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Natural product-inspired aryl isonitriles as a new class of antimalarial compounds against drug resistant parasites

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Abstract

Malaria is a prevalent and deadly disease. The fast emerging of drug resistant malaria parasites makes the situation even worse. Thus, developing new chemical entities, preferably with novel mechanisms of action, is urgent and important. Inspired by the complex and scarce isonitrile-containing terpene natural products, we evaluated a collection of easily prepared synthetic mono- and bis-isonitrile compounds, most of which feature a simple, but rigid stilbene backbone. From this collection, potent antimalarial lead compounds against the Dd2 strain using a blood stage proliferation assay were identified. These compounds include **31** (EC_{50} = 88 nM), **41** (EC_{50} = 68 nM), and **43** (EC_{50} = 27 nM). Preliminary SAR information showed that the isonitrile group is essential for the observed activity against the Dd2 strain and the bis-isonitrile compounds in general perform better than the corresponding mono-isonitrile compounds.

Graphical Abstract:

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INTRODUCTION

Malaria is a prevalent and life-threatening disease caused by *Plasmodium*, a genus of protozoan parasites. While billions of US dollars have been dedicated to malaria control and elimination annually, there were still around 228 million cases of malaria worldwide and about 405,000 malaria deaths in 2018, as estimated by the World Health Organization.¹ Of the five different protozoan *Plasmodium* parasites causing human malaria, *P. falciparum* is the deadliest and *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* still result in fatal illnesses. With the success of medicines derived from artemisinin, quinoline, and others, millions of lives have been saved. However, the emergence of drug-resistant strains to the existing antimalarial drugs like the quinolines (chloroquine, amodiaquine, and mefloquine), artemisinin-based combination therapies (ACTs), and the antifolate combination drugs (sulfadoxine and pyrimethamine) in malaria endemic areas, raises an urgent alarm in our fight against malaria and makes the battle more difficult.² Although there are continued attempts to develop malaria vaccines, this endeavor is still far behind what is desired.³ Therefore, developing new chemical entities, preferably with a novel or diverse mechanisms of action⁴ against the *Plasmodium* parasite, is urgently needed in order to address the serious drug resistant problems.

Small molecules derived from natural sources have had a profound influence on modern pharmacopeia. They served either as molecular blueprints for further development, or as an inspiration for creative exploration of structural motifs found within them.⁵ This notion is particularly evident in the case of antimalarial drug discovery. Naturally produced molecules such as artemisinin⁶ (Nobel prize in 2015 awarded to Tu Youyou for its isolation) and quinine,⁷ which served as the starting points for the discovery and development of those life-saving anti-malarial drugs widely used in the frontier treatment for malaria (see previous paragraph). In addition to the sesquiterpene artemisinin, there are among naturally produced compounds a group of isonitrile-containing terpenes with extremely potent antimalarial activity.⁸ As in the case of artemisinin, the terpene skeleton here also provides a scaffolding role on which the important functionality is held. Even though they are structurally distinct among themselves, all these molecules feature one, two or more isonitrile groups in their molecular structures (Figure 1). The isonitrile group has been shown to be essential for the observed antimalarial activity of these terpene natural products. For example, sesquiterpene axisonitrile inhibits the growth of W2 strain with 71 nM EC₅₀ value,⁹ but its close analog axisothiocyanate is around 200 times less potent against the same strain (EC₅₀ = 11.8 μM). While it is difficult to quantitatively ascribe contributions of individual functional groups to biological activities, bis-isonitrile-containing natural products kalihinol A¹⁰ and 7,20-diisocyanoadociane¹¹ both showed increased potency activity against FCR-3 strain (Kalihinol A EC₅₀ = 1.2 nM) and W2 strain (7,20-diisocyanoadociane EC₅₀ = 13 nM).

