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3-Alkylthio-1,2,4-triazine dimers with potent antimalarial activity

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ABSTRACT

We report on the discovery of 3-alkylthio-1,2,4-triazine dimers that are potently toxic to *Plasmodium falciparum*, with single digit nanomolar activity, and up to several thousand-fold lower toxicity to mammalian cells. They are equipotent against chloroquine-resistant strains of *P. falciparum*.

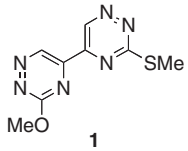
We have previously reported that WEHI 59743, which supposed to be triazine dimer **1**, as purchased from the supplier, was found by us to be potently toxic to *Plasmodium falciparum*, a causative agent of malaria, and that it is highly selective with little activity against a range of other pathogenic parasites as well as mammalian cells.[1] These data are summarized in Table 1. We also remarked that preliminary LCMS analysis indicated that WEHI 59743 comprised predominantly an impurity of M+16. We here reveal the composition of WEHI 59743 and the results of an SAR investigation around the most active component.

WEHI 59743 supplied as 3-methoxy-5-(3-(methylthio)-1,2,4-triazin-5-yl)-1,2,4-triazine and the NMR spectrum appeared to confirm this structure. As shown in Figure 1 (disregarding the two signals at 7.26 ppm and 1.5 ppm which correspond to the solvent peak (chloroform) and water peak, respectively) there appears to be clear SMe and OMe signals at 2.78 and 4.33 ppm, respectively, and each of these integrates cleanly to three protons. Similarly, the aromatic protons downfield near 10 ppm integrate cleanly to two protons as expected.

However, the LCMS trace of WEHI 59743 suggested a major component of longer retention time with a molecular ion of M+16 (Fig. 2). This was unexpected and it was difficult to reconcile the NMR spectrum in Figure 1 with the LCMS trace in Figure 2.

Simple thin layer chromatography initially added further to the confusion because the results suggested that the sample was in fact a mixture of three major components. It was observed that two of these were yellowish in color, with the highest R_f spot being particularly strongly colored. It was hypothesized that any

Table 1
Antiparasitic activity profile of compound WEHI 59743



1

Parasite	EC_{50},[a] (μM)
P. falciparum[b]	0.18
L. infantosum[c]	8.6
T. cruzi[d]	16
T. brucei[e]	5.7
Cytotoxicity[f]	33

[a] Values are means of three experiments, ±50%.
[b] *Plasmodium falciparum* 3D7 strain, erythrocytic stage, chloroquine was used as a control, EC_{50} 30 μg/ml.
[c] *Leishmania infantosum*, amastigote stage, miltefosine was used as a control, EC_{50} 2.4 μg/ml.
[d] *Trypanosoma cruzi* Tulahaen C2C4 strain, amastigote stage, nifurtimox was used as a control, EC_{50} 0.24 μg/ml.
[e] *Trypanosoma brucei rhodesiense* strain STIB 900, bloodstream form. Suramin (EC_{50} 0.13 μg/ml) was used as a control.
[f] Rat skeletal myoblast cell L-6 strain, Tamoxifen was used as a control, EC_{50} 4.9 μg/ml.

