3,3'-Disubstituted 5,5'-Bi(1,2,4-triazine) derivatives with Potent *in vitro* and *in vivo* Antimalarial Activity

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Abstract

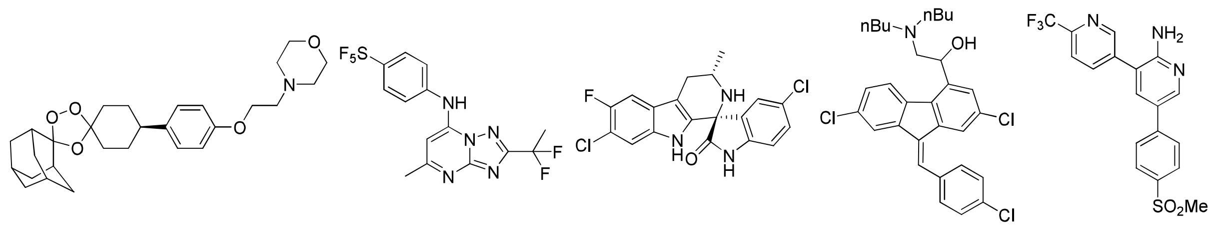
A series of 3,3’-disubstituted 5,5’-bi(1,2,4-triazine) derivatives was synthesized and screened against the erythrocytic stage of *Plasmodium falciparum* 3D7 line. The effects of *S*-alkyl substituents, including those bearing bulky motifs, on 3/3’ positions, replacement of one or both *S*-substituents with *O*-substituents and the introduction of a heteroatom on the alkyl groups at the 3/3’ position were investigated. The presence of *S*- over the *O*-ether group and a side chain with the terminal tertiary amino group at the 3/3’ positions conferred favorable potency. Amongst the unsymmetrical 3,3’-disubstituted dimers evaluated in this study, most offered better potency than the corresponding symmetrical counterparts. The most potent 3,3’-disubstituted dimer, **24**, with an IC_{50} (50% inhibitory concentration) of 0.008 µM, had high *in vitro* potency against *P. falciparum* lines resistant to chloroquine (W2, $IC_{50} = 0.0047 \pm 0.0011$ µM) and artemisinin (MRA1240, $IC_{50} = 0.0086 \pm 0.0010$ µM). Excellent *ex vivo* potency of **24** was shown against clinical field isolates of both *P. falciparum* (IC_{50} = 0.022-0.034 µM) and *P. vivax* (IC_{50} = 0.0093-0.031 µM) from the blood of outpatients with uncomplicated malaria. In the *P. berghei* rodent model, **24** was tolerated at a dose of 32 mg kg^{-1} day^{-1} without any signs of ill-health. Despite **24** being cleared relatively rapidly *in vivo*, it supressed parasitemia in the Peters 4-day test, with mean ED_{50s} values (50% effective dose) of 0.21 mg kg^{-1} day^{-1} and 1.47 mg kg^{-1} day^{-1} following subcutaneous and oral administration, respectively. The disubstituted triazine dimer **24** represents a new class of orally available antimalarial compounds of considerable interest for further development.

■INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* that infect red blood cells (RBCs).[1] If left untreated, malaria can progress to severe disease and death. The introduction of an effective vaccine against malaria remains elusive and chemotherapy thus remains central to malaria control and treatment. Quinoline compounds, such as chloroquine and mefloquine, antifolates and artemisinin derivatives have been critical in the fight against malaria, but parasites have shown development of resistance to these agents.[3]

Over 40% of the world's population is at risk of malaria, with children and pregnant women particularly vulnerable in resource-limited communities.[2] Each year malaria causes more than 400,000 deaths, mostly due to infection with *Plasmodium falciparum* in sub-Saharan Africa.[4] Chemotherapy remains of central importance for the control and ultimate elimination of malaria, with artemisinin combination therapies (ACTs) being the first-line antimalarial treatment in most endemic countries.[4] However, *P. falciparum* resistance to artemisinin is now documented across most of the greater Mekong region, with associated drug resistance emerging to the longer acting partner drugs. The 2016 WHO report emphasizes the importance of continuing investment in research and development to combat malaria and to meet the milestones outlined in Global Technical Strategy (GTS) goals for malaria control and elimination.[5] There is, therefore, an urgent need to develop new medicines able to clear parasites from the blood, prevent recrudescence, and reduce the duration of treatment.

As parasite resistance to conventional antimalarial agents emerge and spread, so must the search for new chemotypes that are capable of killing the parasite through new mechanisms. In this context, the development of OZ439, DSM265, KAE609, KAF156 and MMV390048 are notable (Figure 1).[6] OZ439 (Artefenomel), a relatively newly discovered trioxolane being developed in combination with ferroquine, is being investigated for reduced-frequency dose regimens. This combination is currently in a phase IIb clinical trial, projected to be completed by the end of 2018.[7] DSM265 has been proven to be potentially useful in treating and protecting against malaria in a single dose. Dihydroorotate dehydrogenase (DHODH) – an enzyme in the malaria parasite that is essential for its survival - is selectively targeted by DSM265 without impacting the orthologous human enzyme. Currently, DSM265 is progressing through phase IIa clinical trials.[8] KAE609 (Cipargamin), an inhibitor of P*f*ATP4, is being developed for a single exposure radical cure as it is considered to be very potent, fast acting and retained for more than 8 days in plasma. Cipargamin has completed the first phase IIa clinical trials.[9] KAF156 indues rapid killing of the parasite and mechanistic studies have identified decreased susceptibility associated with mutations in three *P. falciparum* genes: CARL (cyclic amine resistance locus), UDP-galactose and acetyl-CoA transporters.[10] KAF156 has completed phase IIa clinical trials in patients with single species *P. falciparum* or *P. vivax* infections. MMV390048 is a P*f*PI4K inhibitor that has been developed for a single exposure radical cure with the potential of providing chemoprophylaxis.[11] Although these developments are promising, the high attrition rate encountered during clinical development and the inevitability of resistance demand continued efforts to discover and develop new antimalarial drugs.



OZ439 DSM265 KAE609 KAF156 MMV390048

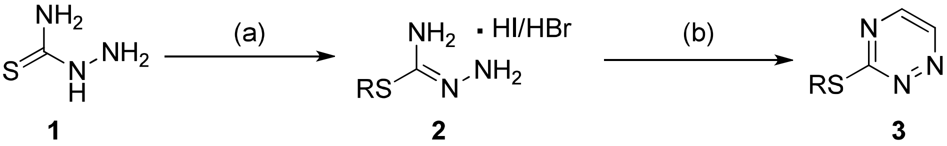
Figure 1. Antimalarial drugs in the pipeline.

Previously, we have reported preliminary observations of related 3,3'-disubstituted 5,5'-bi(1,2,4-triazine) analogues with activity against *P. falciparum*.[12] We now describe our structure-activity relationship (SAR) investigation of this novel chemotype with the most potent compound of this series, **24**, exhibiting an excellent activity against chloroquine and artemisinin resistant *P. falciparum* lines and being efficacious orally at low doses in the Peters 4-day *P. berghei* rodent model. We further provide details of *ex vivo* studies on clinical isolates from patients with malaria in Papua, Indonesia.

■ RESULTS

Chemistry. The synthesis of all dimeric 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s **14–54** was achieved by cyanide-mediated dimerization of equimolar mixtures of the appropriately substituted monomers, as adapted from related work reported by us and others (Scheme 5).[12, 13,14]

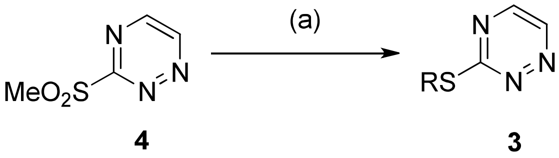
Scheme 1. Synthesis of 3-(alkylthio)-1,2,4-triazines[a]



[a]Reagents and conditions: (a) RBr/NaI or RI, EtOH, 81 °C; (b) aq. glyoxal/aq. $NaHCO_3$, 0 °C then rt.

Monomers utilized included those with thioether (**3**, Schemes 1 and 2), ether (**5**, Scheme 3) and alkyl (**8**, Scheme 4) groups. The synthesis of 3-(alkylthio)-1,2,4-triazine monomers **3** was achieved after *S*-alkylating thiosemicarbazide with alkyl halides, followed by cyclocondensation with glyoxal (Scheme 1). An alternative approach to synthesize 3-(alkylthio)-1,2,4-triazines **3** involved a nucleophilic substitution reaction that was accomplished using 3-(methylsulfonyl)-1,2,4-triazine **4** and mercaptans (Scheme 2).

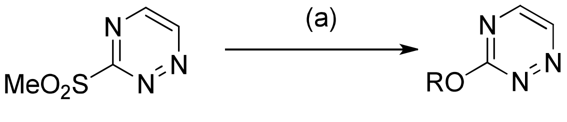
Scheme 2. Synthesis of 3-(alkylthio)-1,2,4-triazines



[a]Reagents and conditions: (a) RSH/Na_2CO_3, ACN, rt.

The synthesis of 3-(alkoxy)-1,2,4-triazine monomers **5** was achieved using the nucleophilic substitution reaction involving alcohols and 3-(methylsulfonyl)-1,2,4-triazine **4** (Scheme 3). This reaction often proved efficient when magnesium alkoxides were employed instead of sodium alkoxides in polar solvents such as DMF.

Scheme 3. Synthesis of 3-(alkoxy)-1,2,4-triazines

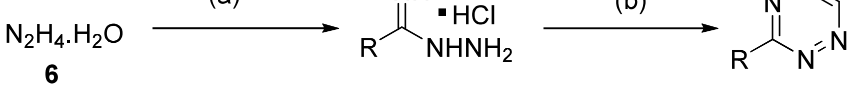


4 **5**

[a]Reagents and conditions: (a) NaH/ROH or MeMgI/ROH, DMF, rt.

The synthesis of 3-alkyl-1,2,4-triazines **8** was accomplished by using procedures adapted from the literature.[13] In this method hydrazine hydrate **6** is reacted amidines to imidohydrazides **7**. The 3-alkyl-1,2,4-triazines **8** can be obtained by a cyclisation reaction involving glyoxal and imidohydrazides **7** (Scheme 4).

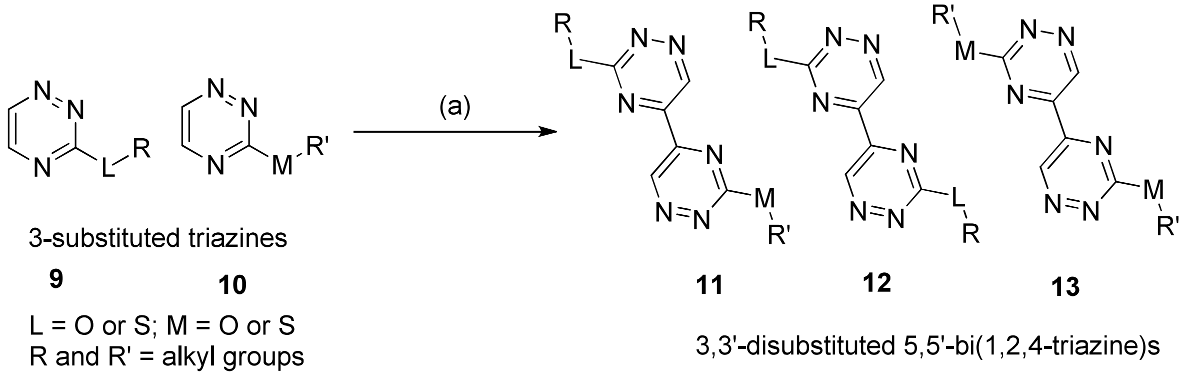
Scheme 4. Synthesis of 3-(alkyl)-1,2,4-triazines



6 **7** **8**

[a]Reagents and conditions: (a) (i) Anhydrous $MgSO_4$ under argon 15min; (ii) acetamidine hydrochloride, 0-5 °C, 4h; (c) aq. gloxal, 0 °C.