While, these terpene isonitrile natural products are promising lead compounds for antimalarial drug discovery, their complex structures characterized by the presence of multiple stereocenters and congested ring systems make their structural elaboration — a necessary part of any drug discovery endeavor — particularly difficult. Isolation of these molecules through a painstaking fractionation of natural sources often yields small quantities

of material, precluding any material-intensive studies on absorption, metabolism, distribution, or excretion. Structure-activity relationships could only be performed on molecules following late-stage functionalization, or through engineering suitable biosynthetic transformations; neither task currently can be trivially performed. Decades of effort and ingenuity by synthetic chemists have progressed the attempts at preparing some of these complex molecules via the total synthesis approach, obviating the need for isolation from natural sources and providing more accessible points for SAR studies.¹² However, often lengthy synthetic sequences and low overall yields (primarily due to the mentioned sequence length) undermine their potential as a solution for the supply problem, whereas the idiosyncrasy of employed transformations often hinders the preparation of synthetic analogs needed for SAR studies. There are hints, however, from these synthetic campaigns that the complex structures are not required for the anti-malarial activity of these compounds. Notably, the Vanderwal group recently developed simplified kalihinol analogs with similar activity as the parent natural products.¹³

Upon review of the what is known about the activity of isonitrile compounds against malaria, we decided to explore the potential of greatly simplified isonitrile-containing compounds towards this goal, and to completely dispense with issues around complex molecule synthesis. Specifically, we recently synthesized a collection of aryl isonitrile compounds, most of them featuring a stilbene (diarylethylene) backbone. From this collection of compounds, we have through screening identified compounds with potent antibacterial¹⁴ and antifungal activity.¹⁵ Our most potent compounds (**43** and **31**) also demonstrated *in vivo* efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) in two different mouse models without showing any toxicity to the mice.¹⁶ Our study further showed that these lead compounds are not toxic to mammalian cells at concentrations as high as 128 μ M or 256 μ M. In addition, they exhibited reasonable stability toward microsomal metabolism, which suggests that the isonitrile group is not an inherent liability for their further development in the pipeline.

Inspired by the antimalarial potency of the terpene isonitrile natural products and encouraged by our success in identifying antibacterial and antifungal leads from our collection of simple aryl isonitrile compounds, it seemed reasonable that they may also possess potent antimalarial activity. These molecules are synthetically easily accessed, which, unlike the complex natural product leads, will allow large-scale production and rapid analoguing if required. There are no reports of similar aryl isonitriles as antimalarial agents. We, therefore, evaluated a series of over 40 aryl isonitrile compounds for antimalarial activity against chloroquine-resistant Dd2 strain of *P. falciparum*. Here, we report our evaluation of the antimalarial activities of these aryl isonitriles as well as the corresponding SAR, which led to the identification of **31** (EC_{50} = 88 nM), **41** (EC_{50} = 68 nM), and **43** (EC_{50} = 27 nM) as three most potent compounds.

RESULTS AND DISCUSSION

Chloroquine resistant *P. falciparum*, has been implicated in over 70% of malarial infections; thus, we decided to evaluate the aryl isonitrile compounds for antimalarial activity against the clinically relevant isolates of *P. falciparum*. In total, 46 compounds were evaluated and

the results are presented in Tables 1, 2 and 3. EC₅₀ values against *P. falciparum* were determined using a blood stage proliferation assay as previously described.¹⁷ *P. falciparum* (Dd2 strain) was obtained from the Malaria Research and Reference Reagent Resource Center (MR4). Parasites were maintained with O-positive human blood in an atmosphere of 93% N₂, 4% CO₂, 3% O₂ at 37 °C in complete culturing medium (10.4 gL⁻¹ RPMI 1640, 5.94 gL⁻¹ HEPES, 5 g L⁻¹ albumax II, 50 mg L⁻¹ hypoxanthine, 2.1 g L⁻¹ sodium bicarbonate, 10% human serum and 43 mg L⁻¹ gentamicin). Parasites were cultured in medium until parasitaemia reached 3–8%. For EC₅₀ value determination, a parasite dilution at 2.0% parasitaemia and 2.0% haematocrit was created with medium. 25 µL of medium was dispensed into 384-well black, clear-bottom plates and 100 nL of each compound in DMSO was transferred into assay plates along with the control compound (mefloquine). Next, 25 µL of the parasite suspension in medium was dispensed into the assay plates giving a final parasitaemia of 1% and a final haematocrit of 1%. The assay plates were incubated for 72 h at 37 °C. 10 µL of detection reagent consisting of 10× SYBR Green I (Invitrogen; supplied in 10,000× concentration) in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.16% (w/v) Saponin, 1.6% (v/v) Triton X-100) was dispensed into the assay plates. For optimal staining, the assay plates were left at room temperature for 24 h in the dark. The assay plates were read with 505 dichroic mirrors with 485 nm excitation and 530 nm emission settings in an Envision (PerkinElmer).

