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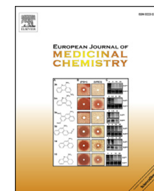

Original article

Antiplasmodial activity: The first proof of inhibition of heme crystallization by marine isonitriles

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A B S T R A C T

Over a decade ago Wright et al., proposed a putative antiplasmodial mechanism of action for marine isonitriles (**1**, and **3**–**6**) and isothiocyanate (**2**) that involved interference in heme detoxification by *Plasmodium falciparum* thus inhibiting the growth of the parasite. In the current paper we describe the successful down scaling of Egan's β-hematin inhibition assay for analyses of small quantities of marine natural products as potential β-hematin inhibitors. The modified assay revealed for the first time that the most active antiplasmodial marine isonitrile **4** (IC_{50} 13 nM) totally inhibited β-hematin crystallization while **5** (IC_{50} 31 nM) and **6** (IC_{50} 81 nM) showed inhibition at lower levels. A cursory *ab initio* molecular dynamics investigation into the relative stabilities of bonded complexes between isocyanate, isothiocyanate and isonitrile groups with the iron center of heme supported our findings that these marine metabolites do indeed interfere with biocrystallization of heme.

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1. Introduction

Each year over 2.2 billion people are exposed to the threat of *Plasmodium falciparum* malaria, resulting in approximately 500–660 million clinical cases and 1–3 million deaths, mostly of children in central and southern Africa [1–5] Consequently, malaria may be considered the pre-eminent parasitic tropical disease and one of the top three global, communicable diseases. The widespread resistance of *P. falciparum* to anti-malarial prophylactics e.g., chloroquine, has necessitated the ongoing search for new anti-malarials and, without a viable malaria vaccine in place, synthetic or natural product based chemoprophylaxis and chemotherapeutic interventions appear to be the only current treatment options [5,6].

The evolution of *P. falciparum*'s resistance to the current armory of anti-malarial drugs requires the development of new chemical entities to combat this disease. Many marine mono- and bicyclic-sesquiterpenes and tri- and tetra-cyclic-diterpenes, possessing isonitrile, isocyanate and/or isothiocyanate functionalities have been shown to possess anti-plasmodial activity, with several exhibiting low nano-molar levels of activity against *P. falciparum* [7,8]. One of the first models proposed to explain the anti-plasmodial activity of these compounds suggested the interference of these compounds in the parasite's heme detoxification pathway [8,9]. In this model a key electrostatic interaction between the iron centre of heme and the isonitrile functionality was proposed. From an *in vitro* study of the heme binding properties of six marine sesquiterpenes and diterpenes bearing either isonitrile or isothiocyanate functionalites (**1**–**6**) we provide evidence to suggest that inhibition of heme crystallization is not common to all marine isonitriles and isothiocyanates with only **4** completely inhibiting the crystallization of heme in a well-established β-hematin inhibition assay. We initiated an exploratory study to probe the binding motif between these marine metabolites and heme and present this as Supplementary data.

Following invasion of a healthy erythrocyte by the *P. falciparum* parasite, rapid growth and eventual release of the parasite from the infected erythrocyte, *P. falciparum* is capable of digesting up to 80% of the hemoglobin within the red blood cell within a 48 h period [10]. The degradation of hemoglobin takes place in *P. falciparum*'s lysosomal food vacuole and is facilitated by the action of aspartic proteases (plasmepsins), [11] a cysteine protease (falcipain 2), [12] and a metallopeptidase [13]. The major by-products of this

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degradation are free-heme [ferriprotoporphyrin IX, (FP)] moieties and hydrogen peroxide. The FP results in colloidal osmotic instability in *P. falciparum* that requires neutralization or removal of this by-product by the parasite, [9,14] while the hydrogen peroxide acts as a source of oxidative toxicity [15]. The parasite protects itself from the majority of the FP through a process of sequestration that involves dimerization of FP into an inert crystalline form of heme known as hemozoin, and partly through non-enzymatic degradation processes [9,16–18]. It has been shown that up to 95% of the heme released from the digestion of the host's hemoglobin, is biocrystallized into hemozoin [19,20]. Chloroquine and a number of other quinoline anti-malarials inhibit the formation of hemozoin and Buller et al., [21] have proposed, through crystallographic studies with synthetic β-hematin (indistinguishable from naturally occurring hemozoin), that a family of non-covalent quinoline drug specific binding sites are located at the developing crystal face associated with fastest crystal growth. The binding of quinolines to these binding sites prevents the sequestration of further heme-dimer (HD) to afford inert hemozoin [17]. Whilst resistance to quinoline anti-malarial drugs, that target the heme detoxification pathways within the food vacuole, is well-entrenched in *P. falciparum*, resistance to chloroquine-type anti-malarials appears to be linked to mutations in the genes responsible for the expression of *P. falciparum*'s trans-membrane multi-drug transporter proteins (e.g., *Pf*CRT) rather than changes in the heme detoxification process itself, that is a chemically fixed target and therefore not subject to mutation [22,23]. The attractiveness of the heme target therefore cannot be ignored as the process of heme detoxification is an integral process in the life of both drug resistant and non-drug resistant *P. falciparum* and will always be necessary for its survival.