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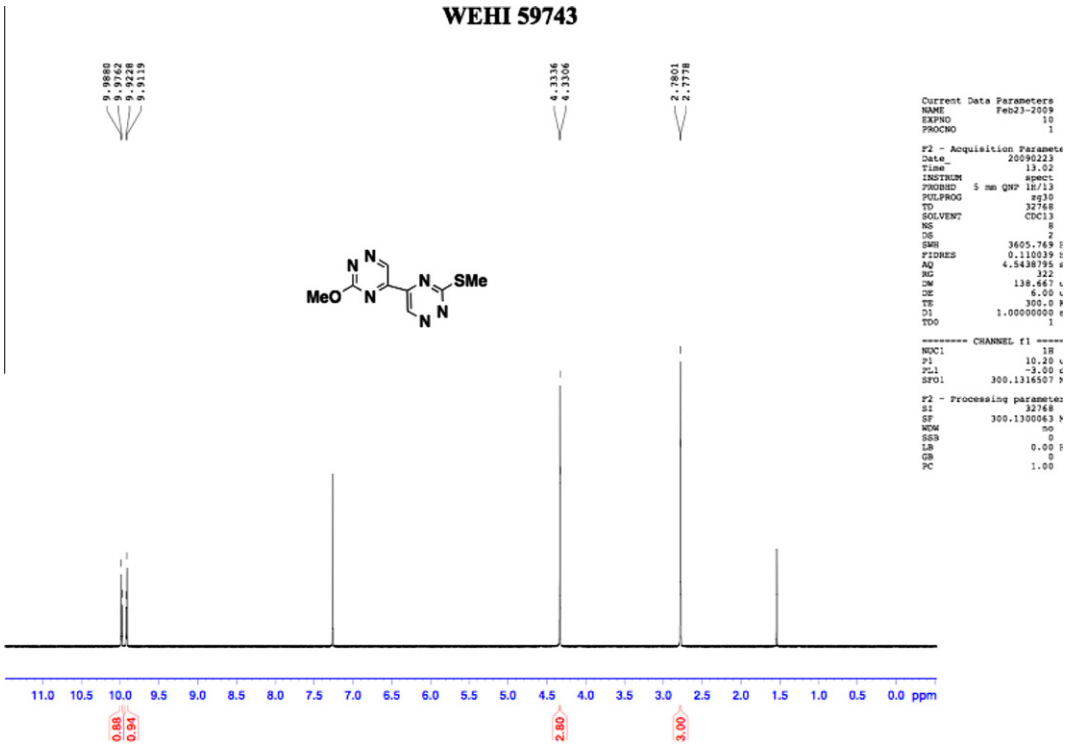


Figure 1. ^{1}H NMR spectrum of WEHI 59743.