Scheme 5. Synthesis of 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s starting with 3-substituted 1,2,4-triazines



[a]Reagents and conditions: (a) aq. KCN, EtOH or Dioxane.

SAR for *P. falciparum*. Testing *in vitro* against the asexual blood stages of *P. falciparum* utilized our optimized and miniaturized assay as previously reported.[15] Relative to the symmetrical thiomethyl dimer **29** (IC_{50} = 0.026 μM), extension of one of the *S*-methyl groups to propyl (**14**, IC_{50} = 0.022 ± 0.004 μM), isopropyl (**15**, IC_{50} = 0.026 μM), isobutyl (**16**, IC_{50} = 0.028 μM), or cyclopentyl (**18**, IC_{50} = 0.015 μM), led to similarly potent compounds. Further branching to the *tert*-butyl (**17**, IC_{50} = 0.053 ± 0.015 μM) or expansion to cyclohexyl (**19**, IC_{50} = 0.041 ± 0.026 μM) led to a marginal drop in bioactivity. The suggestion that steric bulk might become limiting was starkly shown by the relative loss of activity in the *tert*-butylphenyl analogue **20** (IC_{50} = 0.910 ± 0.004 μM) and the 2-pyridyl analogue **21** (IC_{50} = 1.565 ± 0.361 μM). However, it is the *tert*-butyl group in the former and the endocyclic nitrogen atom in the latter that are likely dominating negative influences, rather than an aryl ring per se, as impressive activity was recouped in thioanisole derivative **26** (IC_{50} = 0.086 ± 0.019 μM). Returning to alkyl extensions, incorporation of a heteroatom was less favorable to varying degrees (for example **22** with IC_{50} = 0.951 ± 0.025 μM, **23** with IC_{50} = 0.12 μM, **25** with IC_{50} = 0.11 μM) except for the *N*,*N*-dimethylaminoethyl derivative **24**, which exhibited an impressive IC_{50} of 0.0080 μM. In contrast to the limited tolerance to the nature of the 3-substituent, substitution at the 6-position led to marked abrogation of activity, even with a group as innocuous as methyl (**27** with an IC_{50} 2.4 μM compared with **15** with an IC_{50} of 0.026 μM and **28** with IC_{50} of 1.4 μM compared with **24** with an IC_{50} of 0.0080 μM).

A natural consequence of the synthetic route deployed is provision of homodimers, and a selection of the most interesting of these are shown in Table 1. Here, homodimers with relatively smaller *S*-alkyl groups are generally quite potent, and **29-34** display IC_{50} values that range from 0.017 μM for the *S*-propyl homodimer **31** to 0.12 μM for the *S*-isopropyl dimer **33**. Interestingly, negative steric hindrance effects were markedly increased relative to heterodimeric counterparts, and *S*-cyclopentyl homodimer **36** (IC_{50} = 0.42 μM), *S*-cyclohexyl homodimer **37** (IC_{50} = 1.470 ± 0.170 μM) and especially *S*-*tert*-butyl homodimer **35** (IC_{50} = 3.360 ± 2.376 μM) were markedly less active. Given this, the inactivity of **38** (63% inhibition at 40 μM) was not surprising. In particular, the marked loss in activity observed with the modifications of **18** resulting in **36** suggested a nonsymmetric site of action. Homodimers **39** and **40** were intriguing and while the former showed only a slight activity loss (IC_{50} = 0.41 μM) relative to its heterodimeric counterpart **25**, the latter compound's activity was much weaker (IC_{50} = 1.3 μM) than its heterodimeric counterpart **23** (IC_{50} = 0.12 μM).

Table 1. SAR of 3,3'-Disubstituted 5,5'-bi(1,2,4-triazine)s against *in vitro P. falciparum* proliferation

Cpd	R	R'	IC_{50} (μM) [a]
Unsymmetrical S,S'-alkylthio-5,5'-bi(1,2,4-triazine)			
14	-S-Me	-S-Pr	0.022 ± 0.004
15	-S-Me	-S-*i*Pr	0.026

16	-S-Me	-S-*i*Bu	0.028
17	-S-Me	-S-*t*Bu	0.053± 0.015
18	-S-Me	-S-cyclopentyl	0.015
19	-S-Me	-S-cyclohexyl	0.041± 0.026
20	-S-Me	-S-*para*-*t*Bu-phenylene	0.91± 0.004
21	-S-Me	-S-2-pyridyl	1.565 ± 0.361
22	-S-Me	-S-(CH$_2$)$_2$OH	0.951 ± 0.025
23	-S-Me	-S-(CH$_2$)$_3$OH	0.12
24	-S-Me	-S-(CH$_2$)$_2$NMe$_2$HCl	0.0080[b]
25	-S-Me	-S-(CH$_2$)$_2$OMe	0.11
26	-S-Me	-S-C$_6$H$_4$-*para*-OMe	0.086 ± 0.019
27	-S-*i*Pr	-S-Me, 6-Me	2.4
28	-S-(CH$_2$)$_2$NMe$_2$	-S-Me, 6-Me	1.4
Symmetrical S,S'-alkylthio-5,5'-bi(1,2,4-triazine)			
29	R= R'= -S-Me		0.026
30[c]	R= R'= -S-Et		0.022
31	R= R'= -S-*n*Pr		0.017
32	R= R'= -S-*n*Bu		0.096
33	R= R'= -S-*i*Pr		0.12
34	R= R'= -S-*i*Bu		0.050
35	R= R'= -S-*t*Bu		3.36 ± 2.376
36	R= R'= -S-cyclopentyl		0.42
37	R= R'= -S-cyclohexyl		1.47 ± 0.170
38	R= R'= -S-*para*-*t*-Bu-phenylene		63% at 40 µM

39	R= R’= -S-$(CH_2)_2$-OMe		0.41
40	R= R’= -S-$(CH_2)_3OH$		1.3
	Unsymmetrical S-alkoxy-S’-alkylthio-5,5'-bi(1,2,4-triazine)		
41	-S-Me	-O-Me	0.15
42	-S-Me	-O-Bn	0.039 ± 0.016
43	-S-Me	-O-$(CH_2)_2OH$	0.76
44	-S-Me	-O-$(CH_2)_2$-OMe	0.395 ± 0.025
45	-S-Me	-O-CH_2-CF_3	0.091 ± 0.023
46	-S-Me	-O-$(CH_2)_2$-$SiMe_3$	0.033 ± 0.001
47	-S-Me	-O-$(CH_2)_2$-4-Me-thiazole	0.069 ± 0.024
48	-S-Me	-O-CH(Me)CH_2-OMe	1.1
49	-S-Me	-O-$(CH_2)_2NMe_2$	0.080
50	-S-$(CH_2)_2NMe_2$	-O-Cyclopropyl	0.013 ± 0.001
51	-S-*i*Pr	-O-Me	0.20
52	-S-*i*Pr	-O-$(CH_2)_2$-OMe	0.20
53	-S-*i*Pr	-O-CH(Me)CH_2OMe	0.61
	Symmetrical O,O’-alkyloxy-5,5’-bi(1,2,4-triazine)		
54	R= R’= -O-Me		4.1

[a] Values represent outcomes from one or two experiments (for two experiments, the values represent the mean, with ‘±’ sign representing the standard deviation,) against *P. falciparum* 3D7 strain, erythrocyte stage (chloroquine control, IC_{50} = 0.004 μM). [b] Cytotoxicity was measured for **24**, displaying an IC_{50} against HEK 293 cells of 21 μM, hence a selectivity index of >2600 (puromycin control, IC_{50} = 0.41 μM). [c] See ref. 12.

Our recent success[14] in optimizing a working method to obtain good yields of 3-alkoxy-1,2,4-triazines **5** meant that it was possible for us to synthesize a number of 3-alkoxy-3'-alkylthio heterodimers according to Scheme 5. Our interest in these compounds was influenced by our prior hypothesis that the microsomal instability of **29** could be mediated by the presence of two thioether groups.[14] As the preliminary investigations illustrated, replacement of one thiomethyl group in **29** with a methoxy group resulted in a substantial loss of activity but the resulting heterodimer **41** was nevertheless still relatively potent, exhibiting an IC_{50} value of 0.15 μM against *P. falciparum* 3D7. Furthermore, functional groups with increased lipophilicity in **42**, **43**, and **45-47** such as benzyloxy (**42**, IC_{50} = 0.039 ± 0.016 μM), trifluoroethoxy (**45**, IC_{50} = 0.091 ± 0.023 μM), and trimethylsilylethoxy (**46**, IC_{50} = 0.033 ± 0.001 μM) led to levels of potency approaching that of **29** (IC_{50} 0.026 μM). We were intrigued by **46**, since silicon has unique properties in terms of large covalent radius, enhanced lipophilicity and ability to alter metabolic pathways, and has been put to good use in a myriad of medicinal chemistry applications.[16, 17]

In parallel with the SAR just discussed, higher polarity sidechains such as those in **43** (IC_{50} = 0.76 μM) and **44** (IC_{50} = 0.395 ± 0.025 μM) led to significant activity loss and this was exacerbated by branching, with **48** showing an IC_{50} of 1.1 μM. In agreement is the observation that additional heteroatoms in the sidechain was not necessarily unfavorable; the *N*,*N*-dimethylaminoethoxy sidechain of **49** led to potent activity, with an IC_{50} of 0.080 μM. The related heterodimer **50** was even more potent (IC_{50} = 0.013 ± 0.001 μM), where the basic sidechain was installed in the thioether linkage and the ether alkyl group was cyclopropyl. Analogues **51-53** can be compared with the *S*-isopropyl parent **15**. Here, it is observed that the methoxy counterpart **51** (IC_{50} = 0.20 μM) and methoxyethoxy counterpart **52** (IC_{50} = 0.20 μM) are less tolerated than the methylthio of **15** (IC_{50} = 0.026 μM) and that again, branching of the α-carbon is detrimental, with **53** returning an IC_{50} of 0.61 μM.

ALARM NMR. It is plausible that a thioether group attached to an electron-deficient position in the 1,2,4-triazine harbors some non-specific thiol reactivity. Although not precluding development, it is preferable for drug candidates to not exhibit this trait. ALARM NMR is an industry-developed heteronuclear multiple quantum coherence (HMQC) counter screen to detect non-specific protein thiol reactivity.[18, 19] As shown in Figure 2, **24** did not perturb the La antigen conformation, suggesting it is unlikely to react indiscriminately with protein cysteine thiol residues.

