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Synthesis and antimalarial evaluation of amide and urea derivatives based on the thiaplakortone A natural product scaffold†

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A series of amide (**8–32**, **40–45**) and urea (**33**, **34**, **36–39**) analogues based on the thiaplakortone A natural product scaffold were synthesised and screened for *in vitro* antimalarial activity against chloroquine-sensitive (3D7) and chloroquine- and mefloquine-resistant (Dd2) *Plasmodium falciparum* parasite lines. Several analogues displayed potent inhibition of *P. falciparum* growth (IC_{50} <500 nM) and good selectivity for *P. falciparum versus* human neonatal foreskin fibroblast cells (selectivity index >100). Two of these compounds, **8** and **33**, exhibited good aqueous solubility and metabolic stability, and when administered subcutaneously to mice (32 mg kg^{-1}), plasma concentrations remained above 0.2 μM for at least 8 h. Both **8** and **33** were well tolerated in mice after subcutaneous administration of 32 mg kg^{-1} twice daily for 4 days. Using this regimen blood stage *P. berghei* was suppressed by 52% for **8** and 26% for **33**, relative to the vehicle control.

Introduction

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium* and is a major health and economic problem globally. An estimated 3.2 billion people living in tropical and subtropical regions of the world, such as Sub-Saharan Africa, Central and South America, the Middle East, India and South East Asia are at risk from this disease.[1] The World Health Organisation estimated that in 2010 there were 207 million clinical cases and 627 000 deaths due to malaria, with more than 90% of malaria cases and the majority of malaria deaths occurring in Sub-Saharan Africa.[2] Despite recent data on a partially effective pre-erythrocytic stage vaccine (RTS,S),[3,4] malaria prevention and treatment currently relies on small molecule drugs and vector control. It is predicted that even if the RTS,S vaccine is approved for clinical use, it will be implemented as part of a multi-pronged approach that will also include antimalarial drugs. Whilst a number of drugs are used therapeutically to treat malaria, all have now succumbed to parasite drug resistance or display reduced efficacy.[5–8] Consequently, new antimalarial drugs with novel targets are urgently needed in order to combat the global problem of parasite drug resistance.

In recent efforts to discover new antimalarial leads from nature, a high-throughput antimalarial screening campaign using a pre-fractionated natural product library[9,10] identified four novel antimalarial thiazine-derived alkaloids, thiaplakortones A–D (**1–4**; Fig. 1), from the Australian marine sponge *Plakortis lita*.[11] Thiaplakortone A (**1**) was the most active natural product with IC_{50} values of 51 and 6.6 nM against chloro-

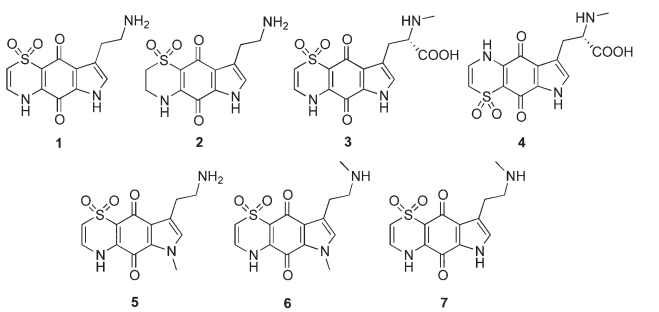

Fig. 1 Chemical structures of the natural products thiaplakortones A–D (**1–4**) and previously synthesised methyl analogues (**5–7**).

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†Electronic supplementary information (ESI) available: ^{1}H and representative ^{13}C NMR spectra of amide (**8–32**, **40–45**) and urea (**33**, **34**, **36–39**) analogues. CCDC 998930 and 998931. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c4ob01849d

quine-sensitive (3D7) and multi-drug resistant (Dd2) *P. falciparum* malaria parasite lines, respectively.[11]

The first total synthesis of **1**, along with a series of mono- and di-methyl analogues (**5–7**), has been reported and some preliminary structure–activity relationships ascertained.[12] While *in vivo* toxicity effects for several of the synthetic compounds indicated potential liabilities associated with this structure class, the limited number of analogues investigated made it difficult to assess their true potential as antimalarial leads. To more thoroughly explore this compound class we generated a series of amide and urea analogues based on the thiaplakortone A scaffold. Herein we report the synthesis of these compounds (**8–46**) along with their *in vitro* antimalarial and cytotoxicity activities, *in vivo* tolerability, ADME profiles and antimalarial efficacy in a murine malaria model.

Results and discussion

Chemistry

With the synthetic route for the lead molecule thiaplakortone A previously optimised,[12] suitable quantities of the natural product were available to commence more extensive structure–activity investigations. To address the possible toxicity associated with the primary amino group of thiaplakortone A, initial analogues focused on replacement of this motif with a series of amides and ureas.

The amide series (**8–32**) was accessed through established procedures, by either exposure of thiaplakortone A to acid anhydrides, acyl chlorides or amide formation under standard HBTU protocols (Scheme 1).[13] On scale-up, methods using HBTU were avoided, since yields were poor and purification was not trivial. These shortcomings were addressed by reaction of **1** with carbonyl imidazoles,[14] that allowed for enhanced yields and undemanding purifications. During the course of these studies, single crystal X-ray structures were obtained on the hydrochloride salt of the natural product **1** and its propyl amide analogue **9**. These data represent the first X-ray structures of this class and confirmed the previously reported NMR-based assignment[11] of the thiazine ring regiochemistry (Fig. 2).

