the untreated macrophages by *C. latifolium* extracts (control).

Discussion

The leaves of CL are widely used (Loi, 200) in traditional Vietnamese medicine for prostate cancer treatment and there are some previous studies that have shown immunostimulatory and anti-inflammatory effects. For the first time, in the present study, the effect of different CL extracts on the polarization of macrophages has been studied. Interestingly, our results suggest that alkaloid and aqueous extracts of CL are able to inhibit tumour growth via macrophage activation while flavonoid and alkaloid extracts directly inhibit the proliferation of the cancer cell line EL4-luc2 in the absence of macrophages.

Macrophages are professional phagocytes that are involved in the body's defence against bacteria, protozoa and viruses, and in anti-tumour immunity, responses to inflammatory signals and the regulation of wound healing (Murray et al., 2011). Depending on the requirements, macrophages can be differentiated into subtypes: classically activated macrophages (M1) and alternatively activated macrophages (M2a), tumour-associated macrophages (TAMs or M2d) and myeloid-derived suppressor cells (MDSCs) (Murray et al., 2011). M1 polarized macrophages are responsible for the production of superoxide anions and other chemically reactive molecules containing oxygen (ROS), (Fairweather et al., 2009; Sindrilaru et al., 2011) for the production of pro-inflammation cytokines (IL-1, TNFα, IL-6), antigen presentation and expression of MHC class II molecules and microbicidal activity in addition attacking cancer cells. M2a polarized macrophages are involved in anti-inflammatory cytokine (IL10, IL1Ra) production, endocytic activity, cell growth and tissue repair. TAMs and MDSCs induce immunosuppression.

Classically activated M1 macrophages also stimulate iNOS induction which catalyses L-arginine transformation into nitric oxide (NO) and citrulline (Odegaard et al., 2008). The interplay between anion superoxide and nitric oxide is the origin of the formation of peroxynitrite, which is a highly active radical responsible for killing bacteria or different types of tumour cells. ROS are a double-edged sword in biological systems. On the one hand, ROS encourage the progression of pathological conditions, including cancer (Rhee, 2006). On the other hand, they play an important role in the host's defence against invading microorganisms (Fialkow et al., 2007) and in intracellular signalling pathways regulating cell function (Rhee, 2006). In cancer, ROS are involved in two inverse functions: cancer promotion via the Ras-Raf-MEK-ERK signalling pathway and cancer suppression via the p38 MAPK pathway (Pan et al., 2009). The Ras-Raf-MEK-ERK signalling pathway is responsible for the inhibition of apoptotic cell death by induced oxidative stress and activation of vascular endothelial growth factor (VEGF), an important protein for tumour angiogenesis (Pan et al., 2009). In contrast, activation of the p38 MAPK pathway results in the induction of apoptotic cancer cell death (Pan et al., 2009). Therefore, an agent with high antioxidant activity could play a central role in suppressing cancer cell proliferation in the case where ROS are involved in cancer promotion.

The antioxidant activity of flavonoid, alkaloid and aqueous extracts of CL shown in section *3.2* and *3.4* that could be critical for tumour cell growth. Importantly, the antioxidant enzymes (NADPH quinoneoxidoreductase 1 (NQO1) mRNA were up-regulated in macrophages polarized with flavonoid and aqueous extracts. Antioxidant enzymes such as glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), glutathione S-transferase (GST), OH-1 and NQO1 are essential for cellular defence mechanisms or phase II detoxification (Surh et al., 2008). The up-regulation of antioxidant enzymes could play a pivotal role in cancer prevention as has been proved in recent studies. Sulforaphane originating from broccoli is able to activate the expression of NQO1 and GST and this is protective against gastric tumors induced by benzo[*a*]pyrene (Fahey et al., 2002). Curcumin isolated from *Curcuma longa* L., Zingiberaceae shows anti-proliferation as well as anti-inflammation and antioxidant activities. Its anti-proliferative action is based on induction of HO-1 expression though the mechanism still remains unclear (Pae et al., 2007). Zerumbone derived from tropical ginger, *Zingiber zerumbet* Smith, which activates phase II enzyme genes including GPx and OH-1, is believed to be effective in the chemoprevention of colon and skin cancer (Nakamura et al., 2004). Thus, the enhanced NQO1 expression in macrophages polarized with flavonoid and aqueous extracts of CL confirmed their antioxidant activity and could contribute to their effectiveness in cancer chemoprevention.

To determine the effect of CL extracts on the growth of tumour cells, the proliferation of EL4 lymphoma cells was assessed in the presence of the different extracts. Flavonoid and alkaloid extracts directly inhibited cell growth. Interestingly, the flavonoid extract showed very high inhibition of lymphoma cell growth. In addition, none of the samples studied showed any toxicity towards

macrophages (Fig. 2). This indicates that the CL flavonoid and alkaloid extracts are specifically toxic toward cancer cells but not towards normal living cells. Flavonoids as well as alkaloids have many important biological activities for instance inactivation of reactive oxygen species (ROS), binding of electrophiles, induction of protective enzymes (enzymes of phase II detoxification), induction of apoptosis, inhibition of cell proliferation, lipid peroxidation, angiogenesis, and DNA oxidation (Chahar et al., 2011; Lu et al., 2012). However, the direct inhibition of lymphoma cell growth of the flavonoid and alkaloid extracts was not only based on their antioxidant properties but also required the modulation of multiple biological signaling pathways to exert their anti-tumour activity. The aqueous extract showed antioxidant activity (section *3.2* and *3.4*) but had no direct inhibitory effect on lymphoma cell proliferation (section *3.3.2*). Moreover, the pure compound, 6-hydroxycrinamidine, isolated from CL, had not antioxidant or inhibitory activities. This indicates that CL exerts its activities due to complex action of many compounds contained in the extracts. Thus, the aqueous, flavonoid and alkaloid extracts naturally contain different type of chemical compounds or different ratios of complex active compounds which results in their differences in the mechanism of their actions.

Because macrophages present different polarization states that have distinct pro-and anti-tumour functions in response to environmental stimuli, we investigated the phenotypic and functional characteristics of monocytes/macrophages after 24 hours of treatment with CL extracts. We particularly studied the modulation of the ability of macrophages to produce ROS ($O_2^{\bullet-}$ and radical hydroxyl) via NADPH-oxidase. Indeed, the oxidative burst is crucial in the microbicidal and tumoricidal functions of different macrophages subtypes. In the study, both alkaloid and aqueous extracts inhibited the oxidative burst in macrophages stimulated with PMA and zymosan. Furthermore, macrophages induced by zymosan showed much stronger inhibition than the macrophage response to PMA. This suggested that zymosan receptors (such as TLR-2 and dectin-1, characteristic markers of M2 macrophages) were down-regulated on the surface of macrophages. Therefore, this macrophage subtype, polarized after treatment with alkaloid or aqueous extracts, was weakly able to mediate phagocytosis of an opsonized yeast containing a □glucan and mannan such as zymosan.