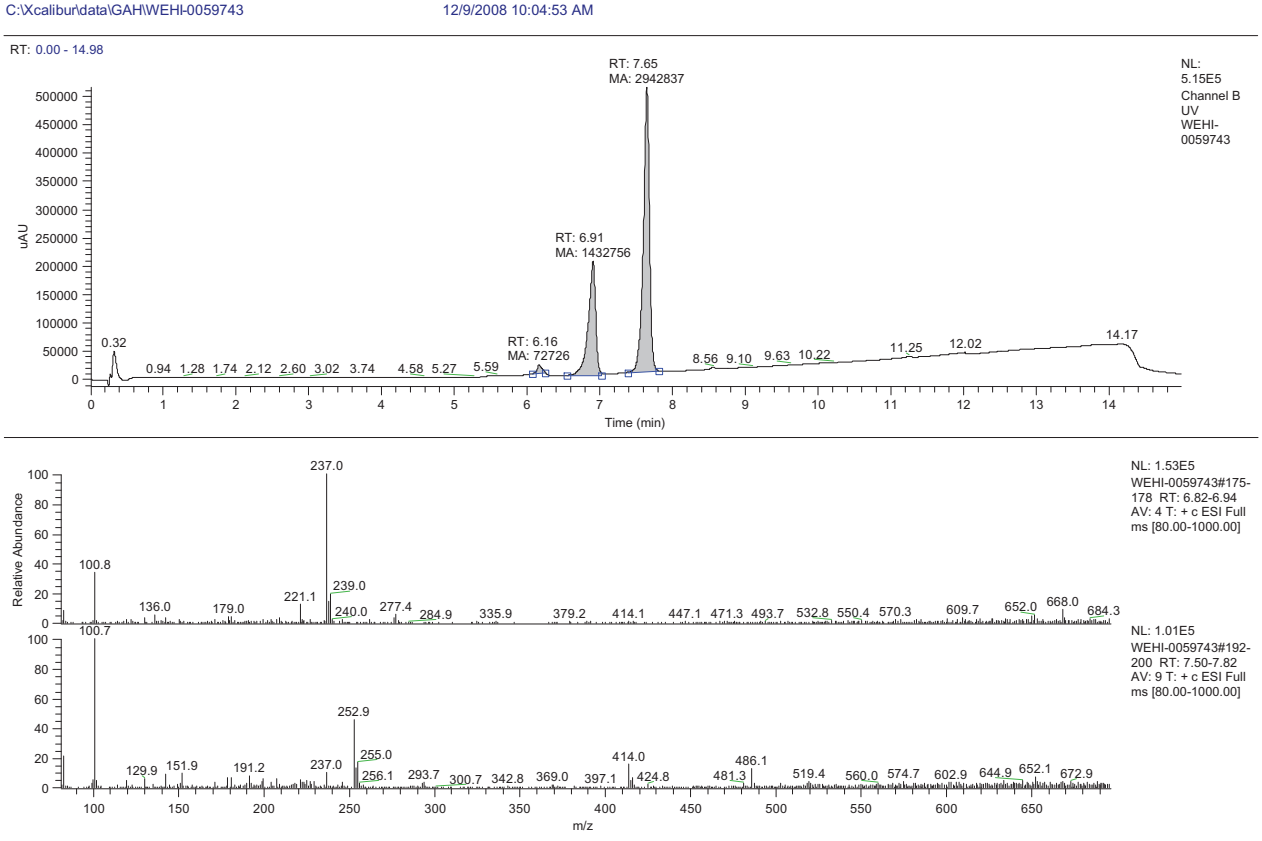


Figure 2. Standard LCMS trace of WEHI 59743.

compound with a strong chromophore might give rise to an artificially intense signal at 254 nm in the LCMS and therefore not be representative of the real concentration in the mixture. Therefore, WEHI 59743 was re-analyzed at a wavelength of 230 nm in order to better detect all impurities. Quite remarkably, as shown in Figure 3, WEHI 59743 now came up as mixture of several

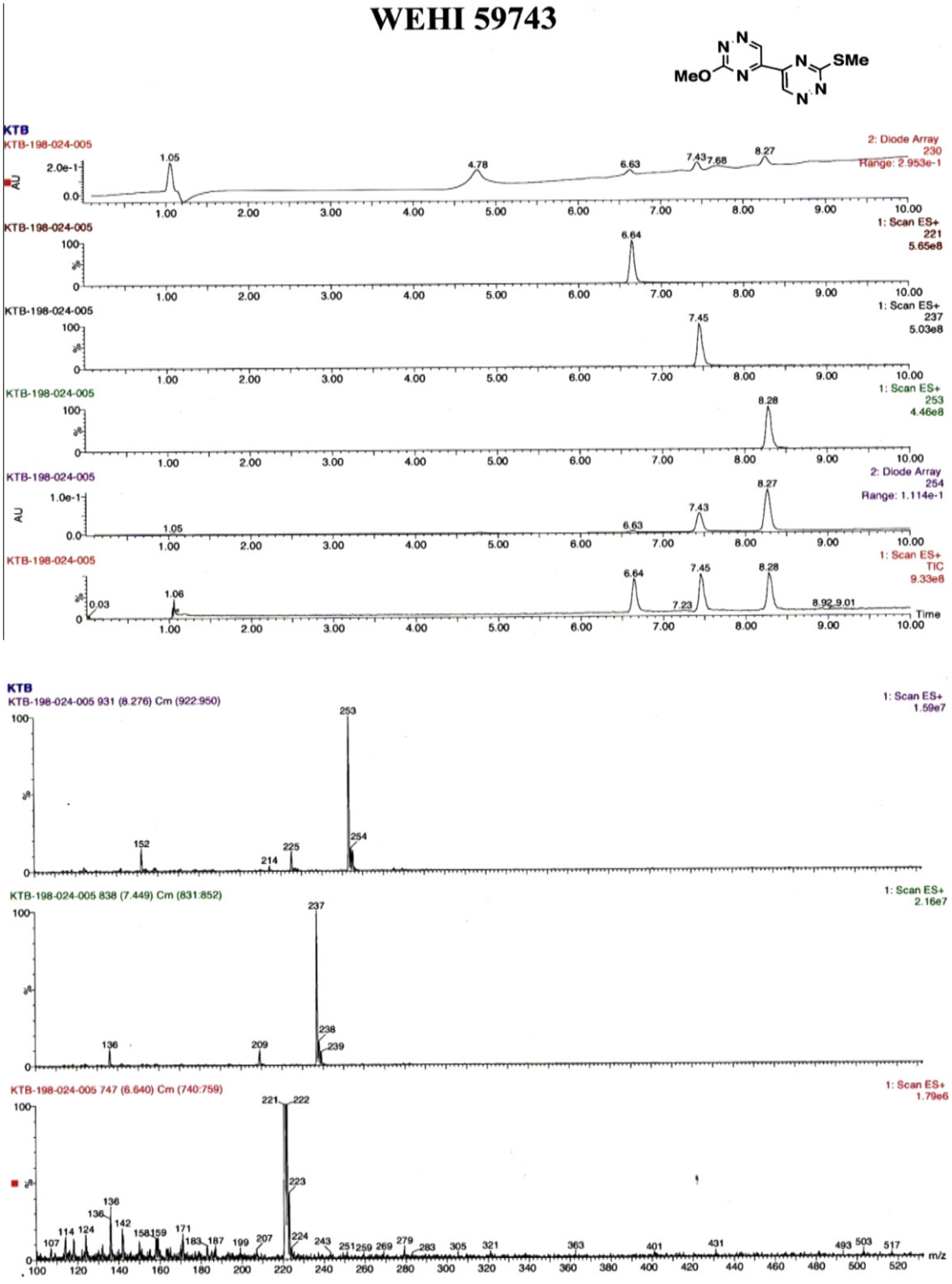
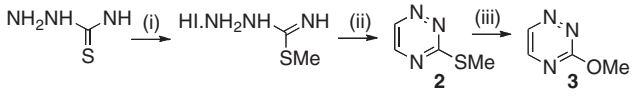
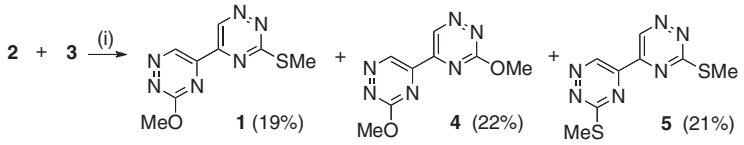