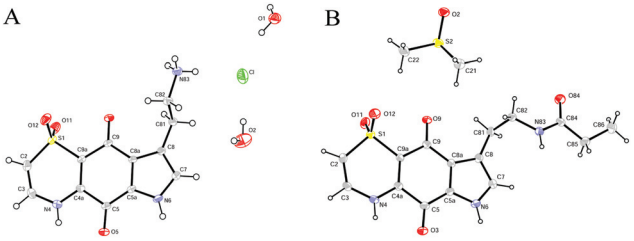


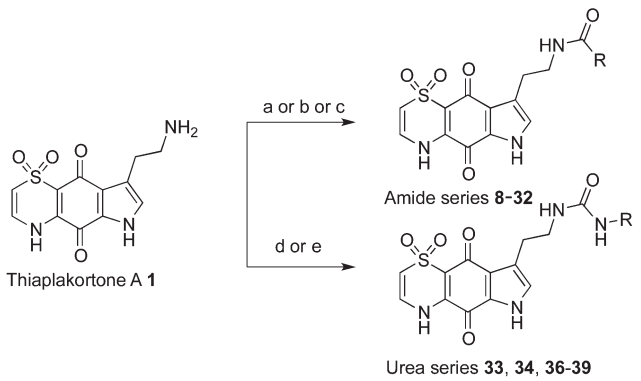
Fig. 2 ORTEP diagrams for (A) the hydrochloride salt of thiaplakortone A (**1**) and (B) the propylamide analogue (**9**).

Synthesis of analogues (**33–34, 36–39**) in the urea series was accomplished by treatment of thiaplakortone A with commercially available isocyanates. In the case of compounds **37** and **39** these molecules were synthesised using their respective carbamoyl imidazoles (Scheme 1).[15,16]

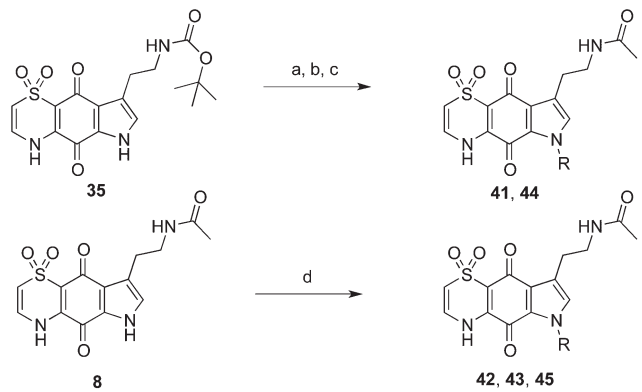
The indole-*N*-alkylated acetamides **40–45** were accessed from either indole-N-alkylation of Boc-protected thiaplakortone A (**35**)[12] followed by subsequent deprotection and acetamide formation with *N*-acetyl imidazole or from direct alkylation of the acetamide **8** (Scheme 2). Compound **40** was accessed from acetylation of the previously reported *N*-methyl analogue **5** [12] with *N*-acetylimidazole.

In vitro antimalarial activity and mammalian cell cytotoxicity

Synthetic analogues **8–45** were all evaluated for *in vitro* antimalarial activity against the chloroquine-sensitive 3D7 and the multidrug-resistant Dd2 *P. falciparum* lines. To compare the selectivity of the compounds for malaria parasites *versus* normal mammalian cells, cytotoxicity tests were carried out using the human neonatal foreskin fibroblast (NFF) cell line. All biological data, including selectivity indices (SI) are detailed in Tables 1–3.



Scheme 1 Synthesis of thiaplakortone A amide and urea analogues. Reagents and conditions: (a) acid anhydride, pyridine, dioxane, rt; (b) HBTU, DIPEA, carboxylic acid, DMF; (c) (i) carbonyl diimidazole, carboxylic acid, THF, reflux; (ii) DIPEA, DMF, rt; (d) DIPEA, isocyanate, DMF, 0 °C; (e) carbamoyl imidazole, DIPEA, DMF, rt.



Scheme 2 Synthesis of thiaplakortone A acetamide analogues. Reagents and conditions: (a) KOH, DMSO, alkyl bromide, rt; (b) (i) HCl/dioxane, 0 °C to 40 °C; (ii) NH_3(aq.) (c) *N*-acetylimidazole, DMF; (d) KOH, DMSO, alkyl bromide, rt.

Table 1 Biological profiles of amide analogues **8–32**

Compound R =		Mean IC_{50} ± SD (nM)			SI[d]	
		3D7[a]	Dd2[b]	NFF[c]	3D7	Dd2
8	Methyl	472 ± 102	182 ± 17.8	46 400 ± 12 700	98	255
9	Ethyl	703 ± 177	420 ± 50	47 000 ± 12 000	67	112
10	*n*-Propyl	439 ± 158	373 ± 41	29 000 ± 15 000	66	78
11	*c*-Propyl	1139 ± 249	783 ± 63	37 600 ± 2070	33	48
12	*i*-Propyl	537 ± 3	473 ± 129	76 800 ± 15 200	143	162
13	*n*-Butyl	644 ± 261	525 ± 98	37 000 ± 16 000	57	70
14	*i*-Butyl	537 ± 68	565 ± 64	43 600 ± 16 000	81	77
15	Cyclopentylmethyl	604 ± 27	408 ± 54	24 500 ± 3900	40	60
16	Phenyl	582 ± 99	330 ± 71	40 800 ± 2000	70	124
17	2-Fluorophenyl	445 ± 37	478 ± 80	36 100 ± 17 100	81	76
18	3-Fluorophenyl	1260 ± 137	1120 ± 329	40 400 ± 6275	34	37
19	3-Bromophenyl	1423 ± 320	888 ± 101	34 900 ± 6550	24	39
20	4-Fluorophenyl	1290 ± 253	805 ± 153	77 300 ± 18 380	60	96
21	2,5-Difluorophenyl	1300 ± 144	986 ± 218	32 724 ± 4633	25	33
22	2,6-Difluorophenyl	1090 ± 39	821 ± 185	73 290 ± 4955	67	89
23	5-Fluoro-2-morpholinophenyl	1210 ± 128	1630 ± 819	82 500 ± 841	68	51
24	2-(2-Fluorophenyl)acetyl	1300 ± 176	909 ± 188	72 120 ± 8015	55	79
25	2-Fluoro-5-(trifluoromethyl)phenyl	1957 ± 203	1490 ± 167	42 792 ± 5587	22	29
26	3-Chloro-4-methoxyphenyl	925 ± 227	619 ± 66	39 600 ± 13 740	43	64
27	2-Hydroxy-4-methoxyphenyl	1412 ± 160	1089 ± 230	35 400 ± 2500	25	32
28	3-(4-Methoxyphenyl)propyl	1633 ± 275	848 ± 235	61 300 ± 4300	38	72
29	Isoquinolin-1-yl	824 ± 136	728 ± 203	>100 000	>121	>137
30	Furan-3-yl	1079 ± 618	509 ± 308	63 600 ± 6000	59	125
31	(*S*)-5-Oxopyrrolidin-2-yl	1120 ± 380	700 ± 215	14 000 ± 2000	13	20
32	1*H*-Indol-2-yl	886 ± 324	378 ± 24	33 900 ± 4100	38	90
CQ[e]		12 ± 4	109 ± 60	46 000 ± 14 000	3833	422