When macrophages were polarized by fraction F, flavonoid, alkaloid and aqueous extracts, we observed dissociation between the respiratory burst stimulated by fMLP and that stimulated by TPA and zymosan. These results suggest that the aqueous and alkaloids extracts on the one hand, and fraction F and flavonoid extract on the other hand, differently modulate the expression of fMLP and zymosan receptors in monocytes/macrophages.

In particular, the enhanced ROS production in macrophages was induced by the ligand fMLP which suggested the formyl peptide receptor (FPR) was up-regulated on the surface of polarized macrophages. FPR plays important roles in innate immunity and host defences via chemotaxis, phagocytosis and the generation of ROS (Gemperle et al., 2012). The activation of the FPR2 receptor limits CCL2 activity, resulting in reduction in macrophage infiltration associated with tumors, suppression of the polarization of TAMs as well as moderation of tumour growth (Liu et al., 2013). Additionally, FPR1 up-regulation is required for host responses to bacterial and viral infection (Gemperle et al., 2012). The increase in FPR1 and FPR2 expression on the surface of macrophages leads to the differentiation of macrophages towards proinflammatory M1 macrophages which enhances the anti-tumour host response (Gemperle et al., 2012), (Liu et al., 2013). Therefore, three extracts (fraction F, flavonoid and alkaloid extracts) were able to induce FPR receptor expression on the surface of polarized macrophages and this could be disadvantageous for tumour progression.

In the gene expression part of the study, high production of pro-inflammatory cytokines (IL1-β, TNFα, IL-6) was observed early on (Fig. 6) which was characteristic of M1 or M2b polarization (Hao et al., 2012). In addition, peritoneal macrophages treated with the aqueous or flavonoid extracts of *C. latifolium* expressed any proteins encoding for markers of M2 macrophage activation, including the mannose receptor (MR) (Fairweather et al., 2009), CD36 (Sindrilaru et al., 2011), dectin-1 (Lefevre et al., 2010), CD11b and TGFβ (Fairweather et al., 2009) (Fig. 7). This finding indicated that the macrophages differentiated towards M1 macrophages in the samples treated with aqueous extract. As M1 macrophages are microbicidal and tumoricidal a stimulation provoking M1 polarization could be considered as a good potential cancer therapy. This is known as macrophage mediated tumor cytotoxicity (MTC) (Deborah, 1982). Thus, the aqueous extract of *C. latifolium* was able to activate macrophages to differentiate into M1, resulting in the suppression of cancer cell growth (section *3.6*.). Furthermore, it was not a surprise when the total alkaloid extract showed direct inhibition of tumour cells (Fig. 3B) as well as indirect suppression via activation of macrophages (Fig. 9B) since some pure alkaloid compounds extracted from CL have been reported to have anti-tumour activity and to be immuno-regulatory (Ghosal et al., 1985; Ghosal et al., 1986).

Conclusion

In summary, CL extracts inhibit the proliferation of lymphoma cells in multiple ways. Aqueous extracts suppress lymphoma cell growth via activation of M1 macrophage polarization without any direct inhibitory activity. This result is important because it suggests a mechanism for the anti-cancer action of aqueous extracts that have been used for a long time in traditional medicine. The alkaloid extract shows a suppressive effect on lymphoma cells in both direct and indirect pathways via activation of macrophages. In addition, the flavonoid extract directly inhibits tumour cell proliferation without macrophage mediation. Further studies will be needed in order to establish a causal relationship between M1 induced polarization of macrophages by aqueous and alkaloid extracts and the activation of their anti-tumour function.

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Keywords: *Crinum latifolium* · Macrophages · Immunomudulation · Reactive oxygen species · Traditional medicine

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3. Conclusion

Différents extraits isolés de *C. latifolium* exercent leur activité anticancéreuse par différentes voies: 1) inhibition de la production de ROS par les cellules cancéreuses grâce à leurs capacités antioxydantes; 2) activation des macrophages en macrophage activé de type M1, 3) induction de l'expression du récepteur de peptide formyle sur la surface des macrophages polarisés qui inhibe la production de peptides chimiotactiques des monocytes-1 (MCP-1 ou CCL2) par les cellules tumorales. Cet effet se traduit par une diminution de l'infiltration des macrophages due aux tumeurs, la répression de la polarisation des macrophages associés aux tumeurs (TAM) et une modération de la croissance tumorale. Il a également été demontré que les macrophages sont activés sous la forme M1 qui est favorable à une suppression tumorale. En outre, les propriétés anticancéreuses des extraits de *C. latifolium* ne sont pas simplement basées sur l'activité de quelques composés. Il s'agit d'une activité résultant de la complexité du mélange moléculaire : i) les extraits de flavonoïdes et les extraits aqueux présentent des activités anti-oxydantes ; l'extrait aqueux n'inhibe pas directement la croissance des cellules tumorales, mais active les macrophages pour qu'ils exercent un effet inhibiteur; ii) bien que l'extrait alcaloïde ait une capacité antioxydante faible dans les essais « DPPH » et « béta-carotène », il montre de manière significative des propriétés antioxydantes sur monocytes humains. Ces effets se présentent de deux façons, un effet inhibiteur direct sur les cellules tumorales et, un effect indirect, par l'intermédiaire d'une activation des macrophages, démontré par l'induction de l'expression du récepteur au peptide formyle à la surface des macrophages polarisés.

Même si l'extrait aqueux ne montre aucune activité anticancéreuse directe, il supprime la croissance des cellules tumorales par l'activation de la polarisation des macrophages en M1. Ce résultat est important car il explique le mécanisme de l'activité antitumorale de l'extrait aqueux et justifie ainsi l'usage traditionnel de cet extrait depuis des milliers d'années.
L'extrait des flavonoïdes présente une activité anti-proliférative sur les lymphomes fortement sans activation des macrophages ou toute autre aide de médiateurs. Ce résultat n'a jamais été signalé avant.