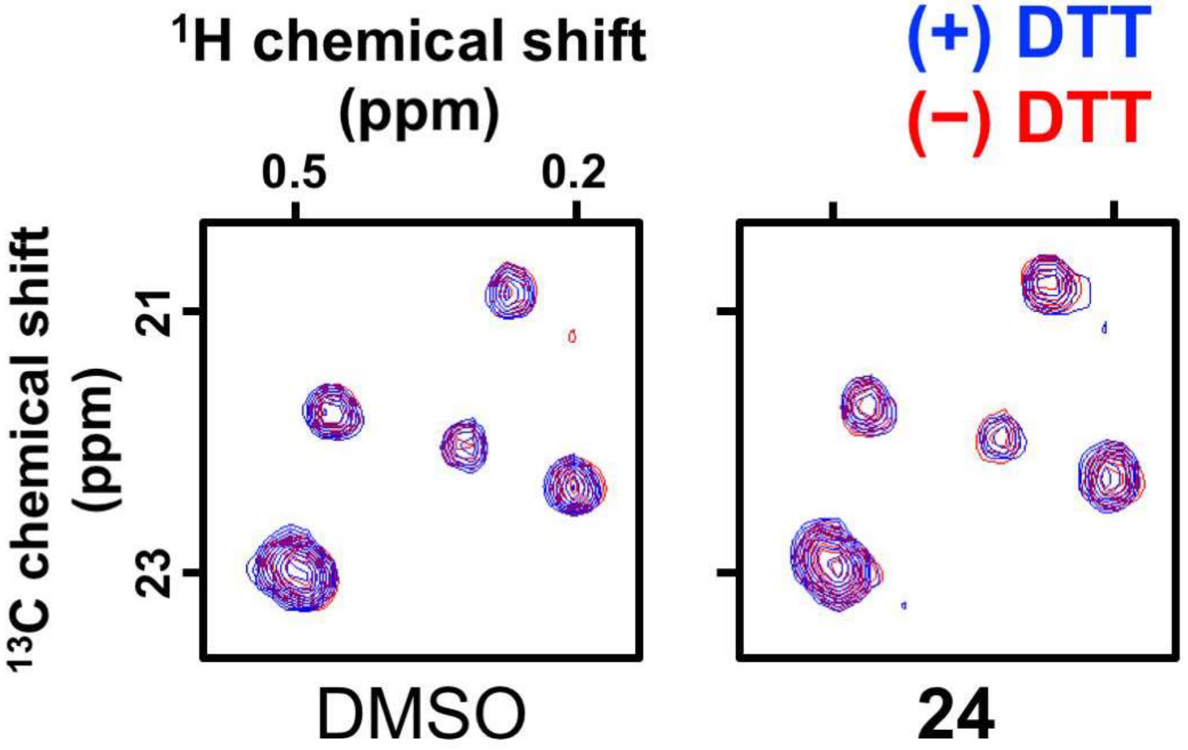


Figure 2. Compound 24 does not perturb the La antigen conformation by ALARM NMR counter-screen for nonspecific compound thiol reactivity. Shown are ^{1}H-^{13}C HMQC spectra of key ^{13}C-labeled methyl groups of the La antigen after incubation with either DMSO or **24**, which was incubated with the La antigen probe in the presence (blue spectra) and absence (red spectra) of excess DTT. Data are normalized to DMSO vehicle control. ALARM NMR-positive compounds perturb the La antigen, as evidenced by pronounced significant peak shifts and/or peak signal attenuations in the absence of DTT.[19]

Physicochemical data and microsomal stability studies. A selection of compounds was subjected to preliminary evaluation of drug-likeness. As shown in Table 2, the molecular weight of each compound was well below 500 g/mol, topological polar surface area ranged from 77 to 118 Å^2, calculated distribution coefficients (clogD) at pH 7.4 ranged from -0.6 to 3.6, and kinetic aqueous solubility in some cases was higher than 100 μg mL^{-1}. These properties are likely to predispose these triazine dimers favorably towards oral bioavailability, albeit the solubility range was wide with two compounds (**32** and **34**) showing poor aqueous solubility. The better solubility for compounds such as **23**, **40**, **49** and **54** can be attributed to a marked influence of the sidechain polarity. It is worth pointing out that some compounds such as **24** and **49**, with a tertiary amino group (–NMe$_2$) attached at the end of the side chain, will be mostly ionized according to pKa calculations (8.2 and 8.4, respectively), aiding solubility. The fact that the solubility of **49** is so much better than that of **24** testifies to the greater polarity of an ether group relative to a thioether group.

Table 2. Key physicochemical parameters and *in vitro* metabolic stability of selected 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s

Cpd	PSA[a]	Partition Coefficients		Solubility (μg mL^{-1})[b]		Degradation half-life (min)[c] [h/m]	*In vitro* CL$_{int}$ (μL / min / mg protein)[c] [h/m]	Microsome-predicted E$_H$[c] [h/m]
		cLog*P*	**cLog*D* 7.4**	**pH 2.0**	**pH 6.5**			
29	77	1.2	1.2	6.3–12.5	3.1–6.3	12 / 3	146 / 655	0.85 / 0.93
31	77	2.8	2.8	3.1–6.3	3.1–6.3	7 /na	252 / na	0.91 / >0.95
32	77	3.6	3.6	< 1.6	< 1.6	19 / 2	92 / 1031	0.79 / 0.96
34	77	3.5	3.5	1.6–3.1	1.6–3.1	46 / 2	38 / 1066	0.60 / 0.96
18	77	2.3	2.3	6.3–12.5	6.3–12.5	4 / na	421 / na	0.94 / >0.95
36	77	3.5	3.5	NA	NA	32 / 3	54 / 516	0.68 / 0.92

23	98	0.5	0.5	50–100	50–100	16 / 5	111 / 350	0.82 / 0.88
40	118	-0.3	-0.3	25–50	60–100	57 / 18	31 / 97	0.55 / 0.68
39	96	0.6	0.6	12.5–25	12.5–25	68 / 14	26 / 124	0.50 / 0.73
24	82	1.0	0.1	6.3–12.5	6.3–12.5	113 / 13	15 / 138	0.38 / 0.75
54	96	-0.4	-0.4	> 100	50–100	> 247 / 189	< 7 / 9	< 0.2 / 0.162
49	91	0.4	-0.6	25–50	50–100	87 / 31	20 / 56	0.44 / 0.55

[a] Calculated using Chem Axon JChem software.
[b] Kinetic solubility determined by nephelometry.
[c] Degradation half-life and *in vitro* intrinsic clearance determined in human (h) or mouse (m) liver microsomes and predicted hepatic extraction ratio calculated therefrom.

Ensuring that compounds are not highly metabolized is important for good oral bioavailability and to achieve a reasonable *in vivo* half-life ($T_{1/2}$). The metabolic stability of compounds shown in Table 2 was evaluated in human and mouse liver microsomes (referred to as HLM and MLM, respectively) at 37 ^{o}C. The metabolic reaction was initiated by the addition of the NADPH-regenerating buffer system. Substrate depletion was used to calculate the first order degradation $T_{1/2}$, *in vitro* intrinsic clearance value (CL_{int}) and the *in vivo* hepatic extraction ratio (E_H), which provides guidance on the structures of 3,3'-disubstituted dimers that are most vulnerable to hepatic metabolism.

From the data in Table 2 it is clear that compounds **18** and **31**, both of which are very potent, were rapidly metabolised in both HLM and MLM. Stability was somewhat improved with an increase in the alkyl chain in going from compound **31** to **32** (HLM $T_{1/2}$ = 19 min), where the *S*-alkyl group increases in length from three to four carbon atoms, although the corresponding IC_{50} values also increased from 0.017 to 0.096 μM. The branching of the side chain appeared to have a favorable effect on the metabolic stability. For example, **34** with an isobutyl group as the *S*-alkyl substituent was more stable in HLM ($T_{1/2}$ = 46 min) when compared with **32** that has an *n*-butyl group as the *S*-alkyl substituent (HLM $T_{1/2}$ = 19 min). However, the increase in the $T_{1/2}$ was also associated with a two-fold reduction in potency. Compounds such as **39** and **40**, with *O*-atoms on the alkyl chain resulted in an HLM degradation $T_{1/2}$ of nearly one hour and a marginally improved MLM $T_{1/2}$ (14-18 min), albeit that these compounds have respective IC_{50} values of 0.408 and 1.347 μM and represent significantly lower potency. Compounds **24** (IC_{50} = 0.0080 μM) and **49** (IC_{50} = 0.080 μM) have significant structural analogy and exhibited higher HLM/MLM $T_{1/2}$ values of 113/13 and 87/31 min, respectively. It is worth noting that both **24** and **49** have good potency against the 3D7 line, with **24** being the most potent in the series of compounds investigated in these studies. In the set of compounds included in this study, **54** is by far the most metabolically stable compound, with a HLM/MLM $T_{1/2}$ greater than 247/189 min but has significantly lower potency (IC_{50} of 4.1 μM). On the basis of these analyses, **24** was chosen for further biological investigation, principally because of its marked potency but also because we deemed its microsomal stability to still be workable.

Drug susceptibility studies *in vitro*: comparison of 24 with chloroquine-resistant and artemisinin-resistant *P. falciparum* lines. Drug resistance is a problem in antimalarial drug therapy. New chemotypes are likely to offer new mechanisms of action and good antimalarial potencies across drug resistant *P. falciparum* lines. Compound **24** was tested in the [^{3}H]-hypoxanthine growth inhibition assay[20] with a 48 h exposure period against the *P. falciparum* D6 line that is sensitive to chloroquine and most other antimalarials, and W2, which is resistant to chloroquine and pyrimethamine. Table 3 shows both IC_{50} and IC_{90} values for the compounds investigated. It is clear that the potency of **24** against the D6 and W2 lines was comparable, with IC_{50} values less than 0.01 μM against both strains, whereas the chloroquine IC_{50} was 10-fold higher in the chloroquine resistant W2 strain compared to D6.

Comparative potency measurements were also carried out against the artemisinin sensitive *P. falciparum* MRA1239 line and the artemisinin-resistant MRA1240 line.[25] MRA1239 is susceptible to dihydroartemisinin and mefloquine but resistant to chloroquine, while MRA1240 is resistant to all of the aforementioned drugs. We note that **24** yielded potent IC_{50} values of 0.0109 μM and 0.0086 μM against MRA1239 and MRA1240, respectively. It is clear from these results using the $[^3H]$-hypoxanthine growth inhibition assay, that **24** has good potency against both chloroquine and artemisinin-resistant strains when tested under these continuous drug exposure conditions.

Table 3. *In vitro* antimalarial activity of 24 and chloroquine against various *P. falciparum* lines[a]

	D6		W2		MRA1239		MRA1240	
Cpd	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)
24	0.0084 ± 0.0008	0.0129 ± 0.0004	0.0047 ± 0.0011	0.0083 ± 0.0012	0.0109 ± 0.0015	0.0155 ± 0.0027	0.0086 ± 0.0010	0.0130 ± 0.0017
Chloro-quine	0.0160 ± 0.0032	0.0196 ± 0.0060	0.150 ± 0.030	0.316 ± 0.010	0.0757 ± 0.0010	0.131 ± 0.002	0.097 ± 0.021	0.217 ± 0.071

[a] Values are mean of two independent experiments with each assay performed with three technical replicates. Results expressed as mean ± SD.

***Ex vivo* drug susceptibility of known antimalarial drugs and 24.** Chloroquine, piperaquine, mefloquine, artesunate and **24** were screened against the chloroquine-sensitive FC27 line and the chloroquine-resistant K1 line. These results were compared with drug susceptibilities measured using *Plasmodium* clinical isolates obtained from patients with symptomatic malaria presenting to a clinic in Papua province in eastern Indonesia. High levels of multidrug-resistant *P. falciparum* and chloroquine-resistant *P. vivax* have been reported in this location previously.[26, 27] Patients with a mono-infection with either *P. falciparum* or *P. vivax* with a peripheral parasitaemia of between 2,000 and 80,000 μL^{-1} were included in the study.

Only blood samples that showed >60% parasites at the ring development stage were used after passing through Plasmodipur® filters for white blood cell depletion. Typically, parasites were incubated with test compound at 37°C for 35–56 h until > 40% of parasites reached the mature schizont stage for the control with no antimalarial. When this *in vitro* assay was performed using the FC27 line, the IC_{50} for **24** was 0.0153 µM, comparable with that for chloroquine (IC_{50} = 0.0139 µM) and much lower than that for piperaquine (IC_{50} = 0.0385 µM) or mefloquine (IC_{50} = 0.0390 µM; Table 4). Against the K1 line, the IC_{50} for **24** was 0.0465 µM, significantly lower than that for chloroquine (IC_{50} = 0.1112 µM), and similar to that for piperaquine (IC_{50} = 0.0508 µM), but higher than that for mefloquine (IC_{50} = 0.0061 µM). As expected, artesunate exhibited good potencies with both FC27 (IC_{50} = 0.0029 µM) and K1 (IC_{50} = 0.0023 µM) lines.