[a] 3D7 = *P. falciparum* chloroquine-sensitive line. [b] Dd2 = *P. falciparum* multi-drug-resistant line. [c] NFF = neonatal foreskin fibroblast cells. [d] SI = selectivity index = NFF cell-line IC_{50}/*P. falciparum* IC_{50}. [e] CQ = chloroquine (positive control).

Table 2 Biological profiles of urea and carbamate analogues **33–39**

Compound R =		Mean IC_{50} ± SD (nM)			SI[d]	
		3D7[a]	Dd2[b]	NFF[c]	3D7	Dd2
33	N(H)*n*-Bu	709 ± 159	495 ± 119	73 000 ± 21 000	103	147
34	N(H)*t*-Bu	685 ± 101	787 ± 97	>100 000	>145	>127
35	O*t*-Bu	753 ± 105	418 ± 88	17 800 ± 2000	24	46
36	N(H)-3,4-dimethoxybenzyl	1110 ± 146	1100 ± 58	>100 000	>90	>91
37	N(Me)OMe	741 ± 127	618 ± 16	57 900 ± 21 800	78	94
38	$N(H)CH_2CO_2Et$	663 ± 98	540 ± 85	>100 000	>150	>185
39	N(H)-adamant-2-yl	631 ± 328	529 ± 194	63 000 ± 8000	100	119
CQ[e]		12 ± 4	109 ± 60	46 000 ± 14 000	3833	422

[a] 3D7 = *P. falciparum* chloroquine-sensitive line. [b] Dd2 = *P. falciparum* multi-drug-resistant line. [c] NFF = neonatal foreskin fibroblast cells. [d] SI = selectivity index = NFF cell-line IC_{50}/*P. falciparum* IC_{50}. [e] CQ = chloroquine (positive control).

Structure–activity relationships

In regards to the amide analogues (**8–32**), the series of aliphatic amides showed minimal differences in potency and selectivity as the aliphatic chain increased in length or branching. For instance, replacing the acetamide side chain in **8** with an *n*-pentylamide unit in **13** resulted in a 1.3-fold and 2.8-fold decrease in potency against the 3D7 and Dd2 lines, respectively. The SI for the straight chain amides (**8–10**, **13**) clearly showed a decreasing trend as the chain was extended from

Table 3 Biological profiles of the indole-*N*-alkylacetamide analogues **40–45**

Compound	R =	Mean IC_{50} ± SD (nM)			SI[d]	
		3D7[a]	Dd2[b]	NFF[c]	3D7	Dd2
40	Methyl	684 ± 104	706 ± 146	>100 000	>146	>142
41	Isopropyl	937 ± 160	845 ± 329	>100 000	>107	>118
42	Cyclopentyl	933 ± 149	666 ± 86	>100 000	>107	>150
43	(3-Methyloxetan-3-yl)methyl	822 ± 56	770 ± 211	>100 000	>122	>130
44	Benzyl	441 ± 69	316 ± 55	78 000 ± 10 000	178	246
45	4-Chlorobenzyl	635 ± 99	564 ± 136	>100 000	>158	>178
CQ[e]		12 ± 4	109 ± 60	46 000 ± 14 000	3833	422

[a] 3D7 = *P. falciparum* chloroquine-sensitive line. [b] Dd2 = *P. falciparum* multi-drug-resistant line. [c] NFF = neonatal foreskin fibroblast cells. [d] SI = selectivity index = NFF cell-line IC_{50}/*P. falciparum* IC_{50}. [e] CQ = chloroquine (positive control).

C_2 to C_5; for example the C_2 amide **8** displayed 3.6-fold (Dd2 line) and 1.7-fold (3D7 line) better SI values compared to the C_5 amide **13**. While substitution with cyclic sidechains, as in the cyclopropyl and cyclobutyl derivatives **11** and **15**, respectively, showed no improvement to the potency or selectivity, the isopropyl and isobutyl amides, **12** and **14**, appeared to improve selectivity. Substituting the aliphatic amide motif with benzamide structures, such as those found in **16–28**, typically resulted in less active antimalarial compounds and lower SI values. The urea/carbamate series (**33–39**), whilst still displaying *in vitro P. falciparum* growth inhibition activity, and showing SI values comparable to some of the better amide analogues, did not result in the identification of any significantly potent compounds. Several indole-*N*-alkylacetamide analogues (**40–45**) were synthesised in order to determine if N-substitution of the indoloquinone improved the parasite growth inhibition activity and/or selectivity. Analogues **40–45** were based on the acetamide scaffold **8**, which had been shown to display the best antimalarial activity and selectivity of the amide series. The indole-*N*-benzyl analogue **44** was shown to have similar or slightly better antimalarial activity and selectivity profiles towards the 3D7 and Dd2 lines compared to the acetamide analogue **8**, however the additional synthetic steps and lower yields associated with **44** precluded this compound from further evaluation.