3. Conclusion

Different extracts isolated from *C. latifolium* exert anti-proferative activities in different pathways: 1) inhibition of the ROS produced by cancer cells through their antioxidant capacities; 2) activation of the macrophages into classically activated macrophages M1; 3) induction of the formyl peptide receptor expression on the surface of polarized macrophages which inhibits monocyte chemoattractant peptide-1 (MCP-1 or CCL2) produced by tumor cells. This effect results in the decrease of macrophage infiltration associated with tumor, the suppression tumor-associated macrophages (TAMs) polarization and the moderation tumor growth. It also indicates that macrophages are activated toward M1 which are favorable for tumor killing. In addition, the anticancer properties of *C. latifolium* are not simply based on some compounds. The activities result from the molecular complexity of the extracts because: i) the flavonoid and aqueous extracts show antioxidant activities but the aqueous extract does not directly inhibit tumor cell growth, it needs to activate macrophages to exert inhibitory effect; ii) although alkaloid extract has a weak antioxidant in the “DPPH” and “beta-carotene beaching” assays, it significantly shows antioxidant properties in human monocyte assay. It also presents supressive effects on two ways, a direct inhibitory effect on tumor cells and an indirect way via activation of macrophages, indicating by the induction of formyl peptide receptor expression on the surface of polarized macrophages.

Even though aqueous extract does not show any direct anticancer activities, it suppresses tumor cell growth through the activation of macrophages polarization toward M1. This result is important because it explains the mechanism of antitumor activity of aqueous extract and justifies its use in traditional way for thousand years.

The flavonoid extract strongly exhibits antiproliferative activity on lymphoma without macrophages activation or any assistance of mediators. This result was never reported before

CONCLUSION GENERALE

Examiner les propriétés redox de composés ou de mélanges moléculaires, pour aboutir à une meilleure compréhension de leurs mécanismes d'action dans des modèles de pathologies liées au stress oxydant, a constitué le cheminement de ce travail de thèse.

Deux sources moléculaires, de synthèse et naturelle, avec des propriétés biologiques avérées, ont été choisies pour ce travail: la série des indolone-*N*-oxydes (INODs), à fortes activités antipaludiques, et les extraits ou substances isolées de *Crinum latifolium*, à réputation traditionnelle sur la longévité et la réduction des symptômes de certains cancers.

La série des indolone-*N*-oxydes (Partie A) comprenait, au démarrage de ce travail, une centaine de représentants issus d'un travail antérieur de pharmacomodulation, et des données physico-chimiques et biologiques parmi lesquelles les valeurs de CI_{50} obtenues *in vitro* sur le modèle de l'érythrocyte humain parasité par *Plasmodium falciparum*. Les activités *in vivo* des meilleurs hits et têtes de séries étaient également disponibles. Plusieurs avancées avaient également été faites sur la compréhension des mécanismes d'action de ces molécules. Ces pistes identifiées ont montré que ces molécules possèdent un mécanisme d'action original en interférant avec les mécanismes de régulation des interactions entre cytosquelette et membrane de la cellule hôte sans affecter celle du globule rouge sain. Les modifications membranaires conduisent à une déstabilisation puis à une vésiculation intense de l'érythrocyte parasité entraînant la mort du parasite par éclatement et destruction du globule rouge. Ce mécanisme de déstabilisation est déclenché par l'activation d'une voie de phosphorylation sensible au stress oxydant qui implique des Syk-kinases et affecte les interactions membrane-cytosquelette. Les travaux antérieurs avaient démontré la bio-réductibilité, enzyme et thiol dépendante, des INODs au sein du globule rouge. Ces premières données sur le mécanisme d'action introduisant la notion de stress oxydant et de bio-réductibilité des molécules actives, nécessitaient d'étudier plus en détail le caractère oxydo-réductible des INODs par des approches physico-chimiques ainsi que leurs interactions avec les composants biochimiques érythrocytaires. Ainsi, dans ce travail, les méthodes électrochimiques et RPE couplée ou non, ont été associées aux méthodes HPLC et LC-MS. Plusieurs résultats majeurs ont été obtenus :

- Les INODs (37 représentants étudiés) présentent, en milieu aprotique, deux étapes de réduction situées autour de - 0,68 ± 0,2 V et - 1,45 ± 0,2 V vs SCE. La première étape de réduction est réversible pour tous les composés étudiés dans les conditions de l'étude et

attribuée à la réduction de la double liaison N=C, tandis que la seconde étape de réduction, irréversible, a été attribuée à la réduction du carbonyle.

- Par couplage électrochimie-RPE, l'analyse a confirmé que cette première réduction donne lieu à la formation d'un radical cationique relativement stable. Au cours du temps, la protonation du radical a lieu sur le carbone en alpha de la fonction nitrone et non sur le groupe NO. L'observation, par RPE, de cet intermédiaire radicalaire, obtenu par simple réduction à un potentiel compatible avec celui du milieu intracellulaire, est une étape importante dans l'analyse du mécanisme d'action de ces molécules antipaludiques. Cet intermédiaire radicalaire pourrait être le premier signal redox généré par ces dérivés au sein du globule rouge, signal redox activant plusieurs cascades d'évènements redox, qui, in fine, génèrent un stress oxydant fatal au globule rouge parasité, fragilisé par Plasmodium, alors que le globule rouge sain résiste à ces évènements redox.

- Une relation entre le comportement électrochimique et la structure des indolone-*N*-oxydes a pu être établie pour les composés ayant des substituants électro-attracteurs. L'insertion d'un groupe électro-attracteur sur le carbone en alpha du groupement *N*-oxyde facilite la réduction du composé. Les composés ayant les potentiels de réduction les plus faibles en valeur absolue sont également les plus actifs in vitro (CI_{50}). Précédemment, nous avions montré que ces composés sont bio-réductibles dans le globule rouge. Les résultats de ce travail sont ainsi concordants avec cette première démonstration puisque l'activité antiplasmodiale est d'autant plus élevée que le composé est plus facilement réductible.

- Les études d'interaction entre INODs et les composants biochimiques érythrocytaires montrent que ces composés i) n'entrent pas dans la sphère de coordination du métal dans le complexe fer-hème à l'inverse de la chloroquine; ii) ne génèrent pas d'espèces radicalaires fer-dépendantes comme le fait l'artémisinine; iii) génèrent des intermédiaires radicalaires après réduction à un électron en milieu polaire ; iv) ne peuvent pas piéger les radicaux libres après réduction. Le caractère pro-oxydant des INODs pourrait être l'initiateur du signal redox qui active les Syk-kinases et induit une hyperphosphorylation de la protéine AE1 (bande 3) dans le globule rouge parasité.