Table 4. *Ex vivo* antimalarial activity of known antimalarial drugs and 24 against laboratory lines and clinical field isolates of *P. falciparum* and *P. vivax*

	P. falciparum lab lines IC_{50} (µM)		*P. falciparum* clinical field isolates		*P. vivax* clinical field isolates	
Compound	**FC27 (CQ^s)**	**K1 (CQ^R)**	**n (%)**	**Median (Range) IC_{50}, µM**	**n (%)**	**Median (Range)) IC_{50}, µM**
Chloroquine	0.0139	0.1112	10 (100)	0.0950 (0.0435-0.3258)[a] p=0.005	11 (100)	0.0484 (0.0193-

						0.2633) *p*=0.003
Piperaquine	0.0385	0.0508	10 (100)	0.0207 (0.0113-0.0570) *p*=0.508	11 (100)	0.0452 (0.0096-0.1096) *p*=0.041
Mefloquine	0.0390	0.0061	10 (100)	0.0187 (0.0100-0.0453) *p*=0.202	11 (100)	0.0440 (0.0191-0.0691) *p*=0.004
Artesunate	0.0029	0.0023	10 (100)	0.0021 (0.0009-0.0052) *p*=0.005	11 (100)	0.0033 (0.0011-0.0089) *p*=0.003
24	0.0153	0.0465	10 (100)	0.0267 (0.0221-0.0346)	11 (100)	0.0158 (0.0093-0.0305)

CQ^S, chloroquine-sensitive laboratory strain
CQ^R, chloroquine-resistant laboratory strain
[a]Comparison with **24** (Wilcoxon rank sum test); *p* values represent the significance of difference with **24**.

In the *ex vivo* drug susceptibility assays using the clinical *P. vivax* isolates, the median IC_{50} for **24** was 0.0158 μM, which was significantly lower than that for chloroquine (IC_{50} = 0.0484 μM), piperaquine (IC_{50} = 0.0452 μM), or mefloquine (IC_{50} = 0.0440 μM). When tested against *P. falciparum* field isolates, the median IC_{50} for **24** was 0.0267 μM, significantly lower than that for chloroquine (IC_{50} = 0.0950 μM), but similar to that for piperaquine (IC_{50} = 0.0207 μM) and mefloquine (IC_{50} = 0.0187 μM). Artesunate was the most potent of the tested antimalarials with a median IC_{50} of 0.0021 μM against *P. falciparum* and 0.0033 μM against *P. vivax* isolates.

Pharmacokinetics (PK) of 24 in mice following intravenous (IV) and oral administration. Although the *in vitro* metabolism half-life of **24** in MLM of 13 min was relatively short, its notably potent *in vitro* antimalarial activity justified further evaluation of *in vivo* potency in a murine malaria model. The pharmacokinetic properties were assessed using non-fasted, male, Swiss outbred mice (24–33 g) that had full access to food and water before and after the sampling period. Compound **24** was administered by a bolus IV injection into the tail vein at a dose of 3 mg kg^{-1} or orally by gavage at doses of 3 and 30 mg kg^{-1}. Groups of two mice were used for each dose. Blood samples were collected into tubes containing heparin and a stabilization cocktail for up to 24 h either by a submandibular bleed (120 μL) or terminal cardiac puncture (600 μL) with a maximum of two samples per mouse. Samples were centrifuged for the collection of plasma which was stored at -20°C until analysis.

After IV administration, measurable (greater than 20 nM) quantities of **24** were observed for only up to 30 min (Figure 3A) indicating very high *in vivo* clearance consistent with the low metabolic stability observed in MLM. Following oral administration at 3 mg kg^{-1}, **24** was detected in only one mouse 30 min after the dose was administered (Figure 3B). At the 30 mg kg^{-1} dose level, measurable quantities of **24** were observed at 60 and 120 min time points for one mouse. To rule out poor permeability as a contributor to the low oral bioavailability, the transport of **24** was assessed across Caco-2 cell monolayers. In both the A-B and B-A directions, **24** exhibited well-defined flux profiles with high mass balance (85% ± 8 for A-B and 90% ± 2 for B-A). Values for P_{app} in the A-B direction ($49 \pm 5 \times 10^{-6}$ cm/s) indicate that permeability across the Caco-2 cell monolayer was high, whilst the efflux ratio (1.1 ± 0.2) suggests that **24** was not subject to polarised transport in this test system. Reasonable aqueous solubility and good permeability across Caco-2 cell monolayers suggests that the limited plasma exposure of **24** following oral administration is likely due to extensive first-pass metabolism. Nonetheless, due to its single digit nanomolar potency against *P. falciparum*, **24** was progressed to efficacy studies in the Peters 4-day murine model.

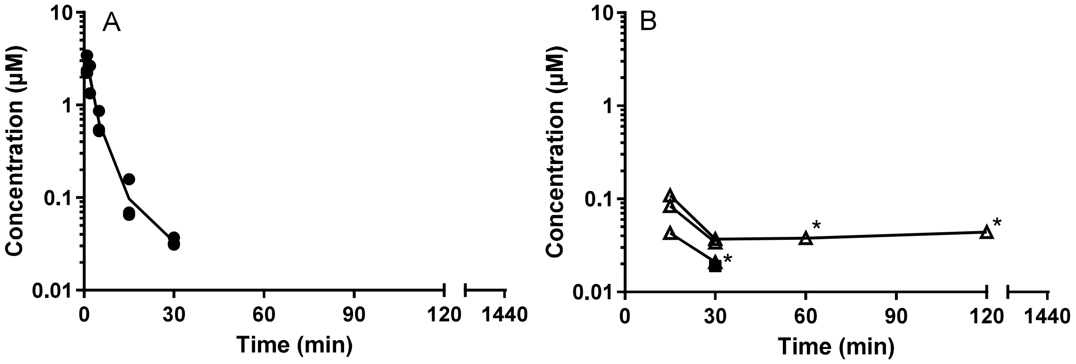
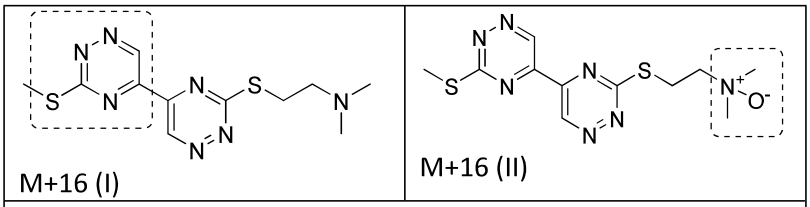


Figure 3. Plasma concentrations of **24** in male Swiss outbred mice following (A) IV administration at 2.8 mg kg^{-1} (B) oral administration at 2.9 mg kg^{-1} (filled square) and 28 mg kg^{-1} (open triangle). Data points represent individual samples with n=2 samples per time point except where shown by * where only one measurement was available.

***In vivo* efficacy of 24 in the Peters 4-day test.** Swiss outbred ARC (Animal Resource Centre, Murdoch, WA) female mice that were 5–7 weeks old with a mean (± SD) body weight of 28.1 ± 2.3 g were used in these studies. A tolerability assessment was first carried out to establish if **24** posed any gross toxicity to mice. Three oral dose regimens were assessed: 4, 8 and 16 mg kg^{-1} day^{-1} for 4 days with mice in a group of three for each dose. Each dose was administered at 24 h intervals. The animals were observed for physical distress 2 h after the administration and at two time points during the day; one between 7–9 am and one between 3–5 pm. Physical adverse events such as inability to move, reduced appetite, extreme pallor, ruffled hair, and loss of body weight were monitored. None of the mice exhibited any of the aforementioned signs indicating that the dose regimens of **24** used in the Peters 4-day test were not likely to be harmful to the mice.

For the Peters 4-day test, mice were inoculated intraperitoneally with 20 × 10^6 *P. berghei*-infected RBCs.[21] Mice were treated with two-fold increasing doses (ranging from 0.125–16 mg kg^{-1} day^{-1} with eight increasing doses) in a group of six. The first dose was administered 2 h after inoculation of infected RBCs (D0). The same dose was administered for three more days after each 24 h period. Blood samples were collected on D+4 for analysis by blood film microscopy. Two different routes of administration (oral and subcutaneous) were evaluated. The subcutaneous route is known to provide good drug absorption and rapid action, while the oral route bioavailability is contingent on the ability of the drug to be absorbed and avoid first pass elimination by the liver. The mean (± SD) ED_{50} and ED_{90} values obtained for subcutaneous administration of **24** were 0.21 ± 0.12 mg kg^{-1} day^{-1} and 0.60 ± 0.02 mg kg^{-1} day^{-1}, respectively. For oral administration, the mean ED_{50} and ED_{90} values of **24** were 1.47 ± 0.01 mg kg^{-1} day^{-1} and 3.43 ± 0.40 mg kg^{-1} day^{-1}, respectively. In contrast, the ED_{50} (oral) values for chloroquine and dihydroartemisinin were 1.1 mg kg^{-1} day^{-1} and 1.3 mg kg^{-1} day^{-1}, respectively, while the untreated vehicle control mice had to be euthanized after 6–7 days post parasite inoculation. Hence, **24** is efficacious when dosed subcutaneously and maintained good oral efficacy. The 6-fold drop in oral efficacy of **24** relative to its subcutaneous efficacy might be expected if **24** is subject to extensive first pass metabolism, assuming that **24** is the active species *in vivo*. The fact that **24** has oral activity, despite its pharmacokinetic profile as shown in Figure 3A, led us to consider what metabolites might plausibly be produced *in vivo* that might contribute to oral efficacy.

Preliminary metabolite identification. We selected five compounds (**24**, **29**, **32**, **49**, **54**) that exhibited low to high rates of degradation in HLM and MLM (Table 2). For most of the compounds, metabolic stability parameters were broadly comparable between species, except for **24** where the rate of degradation was lower in HLM compared with MLM. A preliminary metabolite screen was conducted for each compound by monitoring for the accurate mass of likely metabolites. Mono- and bis-oxygenation, dehydrogenation, demethylations/dealkylations, deaminations (to alcohols or acids), were searched for by MS under positive ionization mode. For the purposes of this preliminary study, full structure elucidation/confirmation using MS/MS was not carried out. While for **29** and **54**, no interpretable metabolite signals were found, for each of **24**, **32** and **49**, a putative metabolite corresponding to mono-oxygenation (mass of parent molecule + 16 Da) was detected following incubation with both HLM and MLM. Plausible sites for mono-oxygenation for all three compounds comprise the thioether sulfur atom or a ring nitrogen atom, while **24** and **49** offer the additional prospect of tertiary amine nitrogen atom mono-oxygenation. It is interesting that **29** did not give rise to an obvious mono-oxygenation peak whereas closely related analogue **32** did. The behavior of **32** was noteworthy in other ways. In the absence of cofactors, **32** showed significant degradation in both HLM and MLM (approximately 41 - 51% loss of parent over the course of incubation), suggesting a contribution of non-specific degradation to the overall degradation rate of this compound. However, none of these metabolic transformations were detected in the control incubations.



M+16 (I)

M+16 (II)

Figure 4. Proposed structures for metabolites of **24**. Dotted lines indicate possible sites of metabolism.