The aryl isonitrile compounds in our collection can structurally be classified into four categories: *ortho*-isonitrile, *para*-isonitriles, *meta*-isonitriles, and bis-isonitriles. The first category consists of compounds with a vinyl or aryl substituent *ortho* to the isonitrile group (*ortho*-isonitriles, Table 1). Most of them have low µM EC₅₀ value. For example, the simplest (*E*)-*I*-isocyano-2-styrylbenzene (**1**) has a modest EC₅₀ value (11.53 µM), which was used as the starting point for the following discussions. Addition of substituents on the second aromatic ring (the one without the isonitrile group) influences their antimalarial activity (cf. compounds **2–10**), but the effect was largely not significant, except for compounds **6** and **8** whose activity went below 1 µM. Adding a CF₃ at the *meta* position (**6**) enhanced the EC₅₀ value to 0.27 µM, about 42 times more potent than compound **1**. The potency of the *para*-fluoride-substituted one (**8**, EC₅₀ = 0.56 µM) increased about 20-fold in comparison to compound **1**. Introducing alkyl (compounds **11–13**) or phenyl group (compound **14**) on the double bond increased the potency about 1 to 3 times. The effects of these substitution, however, are not additive. For example, compound **15** contains both the CF₃ substituent on aryl group and the alkyl group on the double bond, but its potency drops to EC₅₀ = 5.41 µM, whereas compound **17** with the same alkyl substitution and the *para*-fluoride similarly loses potency (EC₅₀ = 9.84 µM). Replacement of the second aromatic ring with a pyridine resulted in potency similar to compound **1** (compound **19**, *meta* pyridine, EC₅₀ = 12.98 µM) or in slight improvement of the potency (compound **18**, *ortho* pyridine, EC₅₀ = 7.53 µM and **20**, *para* pyridine EC₅₀ = 3.70 µM). The pyridine ring improves these compounds' physicochemical properties, primarily observed as improved solubility. Additionally, switching from a stilbene backbone to a biaryl backbone does not cause significant potency change, and compounds **21** (EC₅₀ = 6.21 µM) and **22** (EC₅₀ = 10.82 µM) showed EC₅₀ values not very different from those of compound **1**. Most importantly, the isonitrile group is essential for the observed activity against the Dd2 strain. Complete loss of

activity was observed when the isonitrile group was removed (compound **23** vs compound **11**).

The second and third structural classes of aryl isonitriles are *para*- and *meta*-aryl isonitriles, respectively, shown in Table 2 with their EC₅₀ values given in parentheses. In most of the cases, moving the isonitrile group from the *ortho*-position to the *para*-position led to potency increases except for compound **27** (EC₅₀ = 4.69 μM), which was about 8-fold less active than its *ortho*-analog **8** (EC₅₀ = 0.56 μM). Most notably, compound **31** with an EC₅₀ of 88 nM is about 42 times more potent than its *ortho*-analog **20**. Saturation of the rigid double bond linker to a more flexible 2-carbon alkane linker reduced the potency significantly. The corresponding compound **32** (EC₅₀ = 0.56 μM), while still quite potent, is about 6-fold less active than **31**. Neither a furan (**35**, EC₅₀ = 2.66 μM) nor a quinoline ring (**36**, EC₅₀ = 5.25 μM and **37**, EC₅₀ = 6.98 μM) replicated the properties of the pyridine ring and resulted in a loss of potency. Additionally, moving the isonitrile group to the *meta*-position does not offer much advantage except for compound **40** with an EC₅₀ value of 0.69 μM, which is about 19-fold or 8-fold more potent than its *ortho*- and *para*-analogs **19** and **34**, respectively. In the *para* and *meta*-aryl isonitrile groups, we were delighted to identify compound **31** as the best lead with an EC₅₀ value of 88 nM.