Ces résultats sont en bon accord avec le fait qu'aucune corrélation n'a été observée entre les réponses des INODs et celles de la chloroquine sur isolats frais humains de *P. falciparum*

alors qu'*in vitro* les réponses des INODs et de la dihydroartémisinine sont significativement et positivement corrélées. Ces résultats suggèrent des mécanismes d'action différents ou des cibles moléculaires différentes pour ces trois classes de composés antipaludiques. Ces hypothèses sont renforcées par le fait que les INODs sont équipotentes contre les deux types de souches de *P. falciparum*, résistante et sensible à la chloroquine.
L'ensemble de ces résultats soutient l'hypothèse d'un mécanisme d'action original des INODs à l'origine de leurs activités antipaludiques et encouragent la poursuite des travaux afin de déterminer leurs propriétés pharmacocinétiques et pharmacodynamiques nécessaires aux études pré-cliniques.

Crinum latifolium **(Partie B)** est une plante traditionnellement utilisée en Asie pour ses propriétés anti-inflammatoires et anticancéreuses. Les composants moléculaires ont été largement décrits dans la littérature tandis que les mécanismes par lesquels ils agissent sont peu connus. Dans ce mémoire il a été choisi d'étudier les propriétés des extraits de cette plante par une approche globale, c'est-à-dire par l'identification des activités d'un extrait total ou d'une fraction plutôt que par une approche moléculaire en travaillant sur les composés purs. Cependant, pour des raisons de comparaison, une molécule (6-hydroxycrinamidine) a été également introduite dans les essais pour représenter les alcaloïdes majoritaires de cette plante. Dans une première étape, plusieurs extraits, fractions et molécules ont été isolés. Dans une deuxième étape, il a été choisi d'étudier les capacités d'oxydo-réduction des extraits et des fractions obtenues par différentes méthodes afin d'identifier les fractions à forte activités anti-oxydantes et d'éventuelles fractions à caractère pro-oxydant. Dans une troisième étape, ces extraits ont été étudiés pour leur capacité à activer, voire différencier les macrophages.
Les essais sur les propriétés redox ont montré que l'extrait total de flavonoïdes présente les activités antioxydantes les plus élevées, avec des CI_{50} égales à 107.36 mg/L et à 1010.2 mg/L, obtenues, respectivement, dans l'essai DPPH et dans l'essai de décoloration du beta-carotène. Une activité antioxydante était attendue pour cette fraction. Par contre cette activité antioxydante est remarquablement élevée étant donné qu'il s'agit d'un extrait brut. Aucune autre fraction n'a présenté une telle activité antioxydante. D'autre part, aucun extrait n'a exprimé de caractère pro-oxydant (capacité des molécules à être réduites). La molécule pure isolée (6-hydroxy-crinamidine) et introduite à titre de comparaison dans les essais ne présente aucune activité redox.
Les différents extraits et fractions isolées de *Crinum latifolium* inhibent la prolifération des lymphomes par différentes voies. Les extraits aqueux suppriment la croissance des

lymphomes via l'activation de la polarisation des macrophages de type M1. Ce résultat est important car il explique l'activité anticancéreuse de l'extrait aqueux utilisé depuis très longtemps de façon traditionnelle. L'extrait d'alcaloïdes présente un effet suppressif sur les lymphomes selon deux voies, une directe et une indirecte via l'activation des macrophages. L'extrait de flavonoïdes inhibe directement la prolifération des cellules tumorales, sans médiation des macrophages; ce résultat est original car une telle activité n'avait pas été rapportée dans la littérature jusqu'à présent. Ce travail a démontré que les extraits de flavonoïdes et d'alcaloïdes activaient deux voies différentes conduisant à l'inhibition de la prolifération des cellules tumorales. Le composé pur (6-hydroxycrinamidine) isolé de *C. latifolium* ne présente aucune activité sur les différents modèles étudiés. Les perspectives de ce travail pourraient être d'étudier les synergies potentielles de ces extraits (flavonoïdes, alcaloïdes et extraits aqueux) de *C. latifolium* sur les modèles *in vitro* mises en œuvre dans ce travail ainsi que sur d'autres modèles.

En conclusion, des avancées ont été obtenues sur les mécanismes d'action des indolone-N-oxydes à activités antipaludiques et des extraits de *Crinum latifolium* à réputations traditionnelles, antiinflammatoire et anticancéreuse. Un certain nombre de relations entre propriétés redox et activités biologiques ont pu être obtenues, par des approches moléculaire (substance pure) ou globale (extraits naturels) sur des modèles biochimiques et cellulaires. Dans les deux cas, les résultats ouvrent des perspectives pour poursuivre les travaux de recherche.

GENERAL CONCLUSION

Studying the redox properties of molecular compounds or mixtures, leading to a better understanding of their mechanisms of action in models of pathologies linked to oxidative stress, has been the progress of this work.

Two molecular sources, synthetic and natural, with proven biological properties, were chosen for this work: the series of indolone-*N*-oxides (INODs) with high antimalarial activities and extracts of *Crinum latifolium* or isolated substances with traditional reputation on longevity and on reducing the symptoms of certain cancers.

At the start of this work**, the INODs series (Part A)** included hundred representatives from a previous pharmaco-modulation work, physic-chemical and biological data including the IC_{50} values obtained from the human erythrocyte model parasitized by *Plasmodium falciparum*. The activities *in vivo* of the best hits and leads were also available. Several advances were also made in the understanding of the mechanisms of action of these molecules. Tracks identified showed that these molecules have a novel mechanism of action by interfering with the mechanisms regulating interactions between the cytoskeleton and the membrane of the host cell without affecting those of healthy red blood cells (RBCs). The membrane modifications cause its destabilization and its vesiculation which lead to the death of the parasite and the destruction of the RBCs. This mechanism of destabilization is triggered by the activation of a phosphorylation process sensitive to oxidative stress involving Syk-kinases and affecting membrane-cytoskeleton interactions. Previous work in the laboratory had demonstrated the bio-reducibility of INODs, enzyme and thiol dependent, inside the RBCs. These first data on the mechanism of action, introducing the concept of the oxidative stress and bio-reducibility of the bio-active molecules, required to study in more details the redox character of the INODs, by physic-chemical approaches, and their interactions with the biochemical components of the erythrocyte. Thus, in this work, electrochemical methods, coupled or not to EPR, were associated with HPLC and LC-MS. Several major results were obtained:

- INODs (37 representatives studied) present, in an aprotic medium, two steps of reduction located around - 0.2 V ± 0.68 and - 1.45 ± 0.2 V vs. SCE. The first reduction step is reversible for all tested compounds under the conditions of the study and assigned to the reduction of the

double bond C = N, while the second reduction step, irreversible, was attributed to the reduction of the carbonyl.