In this preliminary study, metabolite standards were not available and LC-MS conditions were therefore optimized for the parent compound only. Incubation conditions were also optimized for assessing the rate of loss of each parent compound (i.e. using low substrate and low microsomal protein concentrations). To more accurately identify and confirm the formation of the major metabolites of **24** in HLM and MLM, incubations were conducted at a high substrate (10 μM) and high protein (1 mg mL^{-1}) concentration to facilitate metabolite identification (SI). Two putative metabolites consistent with mono-oxygenation ([MH+] 326, M+16 (I & II)) were detected. M+16 (I) was detected in HLM only, while M+16 (II) was present in both HLM and MLM. Based on analysis of the MS/MS data, M+16 (I) is likely derived from oxygenation at the methylsulfanyl-triazine region of the molecule, while M+16 (II) is likely an *N*-oxide occurring at the *N*,*N*-dimethylamine (Figure 4). Based on peak area comparison, the putative *N*-oxide, M+16 (II), is the predominant metabolite in both species. In addition, two putative metabolites corresponding to bis-oxygenation ([MH+] 342, M+32) and demethylation ([MH+] 296, M-14) were identified by accurate mass measurement. However, due to poor sensitivity, structure assignment was not possible for either product.

For M+16 (I), four variants are possible if oxidation of any of the ring nitrogen atoms is considered feasible in addition to oxidation of the sulfur atom. Further work is in progress to synthesise these possible metabolites, confirm their structure and test their *in vitro* ADME and activities as well as their *in vivo* efficacy.

■ CONCLUSION

Our analysis of the SAR of 3,3'-disubstituted 5,5'-bi(1,2,4-triazine) derivatives has established that **24** possesses single digit nanomolar *in vitro* antimalarial activity against the 3D7 line, and also against chloroquine-, dihydroartemisinin, and mefloquine-resistant *P. falciparum* lines. In *ex vivo* studies of *P. falciparum* and *P. vivax* clinical field isolates collected in Timika, Indonesia, **24** proved to be more active than chloroquine and comparable with piperaquine when tested against *P. falciparum* isolates, and displayed greater activity than chloroquine, piperaquine and mefloquine, against *P. vivax* isolates. When evaluated in the *P. berghei* rodent model, **24** proved to be efficacious in supressing the development of parasites, with an ED_{50} value of 0.21 mg $kg^{-1}day^{-1}$ when administered subcutaneously and 1.47 mg kg^{-1} day^{-1} when administered orally, making it comparable to chloroquine and dihydroartemisinin.

The relatively poor systemic exposure is somewhat at odds with the oral efficacy exhibited by **24** and may suggest that efficacy is driven by active metabolites. However, at this stage, the possibility that **24** acts by an unusual *in vivo* PK/PD relationship cannot be ruled out and further work is required to better understand these intriguing effects.

This new class of orally active antimalarial bitriazines is an attractive proposition for further optimizing towards a preclinical candidate, and we will report on these efforts in due course**.**

■ EXPERIMENTAL SECTION

Chemistry. *General Experimental Methods***.** For all synthetic endeavours commercially available reagents were used without further purification. Column chromatography was performed using silica gel 60 (40–60 μm). The solvents for chromatography were used without purification. The reactions were monitored by TLC on Silica Gel 60F-254 plates with detection by UV light and/or $KMnO_4$ stain (1.50 g $KMnO_4$, 10.0 g K_2CO_3, and 1.25 mL 10% NaOH in 200 mL water).

Purity. The purity of all compounds submitted for biological testing was >95%, as determined using methods described (*vide infra*).

NMR. ^1H and ^{13}C NMR spectra were recorded at 400.13 and 100.62 MHz respectively, on a Bruker Avance III Nanobay spectrometer with a BACS 60 sample changer. The NMR solvents were purchased from Cambridge Isotope Laboratories. Chemical shifts (δ, ppm) are reported relative to the solvent peak ($CDCl_3$): 7.26 [^{1}H] or 77.16 [^{13}C]; DMSO d_6: 2.50 [^{1}H] or 39.52 [^{13}C]). Proton resonances are annotated as: chemical shift (δ), multiplicity (s, singlet; d, doublet; m, multiplet), coupling constant (J, Hz), and the number of protons.

LCMS (protocol-A). Low resolution mass spectrometry analyses were performed with an Agilent 6100 Series Single Quad LC/MS coupled with an Agilent 1200 Series HPLC, 1200 Series G1311A quaternary pump, 1200 series G1329A thermostated autosampler and 1200 series G1314B variable wavelength detector. The conditions for liquid chromatography were: reverse-phase HPLC analysis fitted with a Phenomenex Luna C8(2) 5 μm (50 x 4.6 mm) 100 Å column; column temperature: 30°C; injection volume: 5 μL; solvent: 99.9% acetonitrile, 0.1% formic acid; gradient: 5–100% of solvent over 10 min; detection: 254 nm. The conditions for mass spectrometry were: quadrupole ion source; ion mode: multimode-ES; drying gas temp: 300°C; vaporizer temperature: 200°C; capillary voltage: 2000 V (positive), 4000 V (negative); scan range: 100-1000 *m/z*; step size: 0.1 sec; acquisition time: 10 min.

LCMS (protocol-B). LRMS $[M+H]^+$ of compounds was analysed on an Agilent UHPLC/MS 1260/6120 system with the following technical information. Pump: 1260 Infinity G1312B Binary pump; autosampler: 1260 Infinity G1367E 1260 HiP ALS; detector: 1290 Infinity G4212A 1290 DAD. LC conditions: reverse-phase HPLC analysis; column: Poroshell 120 EC-C18 3.0 X 50mm 2.7-Micron; column temperature: 35°C; injection volume: 1 μL; flow rate: 1 mL min^{-1}. Solvent A: 99.9% water, 0.1% formic acid, solvent B: 99.9% ACN, 0.1% formic acid, gradient: 5-100% of solvent B in solvent A over 3.8 mins. Gradient takes 4 min to get to 100% solvent B in solvent A; maintain for 3 min and a further 3 min to get back to the original 5% solvent B in solvent A. MS conditions: ion source: Quadrupole, ion mode: API-ES, drying gas temp: 350°C; capillary voltage (V): 3000 (positive); capillary voltage (V): 3000 (negative); scan 52 range: 100-1000; step size: 0.1 s; acquisition time: 5 min. LC/MSD

Chemstation Rev.B.04.03 coupled with Masshunter Easy Access Software managed the running and processing of samples.

HRMS. High-resolution MS was performed with an Agilent 6224 TOF LC/MS coupled to an Agilent 1290 Infinity LC. All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Each scan or data point on the total ion chromatogram (TIC) is an average of 13,700 transients, producing a spectrum every second. Mass spectra were created by averaging the scans across each peak and subtracting the background from the first 10 s of the TIC. Acquisition was performed using the Agilent Mass Hunter Data Acquisition software ver. B.05.00 Build 5.0.5042.2 and analysis was performed using Mass Hunter Qualitative Analysis ver. B.05.00 Build 5.0.519.13. Acquisition parameters: mode, ESI; drying gas flow, 11 L min^{-1}; nebuliser pressure, 45 psi; drying gas temperature, 325°C; voltages: capillary, 4000 V; fragmentor, 160 V; skimmer, 65 V; octapole RF, 750 V; scan range, 100–1500 m/z; positive ion mode internal reference ions, m/z 121.050873 and 922.009798. LC conditions: Agilent Zorbax SB-C18 Rapid Resolution HT (2.1 × 50 mm, 1.8 mm column), 30°C; sample (5 μL) was eluted using a binary gradient (solvent A: 0.1% aq. HCO_2H; solvent B: 0.1% HCO_2H in CH_3CN; 5–100% B [3.5 min], 0.5 mL min^{-1}).

General synthetic procedures. Triazines of three classes were used in cyanide mediated dimerizations, namely 3-(alkylthio)-1,2,4-triazines **3**, 3-(alkoxy)-1,2,4-triazines **5** and 3-alkyl-1,2,4-triazines **8**. The synthesis of each class is fairly general or has been adopted and applied from a specifically described procedure in the literature.

Synthesis of 3-(alkylthio)-1,2,4-triazines 3. The synthesis of 3-(alkylthio)-1,2,4-triazine monomers **3** was achieved through alkylation of thiosemicarbazide with alkyl halides followed by cyclization with glyoxal. This synthetic methodology was the same as what has been previously reported by us for the synthesis of 3-(methylthio)-1,2,4-triazine.[14] An alternative approach to synthesizing 3-(alkylthio)-1,2,4-triazines **3** involved a nucleophilic substitution reaction that was accomplished using 3-(methylsulfonyl)-1,2,4-triazine **4** and mercaptans.[14] 3-(Cyclopentylthio)-1,2,4-triazine; 2-((1,2,4-triazin-3-yl)thio)ethan-1-ol; 3-((1,2,4-triazin-3-yl)thio)propan-1-ol; 3-((2-methoxyethyl)thio)-1,2,4-triazine; 3-((4-methoxybenzyl)thio)-1,2,4-triazine and 3-(isobutylthio)-1,2,4-triazine were all synthesized via this alkylation cyclisation procedure**.** The synthesis of all other 3-(alkylthio)-1,2,4-triazines has been reported by our group in an earlier publication.[14]

Synthesis of 3-(alkoxy)-1,2,4-triazines 5. The synthesis of 3-(alkoxy)-1,2,4-triazine monomers **5** was achieved using the nucleophilic substitution reaction involving magnesium alkoxides and 3-(methylsulfonyl)-1,2,4-triazine **4** in DMF. The syntheses of Mg-alkoxides were accomplished by reacting alcohols with MeMgI in THF solutions. The exact procedural details used in the synthesis of 3-(alkoxy)-1,2,4-triazine **5** have been described in detail in our earlier publication.[14] The synthesis and characterisation of all 3-(alkoxy)-1,2,4-triazines **5** that were utilised in the synthesis of 3,3’-disubstituted 5,5’-bi(1,2,4-triazine)s discussed in this study have been reported by our group in the past.[14]

Synthesis of 3-alkyl-1,2,4-triazines. The synthesis of 3-ethyl- and 3-butyl-1,2,4-triazine was accomplished by using the procedure already documented in the literature for the synthesis of 3-methyl-1,2,4-triazine.[13] The only modification to the procedure was that crude imidohydrazides were used in their cyclisation with glyoxal without isolation and characterization.

Synthesis of 3,3’-disubstituted 5,5’-bi(1,2,4-triazine)s. The synthesis of 3,3’-disubstituted 5,5’-bi(1,2,4-triazine)s **14–58** was accomplished using appropriate mixtures of 1,2,4-triazine monomers and potassium cyanide for coupling reactions as described previously by Courcot *et al* and used by our group.[13] While attempting the synthesis of heterodimers, the homodimers were also obtained. Characterization data of all the unknown monomeric 1,2,4-triazines and dimeric 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s not previously documented in the literature are reported below. Each synthesis was attempted with a view to obtain the heterodimers; but as expected, homodimers were also obtained from these couplings. Unsymmetrical 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s, **14–28** and **41–53** were obtained from the cyanide-mediated couplings of equimolar mixtures of monomers, while the symmetrical 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s **31–40** were obtained from the reactions conducted to obtain respective unsymmetrical bi(1,2,4-triazine)s **14–28**. Symmetrical bi(1,2,4-triazine)s**, 55–58** were obtained from the reaction mixtures there were set to obtain heterodimers of respective 3-(alkoxy)-1,2,4-triazines with 3-(metylthio)-1,2,4-triazines. The isolated yields listed for each 3,3'-disubstituted 5,5'-bi(1,2,4-triazine) are based on the assumption that only the monomer(s) that constituted the formation of the dimer participate in the reaction.

Characterisation data of monomeric 1,2,4-triazines not known in the literature. 3-(Cyclopentylthio)-1,2,4-triazine. Isolated yield: 39% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.89–8.88 (m, 1H), 8.34–8.33 (m, 1H), 4.12–4.09 (m, 1H), 2.27–2.22 (m, 2H), 1.78–1.65 (m, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 175.3, 148.1, 145.2, 43.7, 33.3, 24.9. LCMS (protocol-B**;** EI+): m/z 181.9 (MH)$^+$, t$_R$= 3.28 min. HRMS (EI): calcd for $C_8H_{12}N_3S$ [M+H]$^+$: m/z 182.0746; Found: m/z 182.0743.