In vitro profiling of several analogues towards an atovaquone-resistant *Plasmodium falciparum* line (C2B)

Atovaquone is a synthetic quinone-based antimalarial drug, which is typically co-administered with proguanil for the treatment of *P. falciparum* infection. The mechanism of action for atovaquone involves the inhibition of the cytochrome *bc*1 complex in *Plasmodium* parasites.[17–19] As the thiaplakortone scaffold also contains a quinone motif we examined the activity of several of our more potent compounds, which included the synthesised natural products (**1** and **2**), along with the amide (**8** and **16**) and urea analogues (**33** and **38**) against an atovaquone-resistant *P. falciparum* line (C2B).[20–22] *In vitro* screening results using the C2B line showed that **1**, **2**, **8**, **16**, **33** and **38** had IC_{50} values of 52 ± 9.8, 377 ± 49, 324 ± 70, 286 ± 26, 383 ± 108, and 331 ± 124 nM, respectively. These C2B data when compared to the 3D7 strain (Tables 1 and 2) showed only minimal differences, with most compounds displaying slightly more activity towards the C2B line (*cf.* to 3D7). Compound **39** showed the largest IC_{50} difference, however this equated to only a 2.0-fold change in IC_{50} value. These data indicate that the thiaplakortone structure class does not affect *Plasmodium* parasite growth *via* inhibition of the cytochrome *bc*1 complex.

ADME and pharmacokinetic profiling

A series of eight compounds was assessed for physicochemical (Table 4) and metabolic stability characteristics (Table 5). All compounds exhibited high total polar surface area values in excess of 120 Å^2 and a high number of hydrogen bond accepting groups, suggesting that absorption following oral administration would likely be compromised. With the exception of **32** and **39**, all compounds had relatively low log $D_{7.4}$ values (0.3–1.3) and the majority had good aqueous solubility (50–100 μg mL^{-1} or higher) at pH values representative of the gastric (pH 2.0) and intestinal (pH 6.5) environments. For **32** and **39**, log $D_{7.4}$ values

Table 4 Physicochemical properties of selected thiaplakortone analogues

Compd	PSA[a] (Å^2)	FRB[b]	HBD/HBA[c]	Log $D_{7.4}$	Solubility (μg mL^{-1}) pH 2.0/6.5
8	125	3	3/8	0.3	>100/>100
16	125	4	3/8	1.3	25–50/25–50
32	141	4	4/9	1.9	12.5–25/6.3–12.5
33	137	6	4/9	1.1	>100/>100
34	137	4	4/9	1.1	>100/>100
37	138	4	3/10	0.7	>100/50–100
38	163	7	4/11	0.7	>100/>100
39	137	4	4/9	2.3	6.3–12.5/6.3–12.5

[a] Total polar surface area. [b] Freely rotatable bonds. [c] Hydrogen bond donors/hydrogen bond acceptors.

Table 5 *In vitro* metabolic stability of selected thiaplakortone analogues in NADPH supplemented mouse liver microsomes

Compd	Species	Half-life (min)	*In vitro* CL_{int} (μL min^{-1} mg^{-1} protein)
8	Mouse	>240	<7
16	Mouse	185	9
32	Mouse	17	104
33	Mouse	167	10
34	Mouse	211	8
37	Mouse	>240	<7
38	Mouse	>240	<7
39	Mouse	1	1413
CQ[a]	Mouse	177	10.2

[a] CQ = chloroquine.

were higher (1.9 and 2.3, respectively) and aqueous solubility values were poor at both pH 2 and 6.5 (<12.5 μg mL^{-1}).

The metabolic stability of the same eight compounds was then assessed *in vitro* using NADPH supplemented mouse liver microsomes.[23] All of the compounds exhibited good metabolic stability (*in vitro* intrinsic clearance (CL_{int}) of ≤10 μL min^{-1} mg^{-1} protein) with the exception of **32** and **39**, which both exhibited a high degree of metabolic lability (CL_{int} > 100 μL min^{-1} mg^{-1} protein). Two compounds, the amide analogue **8** and the urea analogue **33**, were progressed to an *in vivo* study where they were administered to mice at a dose of 32 mg kg^{-1} *via* either the oral or subcutaneous route (Fig. 3). The apparent half-lives were approximately 2 h, and as expected given the physicochemical properties, the exposure [area under the curve (AUC) over 8 h and maximum plasma concentration (C_{max})] following oral administration was more than 10-fold lower than that after subcutaneous dosing. Following subcutaneous administration, plasma concentrations for each compound remained above 0.2 μM for at least 8 h.

In vivo tolerability studies in mice

The amide (**8**, **16**, **32**) and urea (**33**, **34**, **37**, **38**) analogues were evaluated for tolerability in mice at 8 mg kg^{-1}, 16 mg kg^{-1} and 32 mg kg^{-1} given twice daily for 4 days by subcutaneous administration. At these doses the analogues were found to be well tolerated in healthy mice with no observable adverse events such as tremors, panting, loss of weight (>20%) and lack of natural movement. These findings are in contrast to the poor tolerance seen with the methyl derivatives of thiaplakortone A reported elsewhere.[12]

In vivo efficacy studies in the *P. berghei* malaria mouse model

Based on the favorable tolerability findings of the thiaplakortone A amide derivatives (**8**, **16**, **32**) and urea (**33**, **34**, **37**, **38**) analogues, the efficacy of these compounds was assessed in the rodent-*P. berghei* model using the highest tested dose of 32 mg kg^{-1}. Compounds were administered subcutaneously twice daily for 4 days. Of the amide analogues tested, **8** was the most active with a mean suppression of blood stage parasitemia of 52% on D + 4 post inoculation of *P. berghei* to mice. The next most active amide analogue was **32** with 11% suppression on D + 4. Fig. 4 compares the mean parasitemia profile of *P. berghei* infected mice treated with either **8**, chloroquine (positive control) or drug-free vehicle (control). Chloroquine suppression at D + 4 was 65% at 1 mg kg^{-1} day^{-1} given subcutaneously, which is comparable to the 50% suppression at 1.5 mg kg^{-1} day^{-1} described by Ridley *et al.*[24]

Of the urea analogues tested, **33** was the most active with 26% suppression of parasitemia on D + 4 following subcutaneous administration. Analogues **37** and **38** had similar suppression values of 16% and 14%, respectively, on D + 4. When orally administered at 32 mg kg^{-1} twice daily for 4 days both compounds **8** and **33** did not significantly inhibit parasitemia (<3%) on D + 4.