- By coupling electrochemistry to EPR, the analysis confirmed that the first reduction gives rise to the formation of a radical cation relatively stable. Over time, the protonation of the radical takes place on the carbon in alpha position to the nitrone function and not on the NO group. The EPR observation of this radical intermediate, obtained by reduction at a potential compatible with an intracellular environment, is an important step in the analysis of the mechanism of action of these antimalarial drugs. This radical intermediate could be the first redox signal generated by these derivatives in the RBC, activating several redox signals in cascade that ultimately generate a fatal oxidative stress to parasitized RBC, weakened by Plasmodium, while healthy RBCs resist to these redox events.

- A relationship between the electrochemical behavior and the chemical structures of INODs has been established for compounds with electron-withdrawing substituents. Inserting an electron withdrawing group on the alpha carbon of the *N*-oxide function facilitates the reduction. Compounds with the lowest reduction potentials are the most active *in vitro* (IC_{50}). Previously, we showed that these compounds are bio-reducible in RBCs, parasitized or not. The results of this work are consistent with this first demonstration since the antiplasmodial activity is higher when the compound is more easily reducible.

- Interaction studies between INODs and biochemical components of the erythrocyte show that these compounds i) are not included in the coordination sphere of the metal in the iron-heme complex unlike chloroquine; ii) do not generate iron-dependent free radical species such as does artemisinin; iii) generate radical intermediates after one-electron reduction in polar medium which is reversible; iv) cannot trap free radicals upon reduction. This pro-oxidant character of INODs could be the initiator of a redox signal that activates Syk-kinases and induces a hyperphosphorylation of the protein AE1 (band 3) in the parasitized erythrocyte.
These results are in good agreement with the fact that no correlation was observed between the responses of INODs and those of chloroquine on human fresh isolates of *P. falciparum in vitro*. Responses of INODS and dihydroartemisinin were significantly and positively correlated. These results suggest different mechanisms of action and molecular targets for these three different classes of antimalarial compounds. These assumptions are reinforced by

the fact that INODs are equipotent against both strains of *P. falciparum*, resistant and sensitive to chloroquine.
Taken together, these results support the hypothesis of an original mechanism of action of INODs behind their antimalarial activities and encourage further work to determine their pharmacokinetic and pharmacodynamic properties necessary for pre-clinical studies.

***Crinum latifolium* (Part B)** is a plant traditionally used in Asia for its anti-inflammatory and anticancer properties. Molecular components have been extensively described in the literature but the mechanisms by which they act are not well known. In this work it was decided to study the properties of the extracts of this plant through a comprehensive approach, that is to say, by identifying activities of total extracts or fractions rather than by a molecular approach working on pure compounds. However, for comparison purposes, a pure molecule (6-hydroxycrinamidine) was also introduced into the assays, as representative of alkaloids which are the most abundant molecules in this plant. In a first step, several extracts, fractions and pure molecules were isolated. In a second step, it was decided to study the redox capacities of extracts and fractions obtained by different methods to identify the fractions with high antioxidant activities and any fractions with pro-oxidant character. In a third step, these extracts were studied for their ability to activate or differentiate macrophages.
Tests on the redox properties showed that the total flavonoid extract presents the highest antioxidant activities with IC_{50} values equal to 107.36 mg / L and 1010.2 mg / L, obtained, respectively, in the DPPH test and the bleaching beta-carotene assay. Although antioxidant activity was expected for this fraction, it can be noted that its antioxidant activity is remarkable high considering that it is a crude extract. No other fraction has presented such antioxidant activity. On the other hand, no extract has expressed pro-oxidant character (ability to be reduced). The isolated pure molecule (6-hydroxycrinamidine) introduced for comparison in testing shows no redox activity.

The different extracts and isolated fractions of *Crinum latifolium* inhibit lymphoma proliferation by different routes: 1) inhibition of the ROS produced by cancer cells through their antioxidant capacities; 2) activation of the macrophages into M1; 3) induction of the formyl peptide receptor on the surface of polarized macrophages which inhibits the expression of monocyte chemoattractant peptide-1 (MCP-1 or CCL2) by tumor cells, leading to reduce the amount of macrophage infiltration associated with tumor, suppress the polarization of tumor-associated macrophages (TAMs) and moderate tumor growth. The

anticancer properties of flavonoid and alkaloid extracts are complex because i) the flavonoid and aqueous extracts show antioxidant activities but the aqueous extract does not directly inhibit tumor cell growth, it needs to activate macrophages to exert inhibitory effect; ii) although alkaloid extract has a weak antioxidant in the DPPH and beta carotene beaching assays, it significantly shows antioxidant properties in human monocyte assay. It also shows a direct inhibitory effect on tumor cells and on activation of macrophages into M1. The aqueous extracts suppress cell growth via the activation of macrophages polarization of type M1. This result is important because it explains the anticancer activity of the aqueous extracts been used in traditional ways for a long time. The extract of alkaloids has a suppressive effect on lymphoma in two ways, one direct on tumor cells without mediation through macrophages or any mediates and one indirect via activation of macrophages and induction of formyl peptide receptor expression on the surface of polarized macrophages, resulting in suppression of TAMs and differentiation of macrophages into M1. Flavonoid extract directly inhibits the proliferation of tumor cells without mediation of macrophages and this result is original because such activity has not been reported in the literature so far. This work has shown that extracts of flavonoids and alkaloids activate two different pathways leading to the inhibition of tumor cell proliferation. The pure compound (6-hydroxycrinamidine) isolated from *C. latifolium* has no activity on the different models studied. The perspectives of this work could be to study the potential synergies of these extracts (flavonoids, alkaloids and aqueous extracts) of *C. latifolium in vitro* models used in this work as well as other models.

In conclusion, progress has been made on the understanding of the mechanisms of action of antimalarial INODs and of *Crinum latifolium* with anti-inflammatory and anticancer reputations. A number of relationships between redox properties and biological activities have been obtained by molecular approaches (pure substance) or global (natural extracts) on biochemical and cellular models. In both cases, the results open perspectives for further research steps.