3-((1,2,4-triazin-3-yl)thio)propan-1-ol. Isolated yield: 32% (column chromatography using 75% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.92–8.93 (d, *J* = 2.4 Hz, 1H), 8.37–8.36 (d, *J* = 2.4 Hz, 1H), 3.76–3.73 (t, *J* = 6.0 Hz, 2H), 3.37–3.34 (t, *J* = 7.0 Hz, 2H), 2.87 (bs, 1H), 2.04–1.98 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.6, 148.4, 145.4, 60.6, 32.1, 27.3. LCMS (protocol-B; EI+): m/z 172.0 $(MH)^+$, $t_R = 1.12$ min. HRMS (EI): calcd for $C_6H_{10}N_3OS$ $[M+H]^+$: m/z 172.0539; Found: m/z 172.0534.

3-((2-methoxyethyl)thio)-1,2,4-triazine. Isolated yield: 63% (column chromatography using 40% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 8.91–8.90 (d, $J = 2.4$ Hz, 1H), 8.35–8.34 (d, $J = 2.4$ Hz, 1H), 3.70–3.67 (t, $J = 6.2$ Hz, 2H), 3.45–3.42 (t, $J = 6.2$ Hz, 2H), 3.36 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.1, 148.3, 145.5, 70.6, 58.8, 30.2. LCMS (protocol-B; EI+): m/z 171.9 $(MH)^+$, $t_R = 2.44$ min. HRMS (EI): calcd for $C_6H_{10}N_3OS$ $[M+H]^+$: m/z 172.0539; Found: m/z 172.0535.

3-((4-methoxybenzyl)thio)-1,2,4-triazine. Isolated yield: 28% (column chromatography using 30% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 8.92–8.91 (d, $J = 2.4$ Hz, 1H), 8.36–8.35 (d, $J = 2.4$ Hz, 1H), 7.38–7.36 (d, $J = 8.4$ Hz, 2H), 6.86–6.84 (d, $J = 8.4$ Hz, 2H), 4.46 (s, 2H), 3.78 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.2, 159.2, 148.3, 145.6, 130.4, 128.3, 114.2, 55.4, 34.7. LCMS (protocol-B; EI+): m/z 121.0 $(M\text{-}C_3H_3N_3S)^+$, $t_R = 2.44$ min. HRMS data could not be obtained for this particular compound due to incompatibility with the ionization or instrumental conditions.

3-(Isobutylthio)-1,2,4-triazine. Isolated yield: 58% (column chromatography using 30% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 8.89¬8.88 (d, $J = 2.4$ Hz, 1H), 8.34–8.33 (d, $J = 2.4$ Hz, 1H), 3.16–3.14 (d, $J = 6.8$ Hz, 2H), 2.06–1.96 (hept, $J = 6.6$ Hz, 1H), 1.06–1.04 (d, $J = 6.8$ Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.9, 148.1, 145.3, 39.2, 28.3, 22.0. LCMS (protocol-B; EI+): m/z 170.0 $(MH)^+$, $t_R = 3.23$ min. HRMS (EI): calcd for $C_7H_{12}N_3S$ $[M+H]^+$: m/z 170.0746; Found: m/z 170.0744.

3-(n-Butyl)-1,2,4-triazine. Isolated yield: 70% (column chromatography using 15% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.09–9.08 (m, 1H), 8.54–8.53 (m, 1H), 3.14–3.09 (m, 2H), 1.88–1.80 (m, 2H), 1.44–1.37 (m, 2H), 0.96–0.91 (m, 3H). ^{13}C NMR (101

MHz, $CDCl_3$) δ 171.0, 148.8, 147.6, 37.2, 30.6, 22.5, 13.9. LCMS (protocol-B**;** EI+): m/z 138.3 $(MH)^+$, t_R = 1.35 min. HRMS (EI): calcd for $C_7H_{12}N_3$ $[M+H]^+$: m/z 138.1026; Found: m/z 138.1022.

Characterisation of 3,3'-disubstituted 5,5'-bi(1,2,4-triazine)s: 3-(Methylthio)-3'-(propylthio)-5,5'-bi(1,2,4-triazine) (14). Isolated yield: 47% (column chromatography using 20% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.88 (s, 2H), 3.35–3.31 (t, J = 7.2 Hz, 2H), 2.77 (s, 3H), 1.92–1.83 (m, 2H), 1.14–1.10 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 174.6, 150.1, 150.1, 142.1, 142.1, 33.1, 22.4, 14.2, 13.6. LCMS (protocol-B**;** EI+): m/z 280.9 $(MH)^+$, t_R = 3.48 min. HRMS (EI): calcd for $C_{10}H_{13}N_6S_2$ $[M+H]^+$: m/z 281.0638; Found: m/z 281.0638.

3-(Isopropylthio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (15). Isolated yield: 47% (column chromatography using 15% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.89–9.88 (m, 2H), 4.24–4.13 (hept, J = 6.8 Hz, 1H), 2.77 (s, 3H), 1.56–1.54 (d, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 174.6, 150.2, 150.1, 142.1, 142.0, 36.6, 22.8, 14.2. LCMS (protocol-B**;** EI+): m/z 281.1 $(MH)^+$, t_R = 3.79 min. HRMS (EI): calcd for $C_{10}H_{13}N_6S_2$ $[M+H]^+$: m/z 281.0638; Found: m/z 281.0637.

3-(Isobutylthio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (16). Isolated yield: 49% (column chromatography using 20% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.89 (m, 2H), 3.28– 3.27 (d, J = 6.8 Hz, 2H), 2.78 (s, 3H), 2.16–2.06 (m, 1H), 1.14–1.12 (d, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, CDCl3) δ 174.8, 174.6, 150.2, 150.0, 142.1, 142.1, 39.5, 28.4, 22.1, 14.2. LCMS (protocol-B**;** EI+): m/z 294.9 (MH)+, t_R = 3.51 min. HRMS (EI): calcd for $C_{11}H_{15}N_6S_2$ $[M+H]^+$: m/z 295.0794; Found: m/z 295.0794.

3-(*tert*-Butylthio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (17). Isolated yield: 37% (column chromatography using 25% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.86–

9.87 (m, 2H), 2.77 (s, 3H), 1.73 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 175.3, 174.7, 150.3, 149.8, 142.1, 141.9, 49.2, 30.1, 14.2. LCMS (protocol-B**;** EI+): m/z 294.8 $(MH)^+$, t_R = 3.46 min. HRMS (EI): calcd for $C_{11}H_{15}N_6S_2$ $[M+H]^+$: m/z 295.0794; Found: m/z 295.0797.

3-(Cyclopentylthio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (18). Isolated yield: 45% (column chromatography using 20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 9.87–9.88 (m, 2H), 4.25–4.18 (m, 1H), 2.77 (s, 3H), 2.38–2.32 (m, 2H), 1.88–1.73 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.3, 174.6, 150.2, 150.0, 142.1, 142.0, 44.1, 33.4, 25.1, 14.2. LCMS (protocol-B**;** EI+): m/z 307.0 $(MH)^+$, t_R = 3.60 min. HRMS (EI): calcd for $C_{12}H_{15}N_6S_2$ $[M+H]^+$: m/z 307.0794; Found: m/z 307.0789.

3-(Cyclohexylthio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (19). Isolated yield: 47% (column chromatography using 15% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 9.85 (bs, 2H), 4.06–3.99 (m, 1H), 2.76 (s, 3H), 2.21–2.18 (m, 2H), 1.85–1.81 (m, 2H), 1.70–1.47 (m, 5H), 1.41–1.35 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 174.6, 150.2, 150.1, 142.1, 141.9, 44.2, 32.8, 26.0, 25.7, 14.2. LCMS (protocol-B**;** EI+): m/z 320.8 $(MH)^+$, t_R = 3.61 min. HRMS (EI): calcd for $C_{13}H_{17}N_6S_2$ $[M+H]^+$: m/z 321.0951; Found: m/z 321.0950.

3-((4-(*tert*-Butyl)phenyl)thio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (20). Isolated yield: 29% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 9.87 (s, 1H), 9.47 (s, 1H), 7.61–7.59 (d, *J* = 8.4 Hz, 2H), 7.54–7.52 (d, *J* = 8.4 Hz, 2H), 2.74 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 175.4, 174.5, 154.0, 150.3, 150.0, 142.4, 142.1, 135.5, 127.0, 123.5, 35.1, 31.4, 14.2. LCMS (protocol-B**;** EI+): m/z 370.9 $(MH)^+$, t_R = 3.73 min. HRMS (EI): calcd for $C_{17}H_{19}N_6S_2$ $[M+H]^+$: m/z 371.1107; Found: m/z 371.1105.

3-(Methylthio)-3'-(pyridin-2-ylthio)-5,5'-bi(1,2,4-triazine) (21). Isolated yield: 26% (column chromatography using 30% EtOAc in hexanes). ^{1}H NMR (400 MHz, DMSO d_6) *δ*

10.04 (s, 1H), 9.53 (s, 1H), 8.68–8.67(d, *J* = 4.0 Hz, 1H), 8.02–7.94 (m, 2H), 7.56–7.53 (m, 1H), 2.73 (s, 3H). ^{13}C NMR (101 MHz, DMSO *d$_6$)* δ 173.3, 172.1, 151.2, 151.0, 150.6, 150.4, 144.0, 142.4, 138.2, 129.9, 124.3, 13.4. LCMS (protocol-B**;** EI+): m/z 315.8 (MH)$^+$, t$_R$ = 3.15 min. HRMS (EI): calcd for $C_{12}H_{10}N_7S_2$ [M+H]$^+$: m/z 316.0434; Found: m/z 316.0431.

2-((3'-(Methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)thio)ethan-1-ol (22). Isolated yield: 32% (column chromatography using 50% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) δ 9.92 (s, 1H), 9.89 (s, 1H), 4.07–4.04 (t, *J* = 5.8 Hz, 2H), 3.60–3.57 (t, *J* = 6.0 Hz, 2H), 2.77 (s, 3H), 1.95 (s, 1H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.7, 173.9, 150.4, 149.8, 142.6, 142.1, 61.5, 34.0, 14.2. LCMS (protocol-B**;** EI+): m/z 282.8 (MH)$^+$, t$_R$ = 2.99 min. HRMS (EI): calcd for $C_9H_{11}N_6OS_2$ [M+H]$^+$: m/z 283.0430; Found: m/z 283.0433.

3-((3'-(Methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)thio)propan-1-ol (23). Isolated yield: 44% (column chromatography using 75% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) δ 9.91 (s, 1H), 9.88 (s, 1H), 3.84–3.81 (t, *J* = 5.8 Hz, 2H), 3.50–3.46 (t, *J* = 7.0 Hz, 2H), 2.76 (s, 3H), 2.25 (s, 1H), 2.13–2.06 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.6, 174.5, 150.3, 150.0, 142.2, 142.1, 60.8, 32.0, 27.7, 14.2. LCMS (protocol-B**;** EI+): m/z 296.9 (MH)$^+$, t$_R$ = 3.13 min. HRMS (EI): calcd for $C_{10}H_{13}N_6OS_2$ [M+H]$^+$: m/z 297.0587; Found: m/z 297.0584.