Figure 3. Fuller LCMS analysis of WEHI 59743. Top segment from top to bottom: UV absorption at 230 nm, ES scan for M+H 221, ES scan for M+H 237, ES scan for M+H 253, UV absorption at 254nm, total ion current trace. Bottom segment from top to bottom: mass spectrum (ES+) for the mw 252 peak, the mw 236 peak, and the mw 220 peak.



Scheme 1. Reagents and conditions: (i) MeI [EtOH], 60 °C, 78%; (ii) 40% aq Glyoxal, $NaHCO_3$, 94%; (iii) NaOMe [MeOH], 55%.

compounds. The total ion current, like the TLC, was suggestive of three major components, of molecular ion peaks M−16, M and M+16, respectively. Analysis at different wavelengths supported the view that the shorter retention time compound (M−16) had the weakest chromophore at 254 nm and the longer retention time compound (M+16) had the strongest chromophore at 254 nm.



Scheme 2. Reagents and conditions: (i) KCN, air [H_2O], 64%.

It was proposed that a logical interpretation was that the M−16 compound was the methoxy homodimeric counterpart and the M+16 compound was the methylthio homodimeric counterpart to the intended heterodimeric structure. A search of the literature revealed that synthetic routes for these compounds could plausibly produce unintended mixtures of compounds.[2] It was further realized that if the two homodimeric components were present in approximately equal amounts (regardless of the concentration of the heterodimer), an NMR spectrum such as that in Figure 1, which superficially looks like pure heterodimer, could arise. Indeed, scrutiny of the peaks supports this and show that each of the methyl peaks, for example, comprises two singlets rather than one. This would make sense if one methoxy signal belongs to the heterodimer and one to the homodimer. Likewise for the methylthio signals (and the aromatic signals). It was briefly considered that the M+16 signal could represent sulfoxide formation, however, this would be very polar and would have a shorter retention time rather than a longer retention time. Also, this does not explain the NMR spectrum.

To summarize, WEHI 59743 was supplied as a mixture of three compounds. This is a good example of why LCMS analysis at 254 nm, however commonly undertaken, can be highly misleading for mixtures containing compounds with chromophores of differing absorbance maxima and extinction coefficients. This is highly pertinent to compound quality control when samples are submitted to bioassays.

Due to the limited availability of the commercial sample, the synthesis and retesting of each proposed component was undertaken as shown in Scheme 1.