Conclusions

In summary, 31 amide and 7 urea derivatives based on the thiaplakortone A tricyclic scaffold were synthesised and evaluated for their *in vitro* antimalarial activity and mammalian cell

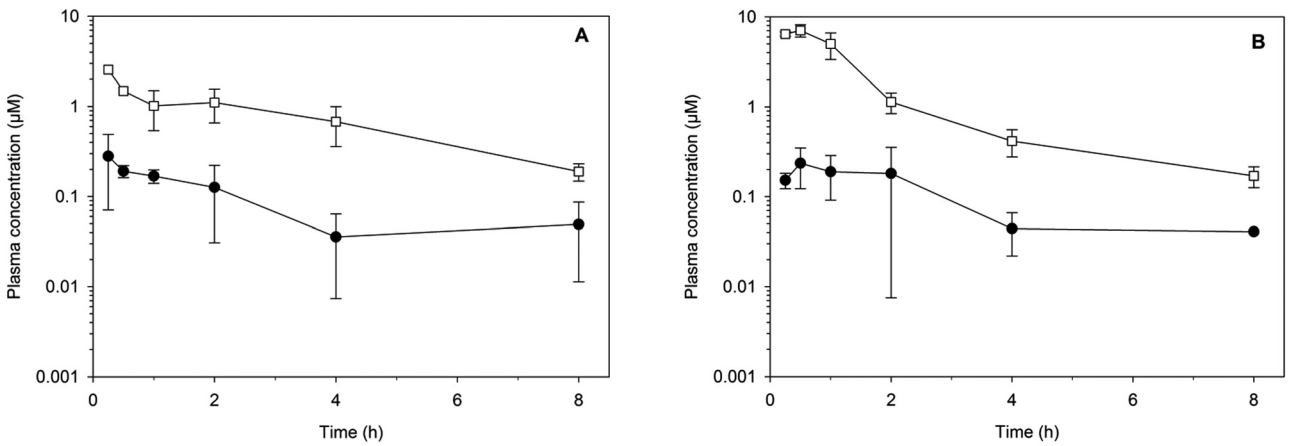


Fig. 3 Plasma concentration *versus* time profiles for (A) **8** and (B) **33**. Symbols represent concentrations after oral (filled circles) or subcutaneous (open squares) administration of 32 mg kg^{-1} (mean ± SD, n = 3).

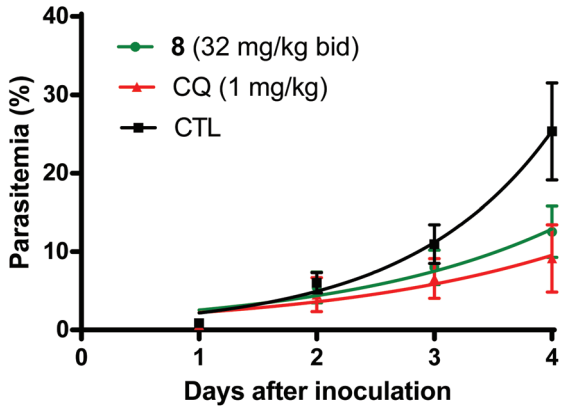


Fig. 4 Mean (±SD, n = 6) parasitemia profile of *P. berghei* infected mice treated with either compound **8** (32 mg kg^{-1} twice daily for 4 days), the positive control chloroquine (CQ, 1 mg kg^{-1} daily for 4 days) or drug-free vehicle (CTL).

toxicity. Of these compounds, eight were chosen for ADME profiling after which several analogues were prioritised for *in vivo* pharmacokinetic analysis, followed by tolerability and antimalarial efficacy in a murine malaria model. Compounds **8** and **33** were both well tolerated in mice when administered subcutaneously at 32 mg kg^{-1} twice daily for 4 days. Furthermore, using this regimen blood stage *P. berghei* was suppressed by 52% for **8** and 26% for **33**, relative to the vehicle control. Unfortunately, oral administration of these compounds at 32 mg kg^{-1} twice daily for 4 days did not suppress parasitemia, most likely due to poor oral bioavailability and limited exposure. While these data highlight the potential of the natural product derived thiazino-quinone scaffold as an anti-malarial starting point, the lack of oral bioavailability currently impinges on the further development of this series.