***N*,*N*-Dimethyl-2-((3'-(methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)thio)ethan-1-amine hydrochloride (24)**. For free base isolated yield: 98% (column chromatography using 7% MeOH in DCM). The hydrochloride salt was obtained by reaction with 4M HCl (200 mol%) in dioxane, followed by ACN-Et$_2$O precipitation. ^{1}H NMR (400 MHz, DMSO *d$_6$)* δ 11.06 (s, 1H), 10.15 (s, 1H), 10.04 (s, 1H), 3.79–3.75 (m, 2H), 3.44–3.42 (m, 2H), 2.83 (bs, 6H), 2.76 (s, 3H). ^{13}C NMR (101 MHz, DMSO *d$_6$)* δ 173.2, 171.5, 151.2, 150.5, 143.4, 143.1, 55.0, 42.1, 23.8, 13.4. LCMS (protocol-B**;** EI+): m/z 309.9 $(MH)^+$, t_R = 2.69 min. HRMS (EI): calcd for $C_{11}H_{16}N_7S_2$ $[M-Cl]^+$: m/z 310.0903; Found: m/z 310.0905.

3-((2-Methoxyethyl)thio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (25). Isolated yield: 41% (column chromatography using 20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.91 (s, 1H), 9.89 (s, 1H), 3.80–3.77 (t, *J* = 6.4 Hz, 2H), 3.60–3.57 (t, *J* = 6.2 Hz, 2H), 3.43 (s, 3H), 2.78 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 174.1, 150.2, 150.0, 142.4, 142.1, 70.6, 59.1, 30.8, 14.2. LCMS (protocol-B**;** EI+): m/z 296.9 $(MH)^+$, t_R = 3.23 min. HRMS (EI): calcd for $C_{10}H_{13}N_6OS_2$ $[M+H]^+$: m/z 297.0587; Found: m/z 297.0589.

3-((4-Methoxybenzyl)thio)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (26). Isolated yield: 31% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.89 (s, 1H), 9.86 (s, 1H), 7.42–7.40 (d, *J* = 8.8 Hz, 2H), 6.88–6.86 (d, *J* = 8.4 Hz, 2H), 4.55 (s, 2H), 3.79 (s, 3H), 2.77 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 174.1, 159.5, 150.2, 150.1, 142.4, 142.2, 130.4, 127.9, 114.4, 55.5, 35.2, 14.1. LCMS (protocol-B**;** EI+): m/z 358.8 $(MH)^+$, t_R = 3.47 min. HRMS (EI): calcd for $C_{15}H_{15}N_6OS_2$ $[M+H]^+$: m/z 359.0743; Found: m/z 359.0755.

3'-(Isopropylthio)-6-methyl-3-(methylthio)-5,5'-bi(1,2,4-triazine) (27). Isolated yield: 56% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.75 (s, 1H), 4.20–4.10 (hept, *J* = 6.8 Hz, 1H), 3.04 (s, 3H), 2.73 (s, 3H), 1.53–1.52 (d, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.6, 171.9, 153.1, 153.0, 148.8, 143.8, 36.3, 23.0, 21.2, 14.1. LCMS (protocol-B**;** EI+): m/z 294.9 $(MH)^+$, t_R = 3.46 min. HRMS (EI): calcd for $C_{11}H_{15}N_6S_2$ $[M+H]^+$: m/z 295.0794; Found: m/z 295.0793.

***N*,*N*-Dimethyl-2-((6'-methyl-3'-(methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)thio)ethan-1-amine (28)**. Isolated yield: 30% (column chromatography using 5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.73 (s, 1H), 3.49–3.45 (t, *J* = 6.8 Hz, 2H), 3.00 (s, 3H), 2.78–2.74 (t, *J* = 7.0 Hz, 2H), 2.70 (s, 3H), 2.33 (s, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) *δ* 173.2, 171.7, 153.0, 153.0, 148.5, 144.0, 57.6, 45.2, 29.0, 21.2, 14.0. HRMS (EI): calcd for $C_{12}H_{18}N_7S_2$ [M+H]$^+$: m/z 324.1060; Found: m/z 324.1062.

3,3'-Bis(propylthio)-5,5'-bi(1,2,4-triazine) (31). Isolated yield: 70% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) *δ* 9.86 (s, 2H), 3.35–3.31 (t, *J* = 7.4 Hz, 4H), 1.92–1.83 (m, 4H), 1.14–1.10 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) *δ* 174.7, 150.2, 142.1, 33.1, 22.4, 13.6. LCMS (protocol-B**;** EI+): m/z 308.9 (MH)$^+$, t_R = 3.68 min. HRMS (EI): calcd for $C_{12}H_{17}N_6S_2$ [M+H]$^+$: m/z 309.0951; Found: m/z 309.0953.

3,3'-Bis(isopropylthio)-5,5'-bi(1,2,4-triazine) (33). Isolated yield: 45% (column chromatography using 7% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) *δ* 9.84 (s, 2H), 4.22–4.12 (hept, *J* = 6.8 Hz, 2H), 1.54–1.53 (d, *J* = 6.8 Hz, 12H). ^{13}C NMR (101 MHz, CDCl$_3$) *δ* 174.6, 150.2, 141.9, 36.6, 22.8. LCMS (protocol-B**;** EI+): m/z 308.9 (MH)$^+$, t_R = 3.61 min. HRMS (EI): calcd for $C_{12}H_{17}N_6S_2$ [M+H]$^+$: m/z 309.0951; Found: m/z 309.0954.

3,3'-Bis(isobutylthio)-5,5'-bi(1,2,4-triazine) (34). Isolated yield: 44% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) *δ* 9.86 (s, 2H), 3.28–3.26 (d, *J* = 6.6 Hz, 4H), 2.16–2.06 (m, 2H), 1.14–1.12 (d, *J* = 6.8 Hz, 12H). ^{13}C NMR (101 MHz, CDCl$_3$) *δ* 174.8, 150.1, 142.0, 39.5, 28.4, 22.1. LCMS (protocol-B**;** EI+): m/z 336.9 (MH)$^+$, t_R = 3.76 min. HRMS (EI): calcd for $C_{14}H_{21}N_6S_2$ [M+H]$^+$: m/z 337.1264; Found: m/z 337.1259.

3,3'-Bis(*tert*-butylthio)-5,5'-bi(1,2,4-triazine) (35). Isolated yield: 52% (column chromatography using 15% EtOAc in hexanes). ^{1}H NMR (400 MHz, CDCl$_3$) *δ* 9.83 (s, 2H), 1.73 (s, 18H). ^{13}C NMR (101 MHz, CDCl$_3$) *δ* 175.3, 150.0, 141.9, 49.2, 30.1. LCMS (protocol-

B**;** EI+): m/z 336.9 $(MH)^+$, t_R = 3.71 min. HRMS (EI): calcd for $C_{14}H_{21}N_6S_2$ $[M+H]^+$: m/z 337.1264; Found: m/z 337.1266.

3,3'-Bis(cyclopentylthio)-5,5'-bi(1,2,4-triazine) (36). Isolated yield: 68% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.85 (s, 2H), 4.25–4.18 (m, 2H), 2.38–2.30 (m, 4H), 1.87–1.71 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.3, 150.2, 141.9, 44.1, 33.4, 25.1. LCMS (protocol-B**;** EI+): m/z 360.8 $(MH)^+$, t_R = 3.82 min. HRMS (EI): calcd for $C_{16}H_{21}N_6S_2$ $[M+H]^+$: m/z 361.1264; Found: m/z 361.1268.

3,3'-Bis(cyclohexylthio)-5,5'-bi(1,2,4-triazine) (37). Isolated yield: 34% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (s, 2H), 4.07–4.00 (m, 2H), 2.22–2.18 (m, 4H), 1.86–1.83 (m, 4H), 1.71–1.34 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.6, 150.2, 141.9, 44.2, 32.8, 26.0, 25.8. LCMS (protocol-B**;** EI+): m/z did not ionise, t_R = 4.00 min. HRMS (EI): calcd for $C_{18}H_{25}N_6S_2$ $[M+H]^+$: m/z 389.1577; Found: m/z 389.1582.

3,3'-Bis((4-(*tert*-butyl)phenyl)thio)-5,5'-bi(1,2,4-triazine) (38). Isolated yield: 38% (column chromatography using 7% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.43 (s, 2H), 7.59–7.57 (d, J = 8.4 Hz, 4H), 7.53–7.50 (d, J = 8.0 Hz, 4H), 1.37 (s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.2, 154.0, 150.3, 142.4, 135.5, 126.9, 123.5, 35.1, 31.4. LCMS (protocol-A**;** EI+): m/z 489.2 $(MH)^+$, t_R = 8.46 min. HRMS (EI): calcd for $C_{26}H_{29}N_6S_2$ $[M+H]^+$: m/z 489.1890; Found: m/z 489.1893.

3,3'-Bis((2-methoxyethyl)thio)-5,5'-bi(1,2,4-triazine) (39). Isolated yield: 39% (column chromatography using 40% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.88 (s, 2H), 3.79-3.76 (t, J = 6.2 Hz, 4H), 3.59-3.56 (t, J = 6.2 Hz, 4H), 3.42 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0, 150.1, 142.3, 70.5, 59.1, 30.8. LCMS (protocol-B; EI+): m/z 340.8

$(MH)^+$, t_R = 3.27 min. HRMS (EI): calcd for $C_{12}H_{17}N_6O_2S_2$ $[M+H]^+$: m/z 341.0849; Found: m/z 341.0847.

3,3'-([5,5'-Bi(1,2,4-triazine)]-3,3'-diylbis(sulfanediyl))bis(propan-1-ol) (40). Isolated yield: 23% (column chromatography using 50% EtOAc in hexanes). ^1H NMR (400 MHz, DMSO *d₆*) δ 9.95 (s, 2H), 4.71–4.68 (t, J = 5.2 Hz, 2H), 3.60–3.55 (m, 4H), 3.40–3.37 (t, J = 7.2 Hz, 4H), 1.95–1.88 (m, 4H). ^{13}C NMR (101 MHz, DMSO d_6) δ 173.0, 150.7, 142.8, 59.3, 31.7, 27.1. LCMS (protocol-B**;** EI+): m/z 340.8 $(MH)^+$, t_R = 2.99 min. HRMS (EI): calcd for $C_{12}H_{17}N_6O_2S_2$ $[M+H]^+$: m/z 341.0849; Found: m/z 341.0849.

3-(Benzyloxy)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (42). Isolated yield: 49% (column chromatography using 10% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.96 (s, 1H), 9.89 (s, 1H), 7.57–7.55 (m, 2H), 7.43–7.36 (m, 3H), 5.72 (s, 2H), 2.76 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.6, 165.1, 153.0, 150.0, 142.2, 141.9, 135.0, 128.9, 128.9, 128.6, 71.1, 14.2. LCMS (protocol-B**;** EI+): m/z 312.9 $(MH)^+$, t_R = 3.37 min. HRMS (EI): calcd for $C_{14}H_{13}N_6OS$ $[M+H]^+$: m/z 313.0866; Found: m/z 313.0869.

2-((3'-(Methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)oxy)ethan-1-ol (43). Isolated yield: 23% (column chromatography using 3% MeOH in DCM). ^1H NMR (400 MHz, $CDCl_3$) δ 9.98 (s, 1H), 9.91 (s, 1H), 4.82–4.80 (t, J = 4.4 Hz, 2H), 4.15–4.12 (m, 2H), 2.78 (s, 3H), 2.27–2.24 (t, J = 6.0 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 165.3, 153.2, 149.9, 142.1, 71.1, 61.1, 14.2. LCMS (protocol-B**;** EI+): m/z 266.8 $(MH)^+$, t_R = 2.85 min. HRMS (EI): calcd for $C_9H_{11}N_6O_2S$ $[M+H]^+$: m/z 267.0659; Found: m/z 267.0657.