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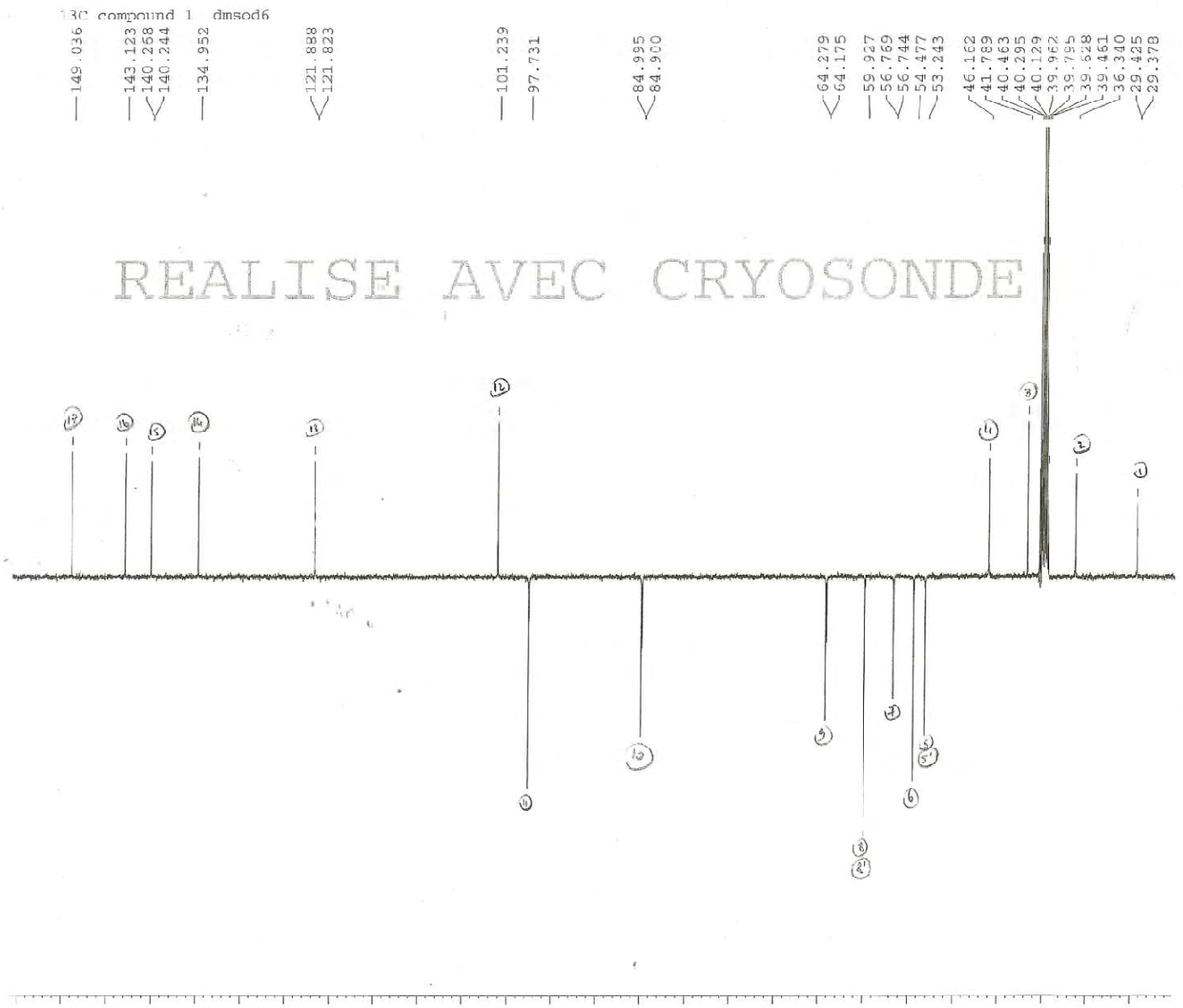
VI. ANNEXES



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DE	6.00 usec
TE	298.0 K
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P2	27.60 usec
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PL1W	141.32804871 W
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PL12	27.00 dB
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PL12W	0.08069114 W
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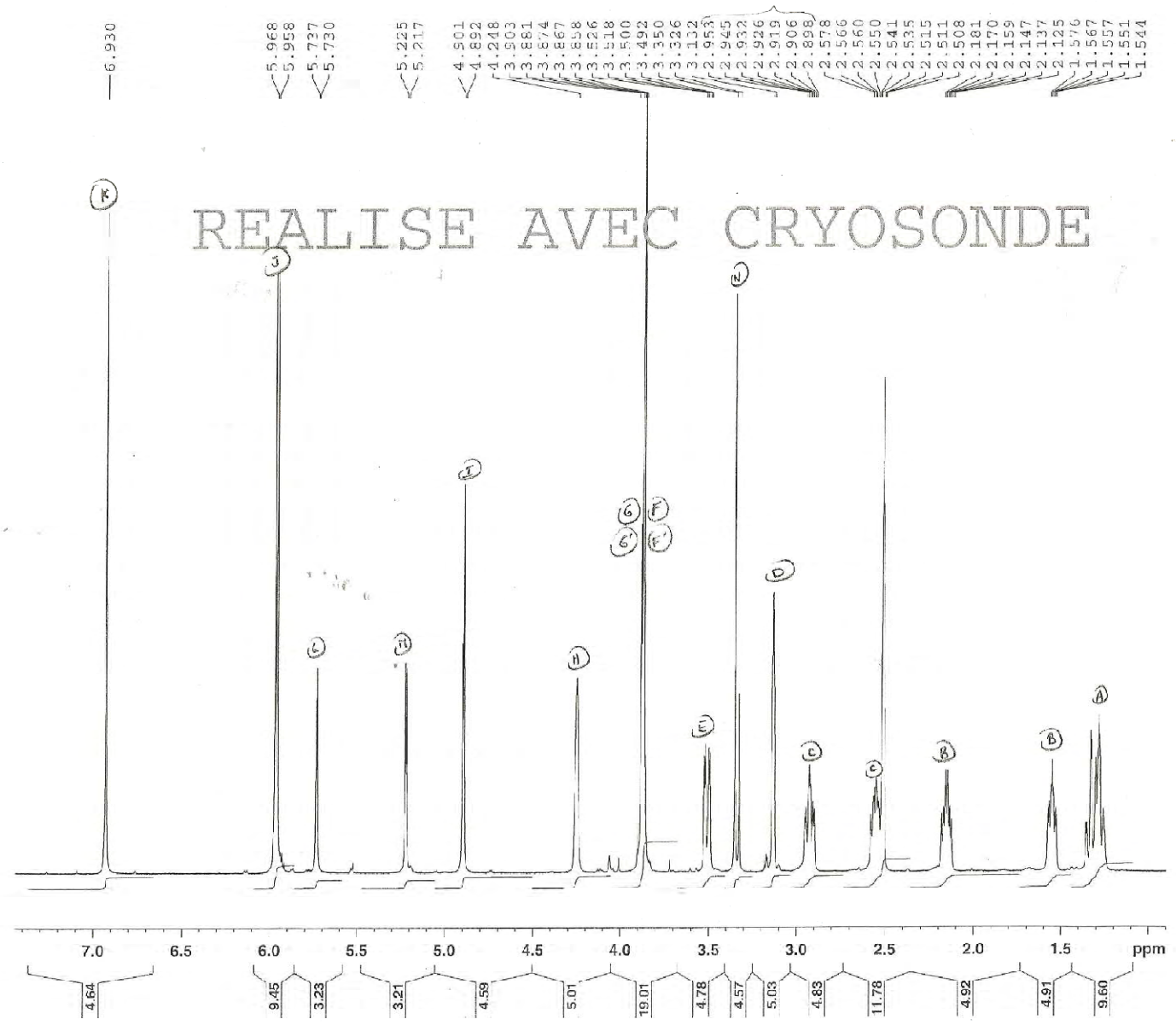

