Discovery of Novel Antimalarial Agents: *In vitro* Biological Evaluation of a Series of Rhodanine Analogues

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Abstract

Discovery of novel antimalarial agents is in urgent demand due to the global emergence of Plasmodium falciparum resistant malaria against first-line drugs. In line with the demand, we studied in vitro antimalarial properties of a series of rhodanine analogues (C-C13) for their bioactive potential against P falciparum. Based on the analysis of screening results and structure-activity relationship (SAR), C11 was found to be a promising bioactive hit molecule (IC_{50}: 4.755 μM).

Keywords: Antimalarial agents, *Plasmodium falciparum*, Rhodanine.

Introduction

The World Malaria Report 2023 states that there were estimated 249 million cases and 608,000 deaths reported worldwide in 2022 due to Malaria[14]. It is a parasitic disease transmitted to humans through the bite of a female *Anopheles* mosquito which is a causative agent of the infection[11]. The five species of parasites that cause human malaria are *Plasmodium falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi*. Notably, *P. falciparum* is the primary species of severe malaria and is associated with a greater case fatality rate due to its resistance to antimalarial drugs[2].

The first-line treatment for malaria involves 3 days of Artemisinin-based combination therapy (ACT) where the artemisinin derivative significantly reduces the number of parasites during the first three days while the partner drug eradicates any remaining parasites, curing the infection. However, artemisinin-resistant *P. falciparum* has emerged and significantly contributed to treatment failure of existing ACT, compounded by partner drug resistance in ACTs[6]. Hence, the need for new antimalarial treatments and drug combinations for clinical use is critical. The end goal is to develop an ideal antimalarial therapy that inhibits all stages of parasite development, acts on novel targets, shows effectiveness at a single dose, shows no cross-resistance with other antimalarial drugs and is clinically safe[7].

Rhodanine (2-thioxothiazolidin-4-one) is a five-membered heterocyclic compound with a thiazolidine core[15] that belongs to a known group of biologically active compounds[10]. Over the years, rhodanine derivatives have garnered significant attention in medicinal chemistry as a valuable scaffold because of their vast spectrum of biological activity[14], chemical diversity and various interactions with different targets[4].

According to numerous *in vitro* studies reported, lead compounds with antimalarial properties were developed by substituting distinct functional groups on the rhodanine ring structure[12]. Furthermore, existing research has conclusively shown that compounds containing rhodanine core exhibit enhanced antimalarial potency compared to the antimalarial drugs that have become resistant[9]. Based on the structure-activity relationship (SAR) analysis of identified lead compounds, the antimalarial activity has significantly increased when the rhodanine scaffold is substituted with a benzylidene group, heterocyclic ring, electronegative group (fluorine, chlorine, bromine etc.), electrostatic group, hydrophobic group, hydrogen-bond donor, or combination with pharmacologically active compounds[16].

Hence, the substitution of different functional groups at the C-5 and N-3 positions of the rhodanine ring is essential for the development of novel rhodanine derivatives[13]. Furthermore, several studies have investigated the roles of compounds containing a tosylurea chemotype where the compounds were reported to exhibit high inhibitory activity against *P. falciparum* in the sub-nanomolar range[1]. Therefore, our study proposed to explore a rational approach to determine the *in vitro* antimalarial activity against *P. falciparum* of a series of 5-benzylidene-rhodanines with a tosylurea chemotype substitution at the N-3 position as prospective antimalarial agents.

Additionally, a structure-activity relationship (SAR) analysis was also derived to understand the role of chemical structural features contribution to their compounds' antimalarial properties. Therefore, the test compounds that were earlier synthesised, purified and characterised by physical and spectral methods (Figure 1) were screened through the SYBR green antimalarial activity screening bioassay[3]. IC_{50} values were determined to calculate the potency with respect to their antimalarial activities.