3-(2-Methoxyethoxy)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (44). Isolated yield: 42% (column chromatography using 50% EtOAc in hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 9.95 (s, 1H), 9.90 (s, 1H), 4.83–4.81 (t, J = 4.4 Hz, 2H), 3.89– 3.87 (t, J = 4.6 Hz, 2H), 3.46 (s, 3H), 2.76 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 165.2, 153.1, 150.0, 142.2, 142.0, 70.3, 68.6, 59.3, 14.2. LCMS (protocol-B; EI+): m/z 280.9 (MH)$^+$, t_R = 3.05min. HRMS (EI): calcd for $C_{10}H_{13}N_6O_2S$ [M+H]$^+$: m/z 281.0815; Found: m/z 281.0817.

3-(Methylthio)-3'-(2,2,2-trifluoroethoxy)-5,5'-bi(1,2,4-triazine) (45). Isolated yield: 75% (column chromatography using 7% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.08 (s, 1H), 9.93 (s, 1H), 5.13–5.07 (q, J = 8.0 Hz, 2H), 2.78 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.8, 164.1, 153.8, 149.3, 143.3, 142.1, 122.8, 64.9, 14.2. LCMS (protocol-A; EI+): m/z 305.3 (MH)$^+$, t_R = 6.24 min. HRMS (EI): calcd for $C_9H_8F_3N_6OS$ [M+H]$^+$: m/z 305.0427; Found: m/z 305.0429.

3-(Methylthio)-3'-(2-(trimethylsilyl)ethoxy)-5,5'-bi(1,2,4-triazine) (46). Isolated yield: 23% (column chromatography using 15% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H), 9.91 (s, 1H), 4.80–4.75 (m, 2H), 2.77 (s, 3H), 1.35–1.30 (m, 2H), 0.13 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.6, 165.2, 152.9, 150.2, 142.2, 141.5, 68.3, 17.7, 14.2, -1.3. LCMS (protocol-B; EI+): m/z 322.9 (MH)$^+$, t_R = 3.58 min. HRMS (EI): calcd for $C_{12}H_{19}N_6OSSi$ [M+H]$^+$: m/z 323.1105; Found: m/z 323.1104.

4-Methyl-5-(2-((3'-(methylthio)-[5,5'-bi(1,2,4-triazin)]-3-yl)oxy)ethyl)thiazole (47). Isolated yield: 34% (column chromatography using 15% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.97 (s, 1H), 9.90 (s, 1H), 8.64 (s, 1H), 4.87–4.83 (t, J = 6.8 Hz, 2H), 3.45–3.41 (t, J = 6.6 Hz, 2H), 2.77 (s, 3H), 2.49 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 165.0, 153.2, 150.4, 150.0, 149.9, 142.2, 142.1, 68.9, 26.1, 15.0, 14.2. LCMS (protocol-B; EI+): m/z 347.8 (MH)$^+$, t_R = 3.17 min. HRMS (EI): calcd for $C_{13}H_{14}N_7OS_2$ [M+H]$^+$: m/z 348.0696; Found: m/z 348.0696.

3-((1-Methoxypropan-2-yl)oxy)-3'-(methylthio)-5,5'-bi(1,2,4-triazine) (48). Isolated yield: 11% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H), 9.91 (s, 1H), 5.76–5.69 (m, 1H), 3.77–3.73 (dd, J = 10.8, 6.8 Hz, 1H), 3.67–3.64 (dd, *J* = 10.8, 3.6 Hz, 1H), 3.42 (s, 3H), 2.77 (s, 3H), 1.52–1.50 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.6, 165.1, 153.0, 150.1, 142.2, 141.7, 75.2, 75.0, 59.5, 16.5, 14.2. LCMS (protocol-B**;** EI+): m/z 295.2 $(MH)^+$, t_R = 3.54 min. HRMS (EI): calcd for $C_{11}H_{15}N_6O_2S$ $[M+H]^+$: m/z 295.0972; Found: m/z 295.0979.

2-((3'-Cyclopropoxy-[5,5'-bi(1,2,4-triazin)]-3-yl)thio)-*N,N*-dimethylethan-1-amine (50). Isolated yield: 29% (column chromatography using 3% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.96 (s, 1H), 9.89 (s, 1H), 4.66–4.62 (m, 1H), 3.51–3.48 (t, *J* = 7.0 Hz, 2H), 2.78–2.75 (t, *J* = 7.0 Hz, 2H), 2.35 (s, 6H), 0.98 (bs, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.3, 166.3, 152.8, 150.1, 142.3, 142.1, 57.8, 53.6, 45.4, 29.1, 6.2. LCMS (protocol-B**;** EI+): m/z 320.2 $(MH)^+$, t_R = 1.66 min. HRMS (EI): calcd for $C_{13}H_{18}N_7OS$ $[M+H]^+$: m/z 320.1288; Found: m/z 320.1291.

3-(Isopropylthio)-3'-methoxy-5,5'-bi(1,2,4-triazine) (51). Isolated yield: 21% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.90 (s, 1H), 9.84 (s, 1H), 4.28 (s, 3H), 4.18–4.08 (hept, *J* = 6.8 Hz, 1H), 1.51–1.49 (d, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 165.5, 152.9, 150.0, 141.9, 141.7, 56.5, 36.5, 22.7. LCMS (protocol-B**;** EI+): m/z 265.1 $(MH)^+$, t_R = 3.66 min. HRMS (EI): calcd for $C_{10}H_{13}N_6OS$ $[M+H]^+$: m/z 265.0866; Found: m/z 265.0868.

3-(Isopropylthio)-3'-(2-methoxyethoxy)-5,5'-bi(1,2,4-triazine) (52). Isolated yield: 26% (column chromatography using 10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.94 (s, 1H), 9.89 (s, 1H), 4.85–4.83 (m, 2H), 4.23–4.13 (hept, *J* = 7.0 Hz, 1H), 3.90–3.88 (m, 2H), 3.48 (s, 3H), 1.55–1.54 (d, *J* = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.6, 165.2, 153.3, 150.0, 142.1, 142.0, 70.3, 68.7, 59.4, 36.6, 22.8. LCMS (protocol-B**;** EI+): m/z 309.2 $(MH)^+$, t_R = 3.66 min. HRMS (EI): calcd for $C_{12}H_{17}N_6O_2S$ $[M+H]^+$: m/z 309.1128; Found: m/z 309.1125.

3-(Isopropylthio)-3'-((1-methoxypropan-2-yl)oxy)-5,5'-bi(1,2,4-triazine) (53). Isolated yield: 19% (column chromatography using 3% MeOH in DCM). ^{1}H NMR (400 MHz, CDCl$_3$) δ 9.86 (s, 1H), 9.84 (s, 1H), 5.71–5.64 (td, J = 6.5, 3.6 Hz, 1H), 4.27 – 4.01 (hept, *J* = 6.8 Hz, 1H), 3.73–3.69 (dd, J = 10.6, 6.7 Hz, 1H), 3.63–3.60 (dd, J = 10.6, 3.6 Hz, 1H), 3.38 (s, 3H), 1.50–1.46 (m, 9H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.4, 165.0, 153.0, 150.1, 142.0, 141.5, 75.1, 74.8, 59.3, 36.4, 22.7, 16.3. LCMS (protocol-B**;** EI+): m/z 323.2 (MH)$^+$, t$_R$ = 3.78 min. HRMS (EI): calcd for $C_{13}H_{19}N_6O_2S$ [M+H]$^+$: m/z 323.1285; Found: m/z 323.1287.

Interference Compounds. All final compounds have been examined for the presence of substructures classified as Pan Assay Interference Compounds (PAINS) using a KNIME workflow.[22, 23]

ALARM NMR. Triazine **24** was tested by ALARM NMR as described previously.[18,24] Briefly, test compounds (400 µM final concentration) were incubated with ^{13}C-methyl-labelled La antigen (50 µM final concentration) at 37°C for 1 h and then 30°C for 15 h prior to data collection. Each compound was tested in the absence and presence of 20 mM DTT. Data were normalized to DMSO vehicle control. Data were recorded at 25°C on a Bruker 700 MHz NMR spectrometer equipped with a cryoprobe (Bruker) and autosampler. Samples were acquired with 16 scans, 2048 complex points in F2, and 80 points in F1 using standard protein HMQC and water suppression pulse sequences. Nonreactive compounds were identified by the absence of chemical shifts or peak attenuations (^{13}C-methyl) independent of the presence of DTT. Reactive compounds were identified by characteristic chemical shifts and peak attenuations in the absence of DTT.

Ethical approval. The ethical approval for an *ex vivo* drug susceptibility study was obtained from the Eijkman Institute Research Ethics Commission, Eijkman Institute of Molecular Biology, Jakarta, Indonesia (EIREC 47 and EIREC 67) and the Human Research Ethics Committee of Northern Territory, Department of Health and Families and Menzies

School of Health Research, Darwin, Australia (HREC 2010-1396). The procedures used for pharmacokinetics studies were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Study protocols were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. The ethical approval to conduct tolerability and efficacy studies of **24** in the *P. berghei* mouse model using the Peters 4-day test was approved by the Animal Ethics Committee, Australian Army Malaria Institute (approval number: 3/2014).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

J.R.H., D.H.S., J.G.B., T.D., K.B. contributed to design, synthesis and manuscript composition. L.X., S.D., L.L., V.M.A. and F.H. contributed to manuscript composition. S.F., S.D., L.L., S.A.C., K.E., S.A.R., R.N., J.M., G.W, M.C., D.C., R.N.P., M.D.E. and V.M.A. all made key contributions to the array of biological testing required. S.T. undertook compound handling and distribution. J.L.D., M.A.W., M.E.C. and J.M.S. carried out ALARM NMR assessment. S.C. oversaw ADMET and PK undertaken by F.C.K.C. and S.B.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ACTs, artemisinin combination therapies; ADME, absorption, distribution, metabolism and excretion; Anh, anhydrous; ARC, Animal Resource Centre, Murdoch, WA; CARL, cyclic amine resistance locus; CL_{int}, intrinsic clearance; CQ, chloroquine; DHODH, dihydroorotate dehydrogenase; DTT, dithiothreitol; ED_{50}, 50% effective dose; ED_{90}, 90% effective dose; E_H, hepatic extraction ratio; ESI, electrospray ionisation; GTS, Global Technical Strategy; HLM, human liver microsomes; HMQC, heteronuclear multiple quantum coherence; IC_{50}, 50% inhibitory concentration; IV, intravenous; MIPS, Monash Institute of Pharmaceutical Science; MLM, mouse liver microsomes; *P. berghei*, *Plasmodium berghei*; *P. falciparum*, *Plasmodium falciparum*; *P. Vivax*, *Plasmodium vivax*; PAINS, Pan Assay Interference Compounds; PK, Pharmacokinetics; RBCs, red blood cells; SAR, structure-activity relationship; SD, standard deviations; SI, selectivity index; TIC, total ion chromatogram; WHO, World Health Organization.

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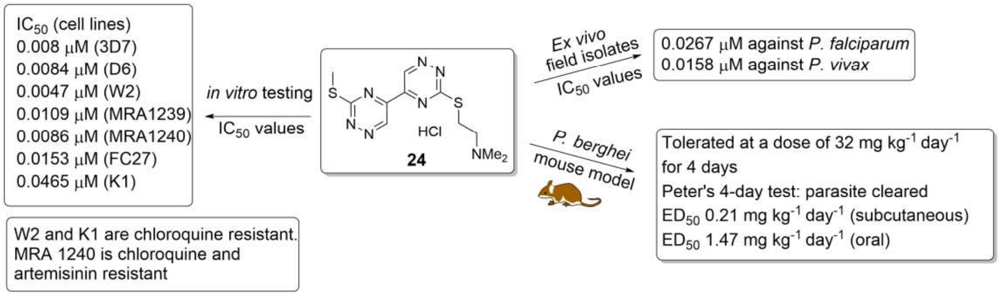

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