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RG	4
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NUC1	1H
P1	8.00 usec
PL1	7.00 dB
PL1W	8.06911469 W
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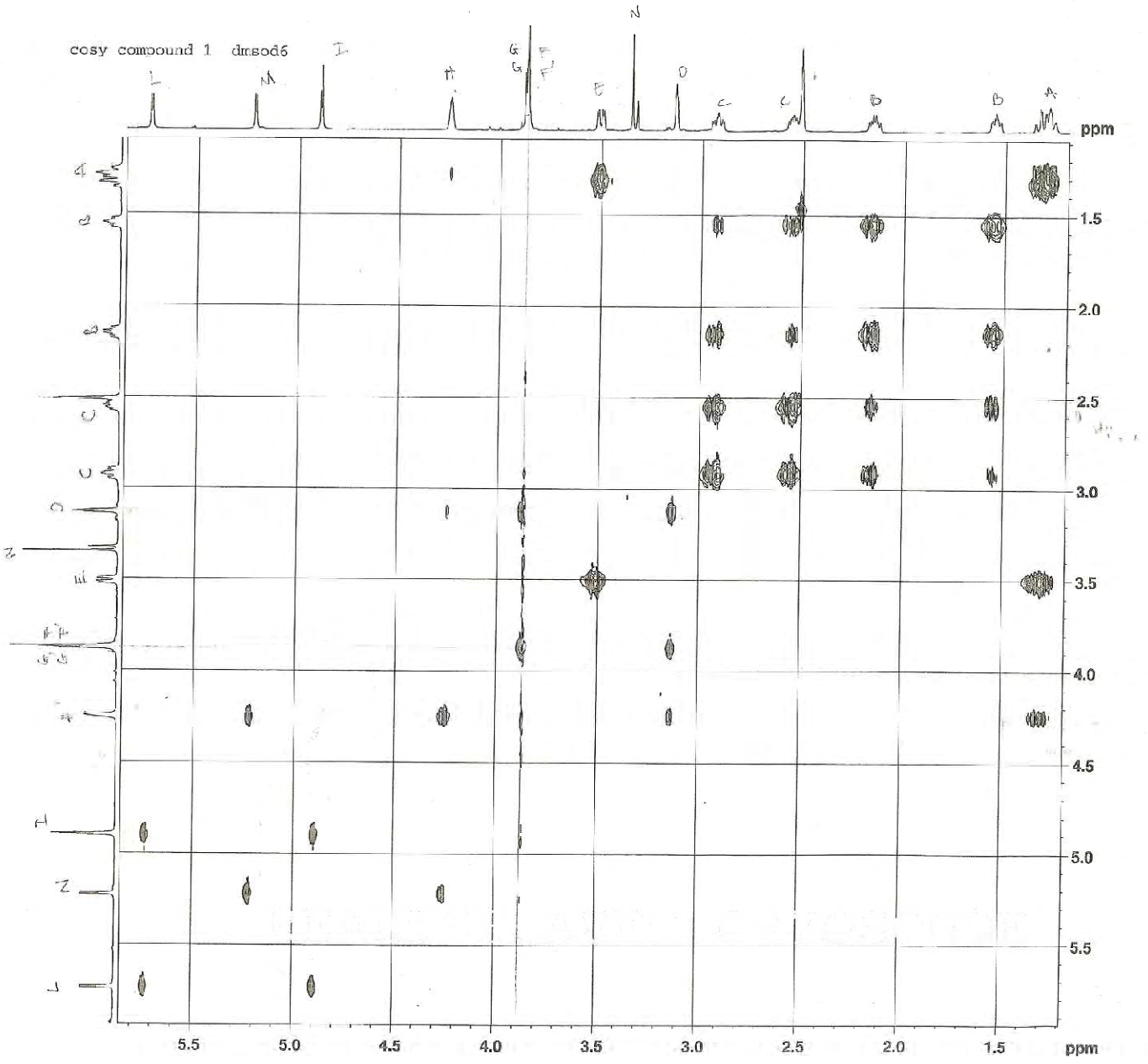


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AQ                  0.5113556 sec
RG                        16
DW                   124.800 usec
DE                     6.00 usec
TE                   298.0 K
D0                  0.00011398 sec
D1                  5.00000000 sec
D13                 0.00300000 sec
D16                 0.00020000 sec
IN0                 0.00024960 sec

======== CHANNEL f1 ========
NUC1                       1H
P1                      8.50 usec
P2                     17.00 usec
PL1                     7.00 dB
PL1W                8.08911459 W
SFO1               500.1317505 MHz