Material and Methods

Medium preparation: An incomplete malaria medium was prepared by dissolving 1 g glucose, 1.35 g sodium

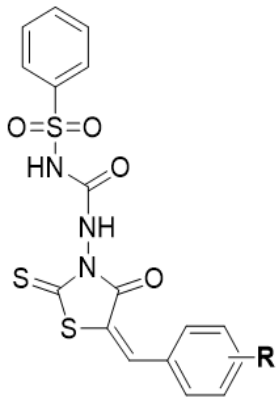
bicarbonate (Thermo Fisher Scientific, USA), 3g of 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) (VWR Life Sciences, USA) in Hyclone RPMI 1640 (Thermo Fisher Scientific, USA) which was then supplemented with albumax II (0.5%, w/v), 30µg/mL gentamicin (Life Technologies, USA) and 50µg/mL hypoxanthine (Merck, USA).

***P. falciparum* parasite culture and maintenance:** The parasite culture method was similar to those previously described by Khaw et al.[6] The *P. falciparum* lab strain 3D7 (provided by University Malaya) was grown in washed human O^+ red blood cells and maintained in culture with the prepared Malaria Complete Medium (MCM). The parasites were incubated in sealed flasks and maintained *in vitro* at 37°C and were subjected to microaerophilic conditions [5% O_2, 5% CO_2 and 90% N_2], supplied in the form of a dedicated gas cylinder (Linde, Germany). Every two days, the medium was changed and the level of parasitaemia was checked. Blood smears were prepared, fixed in methanol and air-dried. The slides were stained with 20% Giemsa for 45 minutes and observed at 100x magnification under oil immersion. Approximately, 1000 red blood cells per smear were counted and the parasitaemia (%) was calculated as:

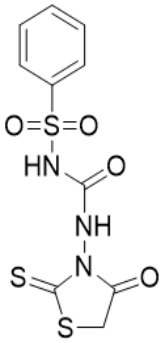
$$\text{Parasitaemia (\%)} = \frac{\text{Number of Parasitised RBCs (iRBCs)}}{\text{Total number of RBCs}} \times 100$$

Preparation of compound dilutions: 100 mM stock solutions of the test compounds (C-C13), which were earlier synthesised and characterised in IMU research laboratories[4], were prepared by dissolving the compounds in 100% dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA). The prepared stock solutions were further diluted in MCM to the desired starting concentrations, after which two-fold serial dilution was performed to achieve concentrations of 100, 50, 25, 12.5 and 6.25 µM. 50 µL of test compounds were pre-dosed in the 96-well flat-bottom black cell culture plate (SPL Life Sciences, Korea) and kept at 4°C to be used within 1 day after preparation.

Malaria SYBR Green Assay: Asynchronous parasites were used, with starting parasitaemia no less than 2.5%. 50 µL of parasitised red blood cells (1% parasitaemia and 5% haematocrit) was added to each well of the pre-dosed 96-well flat bottom black cell culture plate containing test compounds in triplicate at desirable concentrations. DMSO alone without parasitised red blood cells, non-parasitised red blood cells, parasitised red blood cells and artesunate were used as fluorescence control, reference control, negative control and positive control respectively. The final concentration of DMSO in the assay was at 0.1%. Following this, the plate was placed in an airtight Tupperware, gassed with 5% O_2, 5% CO_2 and 90% N_2 and incubated at 37°C for 72 hours. The assay was terminated by freezing the plate at –80°C overnight.