Firstly, the methylthiotriazine **2** was made by cyclocondensation of methylated thiosemicarbazide[3] with glyoxal.[4] The corresponding methoxy counterpart **3** was then made by reaction of **2** with methoxide.[2]

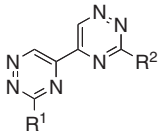
Triazine monomers **2** and **3** were then dimerized through the use of excess cyanide in water[2] as shown in Scheme 2. This gave, after purification, homodimers **4** and **5** heterodimer **1** each in around 20% yield.

Significantly, the ^{1}H NMR spectrum of the crude project mixture was indistinguishable from that in Figure 1, and LCMS data matched identically. These compounds were then tested for activity against *P. falciparum*, using a novel assay developed by Avery and coworkers[5] and as shown in Table 2, it was the methylthio homodimer **5** in which most of the biological activity resides with an EC_{50} of 26 nM.

On this basis, a small set of alkylthio dimers **6**–**8** was assembled using analogous methodology to that shown in Scheme 1 for the synthesis of the methylthio monomer **2**, followed by homodimerization using the same conditions as those in Scheme 2. As shown in Table 2, all dimers were highly potent and activity only started to decrease for the butylthio-containing compound such that **8** had an EC_{50} of 96 nM.

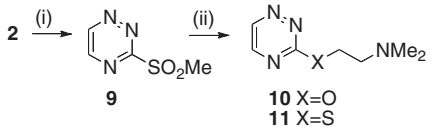
We were next interested in investigating whether a tertiary amine could be tethered to these compounds with maintenance of activity. This was on the basis that such compounds would likely to be more soluble and hence more drug-like but also that this approach has yielded potent antimalarial compounds in other heterocyclic systems.[6] The fact that extended alkylthio groups were reasonably tolerated and readily synthesized highlighted this feature as the logical choice for the incorporation of amino tethers.

Table 2
Biological activity of 1,2,4-triazine dimers[a] against *P. falciparum*

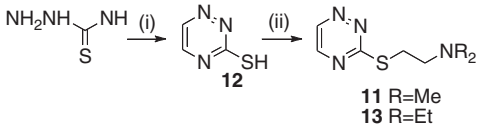


Compd	R^1	R^2	EC_{50}[b] (μM)	
			Pf[c]	Cytotox[d]
1	MeO	MeS	0.15 (0.25)[e]	18
4	MeO	MeO	4.1 (4.4)[e]	36
5	MeS	MeS	0.026 (0.053)[e]	68
6	EtS	EtS	0.022 (0.024)[e]	84
7	PrS	PrS	0.017 (0.022)[e]	>115
8	BuS	BuS	0.096 (0.16)[e]	>115
14	MeS	$Me_2NCH_2CH_2O$	0.080 (0.12)[e]	24
15	MeS	$Me_2NCH_2CH_2S$	0.008 (0.004)[e]	21
16	$Me_2NCH_2CH_2S$	$Me_2NCH_2CH_2S$	0.47 (0.62)[e]	25
17	MeS	$Et_2NCH_2CH_2S$	0.023 (0.012)[e]	9
18	$Et_2NCH_2CH_2S$	$Et_2NCH_2CH_2S$	0.44 (0.79)[e]	16
19	Et	Et	1.6[f]	NT[g]

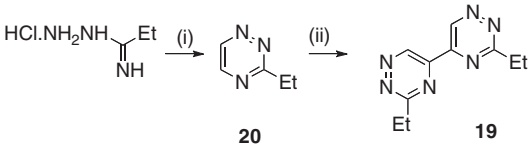
[a] Monomers are inactive.
[b] Values are means of three experiments, ±50%.
[c] *Plasmodium falciparum* 3D7 strain, erythrocytic stage. Chloroquine was used as a control, EC_{50} 0.004 μM.
[d] HEK 293 cells. Puromycin was used as a control, EC_{50} 0.41 μM.
[e] *Plasmodium falciparum* Dd2 strain, erythrocytic stage. Chloroquine was used as a control, EC_{50} 0.17 μM.
[f] Tested in singlicate only.
[g] Not tested.



Scheme 3. Reagents and conditions: (i) mCPBA (2 equiv) [DCM], 0 °C–rt, 2 h; (ii) for X = O, *N*,*N*-dimethylethanolamine, NaH [THF], 3% over two steps; for X = S, *N*,*N*-dimethylethanethiolamine, NaH [THF], 15% over two steps.