The last group of aryl isonitriles as well as the most exciting group were shown in Table 3. They all contain two isonitrile groups, one on each aromatic ring. These molecules were designed to test if the observation made with the bis-isonitrile terpene natural products (vide supra) would also hold with much simpler compound. As we predicated, these compounds did perform remarkably. For example, compound **41** with a combination of a *para* and an *ortho* isonitriles showed an EC₅₀ value of 68 nM, which is over 40-fold more active than **24** with a *mono-para*-isonitrile. Compound **42** (EC₅₀ = 266 nM) with a *para* and a *meta* isonitriles is about 4-fold less potent than compound **41**. Notably, positioning two isonitriles symmetrically at *para* positions of the two aromatic rings produces the most potent compound in this study, compound **43**, with an EC₅₀ value of 27 nM against the Dd2 strain. This is a 100-fold increase of activity in comparison to compound **27** in which one of the isonitriles is replaced by fluorine. Hydrogenation of the stilbene and increased flexibility of compounds **44**, **45**, and **46** decreases their potency about 10 to 15-fold.

Conclusion

By evaluating a collection of easily prepared aryl isonitrile compounds including *mono*-isonitriles and bis-isonitriles, in which we have systematically changed the nature of substituents, and their mutual relationships, we have identified three compounds — compound **31** (EC₅₀ = 88 nM), **41** (EC₅₀ = 68 nM), and **43** (EC₅₀ = 27 nM) — with potent antimalarial activity against the Dd2 strain using a blood stage proliferation assay. Notably, our previous studies¹⁶ showed that compounds **31** and **43** were safe up to the maximum tested concentration of 128 μM against HaCat cells, which gave them a selectivity index (SI) of 1,454 and 4,740, respectively. The structures of these compounds are much simpler than the isonitrile terpene natural products with potent antimalarial activity, which makes them much easier to synthesize and modify. This departure from intricate structures will allow large scale production of these molecules for *in vivo* studies and further evaluations, as well

as quick access of analogs that may improve their drug-like properties. These stilbene bis-isonitriles may also provide useful probe molecules that would shed light on mode of action of these and similar compounds. Wright, Tilley, and co-workers proposed that the isonitrile terpene natural products may act as inhibitors of the heme-detoxification processes by coordinating the heme iron and preventing biocrystallization.¹⁸ Our compounds may function similarly given the fact that the more potent *para*-isonitriles are less sterically hindered and could have a stronger coordination on the iron center than the *meta*- and *ortho*-ones. The bis-isonitriles as well as compound **31** could potentially coordinate on the heme-iron on each end, presumably within an environment of high density of iron. This hypothesis may explain why these dimeric, symmetrical compounds perform significantly better. Our current efforts are directed at exploring this hypothesis and at formulating a more complete understanding of their mode of actions.

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Figure 1.
Selected terpene isonitrile natural products and their antimalarial activity (EC_{50}).

Table 1.

Structure and antimalarial activity (μM) of *ortho*-isonitriles

1 (11.53)	2 (X = F: 3.68) 3 (X = CF ₃ : 4.31) 4 (X = OMe: 21.63)	5 (X = F: 12.44) 6 (X = CF ₃ : 0.27) 7 (X = OMe: 6.45)	8 (X = F: 0.56) 9 (X = CF ₃ : 5.80) 10 (X = Me: 3.07)	11 (R = Et: 10.55) 12 (R = <i>n</i> Bu: 5.25) 13 (R = <i>n</i> Pr: 3.06)

14 (3.62)	15 (5.41)	16 (7.69)	17 (9.84)	18 (7.53)

19 (12.98)	20 (3.70)	21 (6.21)	22 (10.82)	23 (>100)

Table 2.Structure and antimalarial activity (μM) of *para*- and *meta*-isonitriles.

24 (2.77)	25 (1.01)	26 (X = OCH ₃ : 2.27) 27 (X = F: 4.69)	28 (1.62)

29 (2.17)	30 (3.21)	31 (0.088)	32 (0.56)

33 (10.33)	34 (5.18)	35 (2.66)	36 (5.25)

37 (6.98)	38 (5.32)	39 (9.73)	40 (0.69)

Table 3.Structure and antimalarial activity (μM) of aryl bis-isonitriles

41 (0.068)	42 (0.266)	43 (0.027)
44 (0.44)	45 (2.75)	46 (0.22)