General Structure



Intermediate (C)

Code	R
C1	H
C2	3-OCH_3
C3	2,4-diOCH_3
C4	4-$N(CH_3)_2$
C5	2-Cl
C6	3-Cl
C7	4-Cl
C8	2-F
C9	3-F
C10	4-F
C11	2-Br
C12	3-Br
C13	4-Br

Figure 1: Chemical structures of rhodanine analogues (C-C13)

After thawing for 2 hours, each well was added with 50 µL SYBR green I lysis buffer containing 20 mM tris(hydroxymethyl)aminomethane (TRIS) (1st Base, Singapore), 5 mM ethylenediaminetetraacetic acid (EDTA) (R and M chemicals, UK), 0.008 % (W/V) saponin (Sigma Aldrich, Germany), 0.08 % (V/V) triton^{TM} X-100 (Sigma Aldrich) and 1x SYBR green nucleic acid dye (Invitrogen by Thermo Fisher Scientific). The plates were placed on the Stovall Belly Dancer shaker for 30 minutes at room temperature for thorough mixing. This was followed by measurement of the fluorescence intensities at excitation/emission wavelength = 485/528 nm using the Tecan Infinite 200 Pro M Plex microplate reader. The parasite growth percentages were determined using the formula:

$$\text{Parasite Growth (\%)} = \frac{FL_{sample} - FL_{uRBc}}{FL_{iRBC}} \text{ x } 100\%$$

where FL_{sample} is the fluorescence intensity of treated parasites, FL_{uRBC} is the fluorescence intensity of sample background and FL_{iRBC} is the fluorescence intensity of untreated parasitised RBCs.

Statistical Analysis: The concentration that inhibited parasite growth by 50% (IC_{50}) for each test compound was estimated from a dose-response curve by non-linear regression analysis using GraphPad Prism 10 (GraphPad, CA) software.

Results and Discussion

The antimalarial activity of the compounds showcases a range of potencies influenced by their structural features as shown in figure 2. In total, there were 14 compounds (one intermediate and thirteen 5-benzylidene analogues) screened against *in vitro P. falciparum* malaria. All compounds displayed minimal to greater levels of activity at 100µM concentration. The greater potency recorded only in case of compounds (C11-C13) was further studied for their inhibitory potential. The bromine-containing 5-benzylidene rhodanine derivatives (C11-C13) were recognised for their *in vitro* antimalarial efficacy against (3D7) strains of *P. falciparum*, using the malaria SYBER green I nucleic acid staining dye-based fluorescence (MSF) assay.

The bioassay results were shown in figure 2, with the IC_{50} values determined for each compound (C11-C13). Compounds with an IC_{50} greater than 100 µM were considered inactive, while those with an IC_{50} between 1 and 10 µM were classified as bioactive hit molecules. In this case, C11 (2-bromo-5-benzylidene rhodanine) was identified as the most potent compound, with the lowest IC_{50} of 4.755 µM and a high goodness of fit (R^2) value of 0.8374, indicating a good fit to the dose-response model. C12 (3-bromo-5-benzylidene rhodanine) and C13 (4-bromo-5-benzylidene rhodanine) showed low IC_{50} values of 7.130 and 6.876 µM respectively with good R^2 values indicating active compounds for antimalarial activity. Since no dose-dependent response was observed in the case of other compounds (C-C10) below 100µM, the threshold of the concentration was deemed to be inactive (Figure 2).

The SAR was studied based on the functional group modifications introduced on the 5-benzylidene ring of the rhodanine. There are two sets of groups considering EDG (methoxyl and methyl) and EWG (halogens Cl, F and Br) at different positions (ortho, meta and para). This substitution pattern revealed the importance of type of functional group substitution and also its impact on observed antimalarial activity a range of potencies against *P. falciparum* by all compounds initially at 100µM concentration. The substitution of bromine enhances the antimalarial potency as seen in the case of compounds C11-C13. However, the position on the ring system also plays a major role for bromine to be the most active. The ortho-isomer was found to be the most potent analogue in comparison to the meta and para isomers respectively.

Bromine is one of the halogens that has very weak EWG potential; this is a discriminative property of bromo substitution with respect to the observed antimalarial properties[9]. On the other hand, in terms of electronegativity, it is less than chlorine and fluorine, so it has more stability when compared to other halogen atoms.