Scheme 4. Reagents and conditions: (i) 40% aq glyoxal, 80 °C, 82%; (ii) alkyl halide (*N*,*N*-dimethylchloroethylamine for **11** and *N*,*N*-diethylbromoethylamine for **13**), Hünig's base [DMA], reflux 2 h, 20%.



Scheme 5. Reagents and conditions: (i) $NaHCO_3$ [ether] 30 min (RTP) then 40% aq glyoxal, 4 h (RTP), 73%; (ii) KCN [water] 40 deg 3 h, 30%.

Table 3
Physicochemical evaluation and in vitro metabolism in human liver microsomes of representative triazines

Compd no.	In vitro CL_{int}[a] (μL/min/mg protein)	Microsome predicted E_H[a,b]	Metabolites detected[a]	MW	PSA[c] (Å^2)	pK_a[c,f]	Log D[e]		Solubility[d] (μM)	
							pH 3	pH 7.4	pH 2	pH 6.5
5	105.4	0.85	None detected	252	127.9	N/A	2.4	2.4	15.3	14.5
19	11.7	0.39	P+16	216	77.3	N/A	1.6	1.6	426	311

[a] The relative loss of parent compound and formation of metabolic products following incubation in HLM's was determined by LCMS. The concentration of test compound versus time was fitted to an exponential decay function to determine the first-order rate constant for substrate depletion. This rate constant was used to calculate an in vitro clearance (CL_{int}) and a hepatic extraction ratio (E_H) value.
[b] In vitro metabolic stability groupings: very high predicted hepatic extraction ratio E_H >0.95; high = 0.7–0.95; intermediate = 0.3–0.7; low <0.3.
[c] Values calculated with ACD Log *D* suite (version 9.12).
[d] Values measured under equilibrium solubility conditions at 25 °C.
[e] Values measured via a chromatographic Log *D* estimation method.
[f] No physiologically relevant pK_a.

Initially, we investigated activation of the thiomethyl group in **2** towards leaving through oxidation to the sulfone[7] followed by nucleophilic displacement, as shown in Scheme 3.

While the yield over two steps for both **10** and **11** was extremely low (later shown to be due to impure mCPBA), this reaction provided sufficient compound to progress to the next step and so was not optimized. Nevertheless, an alternative method was sought as shown in Scheme 4, involving synthesis of the mercaptotriazine **12** from thiosemicarbazide and glyoxal,[8] followed by alkylation of the thiol group to give either **11** or **13**.

This route proved more efficient for the synthesis of amine-tethered triazines, although the yield for this unoptimized alkylation was still relatively low and could further be improved with optimization.

Triazine monomers **10**, **11** and **13** were each then dimerized with **2** through the use of cyanide as in Scheme 2 to give homo- and hetero-dimeric product mixtures, which were then separated and tested for biological activity. As shown in Table 2, monoalkylamine tethers were all reasonably tolerated and dimers **14**, **15** and **17** had EC_{50} values of less than 100 nM. As before, the oxygen replacement of the sulfur atom was less favorable and so while the EC_{50} of **14** was 80 nM, that of **17** was 23 nM and **15** displayed extremely impressive activity in the single digit nanomolar range with an EC_{50} of 8 nM. Conversely, bis tertiary amine-tethered compounds **16** and **18** were significantly less potent with EC_{50} values in the half micromolar range. While some of these dimers are becoming moderately cytotoxic with EC_{50} values in the mid micromolar range, the selectivity for *P. falciparum* for compound **15** for example is still more than 2500-fold. Dimer **15** is 3–4 times more potent than **5**, though whether this is significant enough to imply an amine-induced localized concentration increase as designed, is hard to say.

It is tempting to speculate that compounds such as **15** could clearly have an affinity with heme and since they also structurally resemble aspects of chloroquine, that a chloroquine-like mechanism of action is responsible for their observed antimalarial activity. If this is the case, then these triazine dimers may be less active against chloroquine-resistant strains of *P. falciparum*. Shown in parentheses in Table 2 are the EC_{50} values of the compounds when tested against the chloroquine-resistant Dd2 strain of *P. falciparum*. Of particular note was that these compounds were essentially equally active against chloroquine-resistant *P. falciparum*, suggesting either a distinctive mechanism of action or that they are not similarly recognized by efflux mechanisms in Dd2 strains.