Marine invertebrates are a widely recognized source of novel potential anti-malarial secondary metabolites [24]. Amongst these are a group of naturally occurring sesquiterpenes and diterpenes (**1**–**6**), originally isolated from the marine sponge *Cymbastela hooperi*, that possess characteristic isonitrile and isothiocyanate functionalities [8,25]. Three of these compounds (**4**–**6**) exhibited potent *in vitro* anti-plasmodial activity at the nano-molar level [9].

Wright et al., used the *in vitro* antiplasmodial activities of **5**–**18**, quantitative structure activity relationship (3D-QSAR) and receptor modeling methodologies to construct a putative hydrophobic pharmacophore for this cohort of marine compounds [9]. They further linked this hypothetical pharmacophore to heme from the results of a series of molecular modeling, mass spectrometry and optical activity studies of selected marine isonitrile secondary metabolites in the presence of heme moieties [9]. From these studies they concluded that isonitrile functionalities have the ability to coordinate with the iron atom in the centre of heme, thus replacing the hydroxyl moiety in hematin or aqua/hydroxyferriprotoporphyrin IX (the facile metabolic oxidation product of heme). They further postulated that the binding of the iron to the isocyano carbon of the marine natural product would prevent the formation of the peroxidase-like ferryl Fe (IV) intermediate complex, thereby hindering H_2O_2 removal processes.

Since the original studies undertaken by Wright et al.,[9,25] a new modeling study has been undertaken by Wright et al.,[26] the results of which point towards the most active compounds binding to hemoglobin in a cavity close to heme and not directly bound to the iron of heme as first proposed [26]. The identification of hemoglobin as a possible targeted stemmed from the observation that these marine isonitriles showed inhibition in the photosynthetic process and antibacterial activity, possibly due to binding of these ligands to the heme-containing proteins cytochromes b and f, essential in the carbon fixation cycle of photosynthesis, and inhibition of heme-oxygenase found in Gram -ve bacteria [26].

The unfortunate lack of solid experimental data to support this deduction in the form of a crystal structure or the like still made these deductions speculative. Also, the use of an antiplasmodial compound which targets hemoglobin does not make a very attractive drug target as it may have adverse effects on basic human biological functioning.

In this paper we return to the original model for the interaction between marine isonitriles and heme to explore the capacity of a select group of marine isonitriles and an isothiocyanate (**1**–**6**) to bind to heme and disrupt the formation of β-hematin *in vitro*. Two commercial heme compounds are available *viz.* hematin [either H_2O–Fe(III)PPIX or HO–Fe(III)PPIX depending on the pH] and hemin [Cl–Fe(III)PPIX] in which H_2O- and Cl-ligands are bound to the central iron atom of heme respectively (FP has no ligand bound to the iron atom at the centre of the porphyrin ring). *P. falciparum* is able to facilitate the dimerization of heme to form a heme-dimer and then undergo further elongation, often also referred to as sequestration, to form a crystalline substance known as hemozoin. β-Hematin, shown by three different research groups to be identical in all respects to hemozoin, [18,27–29] is a synthetic analog of hemozoin prepared from either hematin or hemin.

The discovery that β-hematin (the synthetic equivalent of hemozoin) may be synthesized in a laboratory has initiated a debate as to whether the crystallization *in vivo* is a spontaneous chemical or biological facilitated process, as many of these *in vitro* methods are conducted in supraphysiological conditions, such as high temperature (60 °C) in the Egan's β-hematin inhibitory assay (EBHIA) and pyridine hemichrome inhibition of β-hematin (Phi-β) or lengthy incubation times (18–24 h) in both the heme-crystallization inhibitory activity (HPIA) and the β-hematin inhibitory activity (BHIA) assays. However, the consensus view is that the sequestration of heme into hemozoin *in vivo* occurs by a multifarious mechanism, where lipids may play a major role in mediating the crystallization of heme to hemozoin [30,31]. A summary of known mechanisms of heme-crystallization and valid criticisms of each mechanism is presented in Table 1. We have included an overview of biological assays used to identify inhibitors of heme detoxification. This overview can be found in Supplementary materials for this manuscript.

Egan and co workers developed two methods for determining a compound's ability to interfere with the formation of hemozoin, namely the Phi-β [32] and β-hematin inhibition assays (which we have abbreviated to EBHIA) [33]. In both methods the β-hematin crystals are grown in an acetate buffer (pH 5.0) in the presence of potential inhibitors, thus providing a means to assay the inhibitors ability to hinder the biocrystallization of heme under similar pH conditions to those found in the plasmodial food vacuole. We used the EBHIA to study the inhibition of heme crystallization, in the presence of **1**–**6**, because of the accessibility of these methods with regards to availability of reagents, analytical techniques, cost and incubation times. Analysis of the *in vitro* formation of hematin has progressed recently to incorporate detergent [34] or lipid catalyzation, [35,36] thus theoretically aligning experimental conditions with physiological conditions. At the time this investigation was carried out the reported use of lipids in a β-hematin inhibition assay was in its infancy and therefore the addition of lipids was omitted from our experimental procedure. Repetition of these experiments with added lipids as suggested by contemporary practice is not possible given that no further supplies of several of the naturally occurring compounds screened are available. While others have reported alternative methods for determining the presence of β-hematin, including fluorometry [37] and HPLC, [38] spectroscopy still remains the most convenient and widely used analytical technique.

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