====== GRADIENT CHANNEL =====
GPNAM1               SINE.100
GPNAM2               SINE.100
GPZ1                    10.00 %
GPZ2                    20.00 %
P15                   1000.00 usec
NDO                        1
TD                       256
SFO1               500.1318 MHz
FIDRES              15.650023 Hz
SW                      8.011 ppm
FnMODE                  TPPI
ST                      2048
SP                 500.1300000 MHz
WDW                     QSINE
SSB                        2
LB                      0.00 Hz
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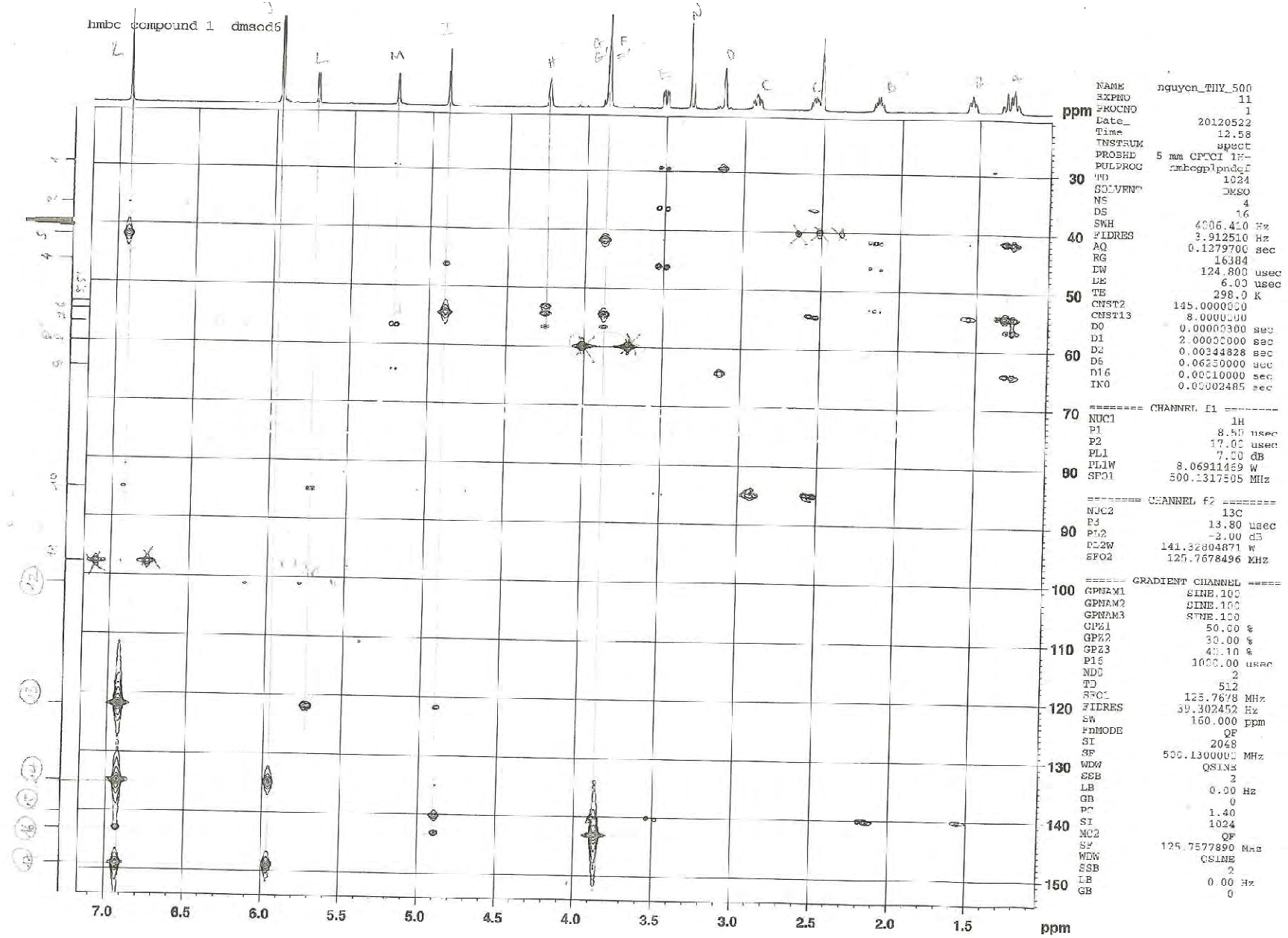
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1.5
ppm
NAME nguyen_TIIY_500
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Date_ 20120522
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SFO1 500.1317505 MHz
======== CHANNEL f2 ========
NUC2 13C
P3 13.80 usec
PL2 -2.00 dB
PL2W 141.32804871 W
SFO2 125.7678496 MHz
====== GRADIENT CHANNEL =====
GPNAM1 SINE.100
GPNAM2 SINE.100
GPNAM3 SINE.100
GPZ1 50.00 %
GPZ2 30.00 %
GPZ3 40.10 %
P16 1000.00 usec
NDO 2
TD 512
SFO1 125.7678 MHz
FIDRES 39.302452 Hz
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SSB 2
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GB 0
PC 1.40
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WDW QSINE
SSB 2
LB 0.00 Hz
GB 0

Auteur : NGUYEN Thi Hoang Yen

Titre : Propriétés redox des indolone-*N*-oxydes et des extraits de *Crinum latifolium* en relation avec leurs propriétés biologiques

Discipline : Chimie-Biologie-Santé

Directrices de Thèse : Pr Françoise NEPVEU et Pr Bach Hue VO THI

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Résumé

Le travail de thèse porte sur l'examen des liens existants entre les propriétés d'oxydo-réduction de molécules de synthèse (indolone-*N*-oxydes) et d'extraits naturels (*Crinum latifolium*) et leurs activités biologiques, respectivement antipaludiques et anticancéreuses.

Les indolone-*N*-oxydes présentent de fortes activités antipaludiques *in vitro* et *in vivo*. Les molécules sont bioréductibles en milieu biologique et le signal redox qu'elles induisent dans le globule rouge parasité permet de détruire la cellule hôte infectée par *Plasmodium falciparum* sans dégrader le globule sain. Les travaux menés à l'aide, notamment, de méthodes biochimiques, électrochimiques et techniques couplées RPE-électrochimie, ont démontré le lien existant entre le potentiel de réduction et l'activité antiplasmodiale des molécules dans la série indolone-*N*-oxyde ainsi que le rôle joué par différents composants érythrocytaires. Les travaux ont également permis de différencier les mécanismes d'action de ces composés comparativement aux antipaludiques de référence, chloroquine et artémisinine.

Les extraits de *Crinum latifolium* sont largement utilisés en Médecine Traditionelle en Asie, notamment au Viet Nam, pour leurs effets bénéfiques sur la longévité et leurs activités anticancéreuses dans le cas du cancer de la prostate, notamment. Les mécanismes d'action de ces extraits ne sont pas encore bien élucidés. En partant de l'examen des propriétes redox (capacité de réduction, caractère pro-oxydant), les travaux ont permis d'établir que plusieurs extraits sont capables d'activer les macrophages et d'inhiber la prolifération de certaines cellules du lymphome (EL4-luc2). D'autres extraits activent la differenciation des macrophages de type M1.

Mots clés: anticancéreux, antipaludéens, *Crinum latifolium*, indolone-*N*-oxydes, propriétés redox.