We were interested as to whether we could move away from thioethers to more drug-like systems. We have shown that an oxygen atom is not bioisosteric with a sulfur atom in this instance and wondered whether better results might be obtained with methylene replacement, which could be the case if a less electronegative and more hydrophobic atom than oxygen in the 3-position was favorable for activity.

Shown in Scheme 5 is the synthesis of the 3-ethyl dimer **19**, which was achieved by the reaction of propionimidohydrazide with glyoxal to give monomer **20**, followed by cyanide-induced dimerization.

As shown in Table 2, this dimer lost significant activity against *P. falciparum* with an EC_{50} of 1.6 μM and so it would seem that sulfur atoms are very important in the 3-position.

Finally, we were interested to investigate whether the small and relatively polar nature of these compounds may confer favorable metabolic properties to what would otherwise appear to be inherently liable systems. Shown in Table 3 are the physicochemical properties of **5** and **19**, as well as stability data from incubation with human liver microsomes. Here, it can be seen that these compounds have great potential for optimization as they are very small and simple. With their low Log *D* and high solubility at both acidic and near neutral pH, one can envisage the potential for very high systemic exposure.

However, somewhat unsurprisingly, our most active system in compound **5** with the thioethers installed is relatively unstable in the environment of the microsome with a relatively high predicted hepatic extraction ratio (E_H) of 0.85. Conversely, **19** was significantly more stable with a corresponding E_H of 0.39. Intriguingly, then, it was only **19** for which metabolites were detected, this being M+16 and therefore a likely product of oxidation (N oxide or less likely ring epoxide or alkyl hydroxyl).

In summary, we have reported the discovery of dimers of 3-substituted 1,2,4-triazines that are potently toxic to *P. falciparum* with single digit nanomolar activity and up to several thousand-fold lower toxicity to mammalian cells. They are equipotent against chloroquine-resistant strains of *P. falciparum*. They are small and highly optimizable with low Log *D* and excellent water solubility and can tolerate side-chain extension with a tertiary amino group. However, thioether groups are required for potent activity and these are a metabolic liability, as assessed in human liver microsomes. The major challenge for these compounds going forward will therefore be the maintenance of potent antimalarial activity while improving metabolic stability.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.065.

References and notes

1. Nhu, D.; Duffy, S.; Avery, V. M.; Hughes, A.; Baell, J. B. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4496.
2. Krass, D. K.; Chen, T.-K.; Paudler, W. W. *J. Heterocycl. Chem.* **1973**, *10*, 343.
3. Emilsson, H. *J. Heterocycl. Chem.* **1989**, *26*, 1077.
4. Paudler, W. W.; Chen, T.-K. *J. Heterocycl. Chem.* **1970**, *7*, 767.
5. Guiguemde, W. A.; Shelat, A. A.; Bouck, D.; Duffy, S.; Crowther, G. J.; Davis, P. H.; Smithson, D. C.; Connelly, M.; Clark, J.; Zhu, F.; Jimenez-Dıaz, M. B.; Martinez, M. S.; Wilson, E. B.; Tripathi, A. K.; Gut, J.; Sharlow, E. R.; Bathurst, I.; El Mazouni, F.; Fowble, J. W.; Forquer, I.; McGinley, P. L.; Castro, S.; Angulo-Barturen, I.; Ferrer, S.; Rosenthal, P. J.; DeRisi, J. L.; Sullivan, D. J., Jr.; Lazo, J. S.; Roos, D. S.; Riscoe, M. K.; Phillips, M. A.; Rathod, P. K.; Van Voorhis, W. C.; Avery, V. M.; Guy, R. K. *Nature* **2010**, *465*, 311.
6. Kelly, J. X.; Smilkstein, M. J.; Brun, R.; Wittlin, S.; Cooper, R. A.; Lane, K. D.; Janowsky, A.; Johnson, R. A.; Dodean, R. A.; Winter, R.; Hinrichs, D. J.; Riscoe, M. K. *Nature* **2009**, *459*, 270.
7. Taylor, E. C.; Pont, J. L.; Warner, J. C. *Tetrahedron* **1987**, *43*, 5159.
8. Heravi, M. M.; Nami, N.; Oskooie, H. A.; Hekmatshoar, R. *Phosphorus, Sulfur Silicon Relat. Elem.* **2006**, *181*, 87.