Code	IC_{50}(µM)	Code	IC_{50}(µM)
C	>100	**C7**	>100
C1	>100	**C8**	>100
C2	>100	**C9**	>100
C3	>100	**C10**	>100
C4	>100	**C11**	4.755
C5	>100	**C12**	7.130
C6	>100	**C13**	6.876

Figure 2: *In vitro* antimalarial activity of compounds (C-C13) against *P. falciparum*

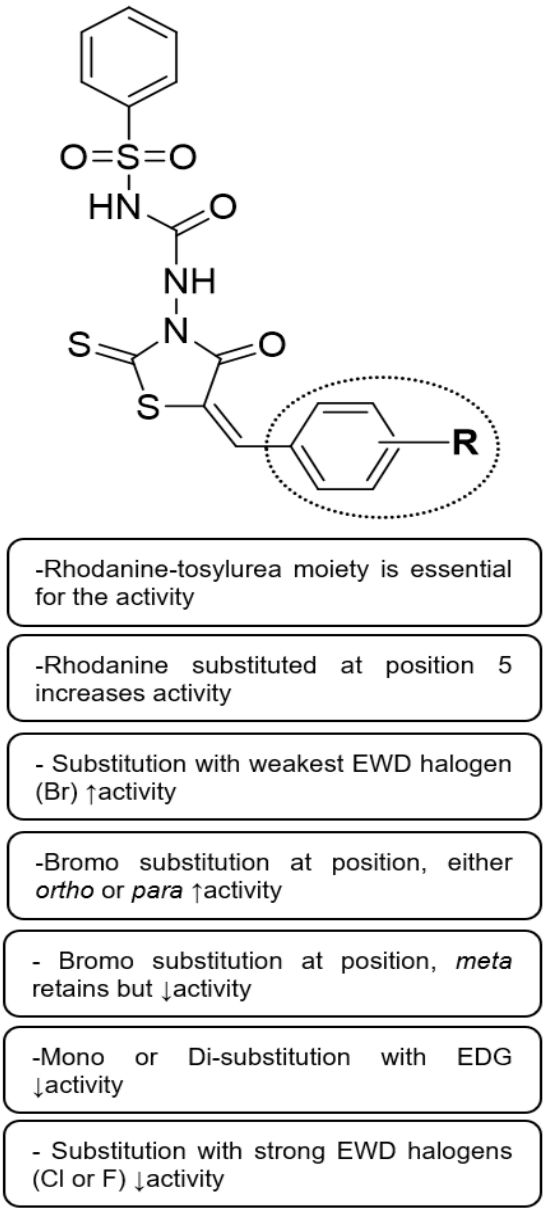


Figure 3: Structure-activity relationship (SAR) analysis of rhodanine analogues (C-C13) with respect to their *in vitro* antimalarial activity against *P. falciparum*.

Our study result aligned with the outcomes obtained in some previously reported studies that state that the EWG substituent on the phenyl ring of rhodanine derivatives plays an important role in the enhancement of antimalarial activity[8]. A close look into the SAR of rhodanines (Figure 3) indicates that the antimalarial activity is more stable for the bromine group at positions ortho and para compared to the meta position. This observation is consistent with the SAR outcomes of a study highlighting that the substitution of halogens at the para position of the phenyl ring in rhodanine derivatives appeared to enhance antimalarial activity[9].

Conclusion

In summary, bromine-containing 5-benzylidene rhodanine derivatives (C11-C13) were identified as the hit molecules that demonstrated a significant range of antimalarial properties based on the *in vitro* antimalarial activity against *P falciparum*, demonstrating potent IC_{50} values ranging from 3.740 to 6.145 µM. Furthermore, the modification of the phenyl ring to other aromatic six membered rings with bromine substitution could be remarkable to optimise the hit molecule to be a lead molecule with a more desirable drug-like property. In continuation, *in vivo* studies in animals are needed to elucidate the molecular mechanism of the chemotype discovered in this study.

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