Experimental

General procedures

Melting points were recorded on a capillary melting point apparatus and are uncorrected. Unless otherwise specified, ^{1}H and ^{13}C NMR spectra were recorded at 30 °C in DMSO-d_6 on a Varian INOVA 500 NMR spectrometer. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.50 and δ_C 39.5. LRESIMS was obtained from LC-MS data generated using a Waters Alliance 2790 HPLC equipped with a Waters 996 photodiode array detector and an Alltech evaporative light scattering detector that was attached to a Water ZQ mass spectrometer. HRESIMS were recorded on a Bruker MicrOTof-Q spectrometer (Dionex UltiMate 3000 micro LC system, ESI mode). Analytical thin layer chromatography (TLC) was performed on aluminum-backed 0.2 mm thick silica gel 60 F_{254} plates as supplied by Merck. Eluted plates were visualised using a 254 nm UV lamp and/or by treatment with a suitable dip followed by heating. These dips included phosphomolybdic acid–$Ce(SO_4)_2$–H_2SO_4 (conc.)–H_2O (37.5 g : 7.5 g : 37.5 g : 720 mL) or $KMnO_4$–K_2CO_3–5% NaOH aqueous solution–H_2O (3 g : 20 g : 5 mL : 300 mL). Flash chromatographic separations were carried out following protocols defined by Still *et al.*[25] with silica gel 60 (40–63 mm, supplied by GRACE) or amino bonded silica gel (Davisil®) as the stationary phase and using the AR- or HPLC-grade solvents indicated. Semi-preparative HPLC work was performed using a Waters 600 pump and 966 PDA detector, a Gilson 715 liquid handler and a C_{18}-bonded silica Betasil 5 μm 143 Å column (21.2 × 150 mm). Alltech sample preparative C_{18}-bonded silica (35–75 μm, 150 Å) and an Alltech stainless steel guard cartridge (10 × 30 mm) were used for preadsorption and HPLC work. A Phenomenex C_{18}-bonded silica Luna 3 μm 100 Å (4.6 × 50 mm) column was used for LC-MS studies. All compounds were analysed for purity using LC-MS and shown to be >95% pure, unless otherwise stated. Starting materials and reagents were generally available from the Sigma-Aldrich, Merck, AK Scientific Inc., Matrix Scientific Chemical Companies and were used as supplied. THF, MeOH and DCM were dried using a Glass Contour solvent purification system that is based upon a technology originally described by Grubbs *et al.*[26] Triethylamine (TEA) was freshly distilled over calcium hydride before use. Where necessary, reactions were performed under a nitrogen atmosphere and glassware was heated in an oven at 140 °C then dried under vacuum prior to use. Temperatures quoted as 0 °C and −78 °C were obtained by cooling the reaction vessel in baths of ice/H_2O and $CO_{2(s)}$/acetone, respectively. Compounds for biological studies were place under high vacuum (0.05 mmHg) for several hours before testing to remove trace, residual solvents.

General procedure A – HBTU mediated amide formation. A solution of carboxylic acid (46 μmol) in DMF (1 mL) and *N*,*N*-diisopropylethylamine (DIPEA) (25 μL, 182 μmol), was treated with HBTU (21 mg, 54 μmol) in one portion and stirred for 1 h at rt. Thiaplakortone A hydrochloride (15 mg, 46 μmol) was added and stirred for a further 2 h. The mixture was concentrated *in vacuo* and then the residue was triturated with a solution of DCM–ether (1 : 3, 3 × 1 mL). The remaining solid was purified by flash chromatography (silica, 1 : 5 v/v MeOH–DCM elution) to afford the amide.

General procedure B – acid anhydride mediated amide formation. A mixture of thiaplakortone A hydrochloride (20 mg, 61 μmol), acid anhydride (100 μL), pyridine (1 mL) and 1,4-dioxane (1 mL) was magnetically stirred for 24 h. The mixture was concentrated *in vacuo* to afford a residue that was purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford the amide.

General procedure C – isocyanate mediated urea formation. A suspension of thiaplakortone A hydrochloride (10 mg 30 μmol) in DMF (1 mL), was treated with DIPEA (10 μL) and stirred vigorously for 5 min. The mixture was then cooled to 0 °C and treated with isocyanate (33 μmol) in one portion. After 1 h the solution was concentrated *in vacuo* and the residue purified by flash chromatography (amino-bonded silica, 1 : 10 v/v MeOH–DCM elution) to afford the urea.

General procedure D – carbonyl imidazole mediated amide formation. A solution of carboxylic acid (0.66 mmol) and 1,1′-carbonyldiimidazole (98 mg, 0.60 mmol) in anhydrous THF

(2 mL) was refluxed for 1 h and then cooled to rt. This solution was then transferred *via* syringe to a mixture of thiaplakortone A hydrochloride (200 mg, 0.60 mmol) and DIPEA (93 μL, 0.66 mmol) in DMF (6 ml) and the reaction was magnetically stirred for 18 h. The mixture was concentrated to dryness *in vacuo* and the residue purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford the amide.

General procedure E – freebasing protocol. A solution of amine hydrochloride (0.4 mmol) in H_2O (2 mL) was treated with aqueous NH_3 (1 mL, 30% w/w) and stirred for 0.25 h. The solution was then concentrated *in vacuo* to afford the amine that was used without further purification.

General procedure F – Boc deprotection protocol. Boc-protected amine (45 μmol) was treated with HCl (1 mL, 4 M solution in 1,4-dioxane) at 0 °C and stirred for 0.5 h. The mixture was warmed to 40 °C and then concentrated with a gentle stream of nitrogen (CAUTION! Highly corrosive vapours). The resulting residue was then triturated with ether (3 × 1 mL) and dried *in vacuo* to afford the amine that was used without further purification.

General procedure G – indole N-alkylation protocol. A solution of indole-quinone (39 μmol) in anhydrous DMSO (1 mL) was treated with fine, freshly ground KOH (4.4 mg, 78 μmol) and alkyl halide (47 μmol) and stirred vigorously for 18 h. The DMSO was removed *in vacuo* and the residue purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford the *N*-alkylated analogues.

Reversed-phase HPLC method 1. Isocratic conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) were employed for the first 10 min, then a linear gradient to MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of MeOH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL min^{-1}. Sixty fractions (60 × 1 min) were collected by time from the start of the HPLC run. All UV active fractions were dried down and analyzed by ^{1}H NMR spectroscopy, with relevant fractions combined.

Reversed-phase HPLC method 2. Isocratic conditions of 95% H_2O (0.1% TFA)/5% MeOH (0.1% TFA) were employed for the first 10 min, followed by a 40 min linear gradient to 50% H_2O (0.1% TFA)/50% MeOH (0.1% TFA) then isocratic conditions of MeOH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL min^{-1}. Sixty fractions (60 × 1 min) were collected by time from the start of the HPLC run. All UV active fractions were dried down and analyzed by ^{1}H NMR spectroscopy, with relevant fractions combined.

Compound 1. The hydrochloride salt of thiaplakortone A was prepared in an analogous method to that previously reported in the literature.[12] The NMR and MS data of **1** were identical to the literature values.[12] Recrystallised from H_2O to afford brown crystals; mp decomp. >280 °C.

Compound 8. Prepared using a method analogous to General Procedure B, orange powder (7 mg, 59%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.92 (br s, 1H), 10.99 (br s, 1H), 7.86 (t, J = 5.5 Hz, 1H), 7.29 (s, 1H), 7.05 (d, J = 9.0 Hz, 1H), 6.41 (d, J = 9.0 Hz, 1H), 3.26 (dt, J = 5.5, 7.0 Hz, 2H), 2.82 (t, J = 7.0 Hz, 2H), 1.77 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 169.1, 167.6, 139.9, 130.2, 128.8, 127.7, 124.6, 123.3, 114.2, 111.6, 38.4, 25.4, 22.6; (+)-LRESIMS m/z (rel. int.) 336 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{14}H_{13}N_3NaO_5S$ $[M + Na]^+$ 358.0468, found 358.0472.

Compound 9. Prepared according to General Procedure B, yellow powder (11 mg, 52%). Recrystallised from DMSO to afford orange crystals; mp decomp. >280 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.63 (br s, 1H), 11.26 (br s, 1H), 7.77 (t, J = 5.4 Hz, 1H), 7.28 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.29–3.26 (m, 2H), 2.82 (t, J = 7.1 Hz, 2H), 2.04 (q, J = 7.6 Hz, 2H), 0.97 (t, J = 7.6 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 172.7, 167.7, 140.1, 130.4, 128.7, 127.7, 124.6, 123.3, 114.2, 111.5, 38.3, 28.5, 25.3, 9.9. (−)-LRESIMS m/z (rel. int.) 348 (100) $[M - H]^-$; (+)-HRESIMS calcd for $C_{15}H_{15}N_3NaO_5S$ $[M + Na]^+$ 372.0625, found 372.0624.

Compound 10. Prepared according to General Procedure B, yellow powder (11 mg, 51%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.84 (br s, 1H), 11.05 (br s, 1H), 7.79 (t, J = 5.5 Hz, 1H), 7.28 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.29–3.26 (m, 2H), 2.82 (t, J = 7.1 Hz, 2H), 2.01 (t, J = 7.3 Hz, 2H), 1.53–1.43 (m, 2H), 0.82 (t, J = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 171.8, 167.5, 139.9, 130.1, 128.8, 127.7, 124.6, 123.2, 114.2, 111.5, 38.2, 37.3, 25.4, 18.6, 13.5; (−)-LRESIMS m/z (rel. int.) 362 (100) $[M - H]^-$; (−)-HRESIMS calcd for $C_{16}H_{16}N_3O_5S$ $[M - H]^-$ 362.0816, found 362.0813.

Compound 11. A magnetically stirred mixture of thiaplakortone A hydrochloride (10 mg, 30 μmol), pyridine (0.5 mL) and dioxane (0.5 mL) maintained under nitrogren at 0 °C was treated with cyclopropanecarbonyl chloride (6.3 mg, 61 μmol) and warmed to rt. The mixture was stirred for 18 h then concentrated *in vacuo* to afford a residue that was purified by flash chromatography (silica, 1 : 5 v/v MeOH–DCM elution) to afford compound **11** (1.5 mg, 14%) as a brown powder. ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed.* δ 8.08 (t, J = 5.6 Hz, 1H), 7.27 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.40 (dd, J = 8.8, 1.4 Hz, 1H), 3.32–3.28 (m, 2H, *CH_2N– obscured by H_2O*) 2.84 (t, J = 7.2 Hz, 2H), 1.54–1.45 (m, 1H), 0.68–0.57 (m, 4H); (−)-LRESIMS m/z (rel. int.) 360 (100) $[M - H]^-$; (+)-HRESIMS calcd for $C_{16}H_{15}N_3NaO_5S$ $[M + Na]^+$ 384.0625, found 384.0622.

Compound 12. Prepared according to General Procedure B, yellow powder (9 mg, 41%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.86 (br s, 1H), 11.02 (br s, 1H), 7.73 (t, J = 5.5 Hz, 1H), 7.26 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.28–3.25 (m, 2H), 2.83 (t, J = 7.1 Hz, 2H), 2.30 (hept, J = 6.8 Hz, 1H), 0.96 (d, J = 6.8 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 175.9, 167.5, 139.9, 130.1, 128.8, 127.7, 124.6, 123.3, 114.2, 111.5, 38.1, 33.9, 25.3, 19.51 (2C); (−)-LRESIMS m/z (rel. int.) 362 (100) $[M - H]^-$; (−)-HRESIMS calcd for $C_{16}H_{16}N_3O_5S$ $[M - H]^-$ 362.0816, found 362.0819.

Compound 13. Prepared according to General Procedure B, orange powder (8 mg, 35%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.90 (br s, 1H), 11.07 (br s, 1H), 7.78 (t, J = 5.5 Hz, 1H), 7.27 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.32–3.26 (m, 2H), 2.82 (t, J = 7.0 Hz, 2H), 2.02 (t, J = 7.4 Hz, 2H), 1.48–1.40 (m, 2H), 1.26–1.17 (m, J = 14.6, 7.4 Hz, 2H), 0.84 (t, J = 7.4 Hz, 3H); (+)-LRESIMS m/z (rel. int.) 378 (100) [M

+ H]$^+$; (+)-HRESIMS calcd for $C_{17}H_{19}N_3NaO_5S$ [M + Na]$^+$ 400.0938, found 400.0937.

Compound 14. Prepared according to General Procedure B, orange powder (7 mg, 31%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.87 (br s, 1H), 11.03 (br s, 1H), 7.80 (t, J = 5.6 Hz, 1H), 7.29 (s, 1H), 7.06 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.33–3.24 (m, 2H), 2.84 (t, J = 7.0 Hz, 2H), 1.98–1.90 (m, 3H), 0.84 (d, J = 6.2 Hz, 6H); (−)-LRESIMS m/z (rel. int.) 376 (100) [M − H]$^-$; (−)-HRESIMS calcd for $C_{17}H_{18}N_3O_5S$ [M − H]$^-$ 376.0973, found 376.0970.

Compound 15. Prepared according to General Procedure A, yellow-orange powder (8 mg, 41%); ^{1}H NMR (500 MHz, DMSO-d_6) 12.93 (br s, 1H), 10.99 (br s, 1H), 7.77 (t, J = 5.6 Hz, 1H), 7.28 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.29–3.26 (m, *CH_2N– obscured by H_2O*, 2H), 2.82 (t, J = 7.0 Hz, 2H), 2.11–2.06 (m, 1H), 2.02 (d, J = 7.3 Hz, 2H), 1.67–161 (m, 2H), 1.58–1.50 (m, 2H), 1.49–1.43 (m, 2H), 1.11–1.53 (m, 2H); (+)-LRESIMS m/z (rel. int.) 404 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{19}H_{21}N_3NaO_5S$ [M + Na]$^+$ 426.1094, found 426.1088.

Compound 16. A mixture of thiaplakortone A hydrochloride (100 mg, 0.30 mmol), anhydrous DMF (5 mL) and benzoic anhydride (460 mg, 1.82 mmol, 90% grade) was treated with TEA (500 μL, excess) and stirred overnight under an atmosphere of nitrogen. The mixture was concentrated *in vacuo* and purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford the compound **16** (54 mg, 45%) as a yellow powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.91 (br s, 1H), 11.00 (br s, 1H), 8.48 (t, J = 5.6 Hz, 1H), 7.81 (d, J = 7.3 Hz, 2H), 7.52–7.48 (m, 1H), 7.46–7.42 (m, 2H), 7.31 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.7 Hz, 1H), 3.53 (app. q, J = 6.8 Hz, 2H), 2.98 (t, J = 7.0 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.5, 167.5, 166.1, 139.9, 134.5, 130.9, 130.1, 128.8, 128.2 (2C), 127.7, 127.1 (2C), 124.6, 123.3, 114.2, 111.6, 39.1, 25.2. (+)-LRESIMS m/z (rel. int.) 398 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{19}H_{15}N_3NaO_5S$ [M + Na]$^+$ 420.0625, found 420.0623.

Compound 17. Prepared according to General Procedure A, yellow powder (11 mg, 58%); ^{1}H NMR (500 MHz, DMSO-d_6) 12.95 (br s, 1H), 11.00 (br s, 1H), 8.34 (t, J = 5.1 Hz, 1H), 7.57–7.54 (m, 1H), 7.52–7.48 (m, 1H), 7.33 (s, 1H), 7.29–7.21 (m, 2H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.51 (app. q, J = 7.0 Hz, 2H), 2.96 (t, J = 7.0 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 416 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{19}H_{14}FN_3NaO_5S$ [M + Na]$^+$ 438.0530, found 438.0527.

Compound 18. Prepared using a method analogous to General Procedure A, yellow powder (2.7 mg, 51%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.76 (br s, 1H), 11.13 (br s, 1H), 8.59 (t, J = 4.8 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.60 (d, J = 10.1 Hz, 1H), 7.52–7.48 (m, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.31, (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.54–3.50 (m, 2H), 2.97 (t, J = 6.8 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 438 (100) [M + Na]$^+$; (+)-HRESIMS calcd for $C_{19}H_{14}FN_3NaO_5S$ [M + Na]$^+$ 438.0530, found 438.0526.

Compound 19. Prepared using a method analogous to General Procedure A, brown powder (6 mg, 42%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.88 (br s, 1H), 10.98 (br s, 1H), 8.63 (t, J = 5.5 Hz, 1H), 7.99 (t, J = 1.6 Hz, 1H), 7.82 (d, J = 7.9 Hz, 1H), 7.71 (d, J = 8.2 Hz, 1H), 7.42 (t, J = 7.9 Hz, 1H), 7.31 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 9.0 Hz, 1H), 3.52 (app. q, J = 6.9 Hz, 2H), 2.97 (t, J = 6.9 Hz, 2H); (−)-LRESIMS m/z (rel. int.) 474 (100), 476 (100) [M − H]$^-$; (+)-HRESIMS calcd for $C_{19}H_{14}{}^{79}BrN_3NaO_5S$ [M + Na]$^+$ 497.9730, found 497.9728; (+)-HRESIMS calcd for $C_{19}H_{14}{}^{81}BrN_3NaO_5S$ [M + Na]$^+$ 499.9709, found 499.9702.

Compound 20. Prepared using a method analogous to General Procedure A, yellow powder (2 mg, 17%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.87 (br s, 1H), 10.97 (br s, 1H), 8.51 (t, J = 5.2 Hz, 1H), 7.89 (dd, J = 8.6, 5.4$_{HF}$ Hz, 2H), 7.31 (s, 1H), 7.27 (app. t, J = 8.8 Hz, 2H), 7.06 (d, J = 8.9 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.54–3.49 (m, 2H), 2.97 (t, J = 6.9 Hz, 2H). (+)-LRESIMS m/z (rel. int.) 416 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{19}H_{14}FN_3NaO_5S$ [M + Na]$^+$ 438.0530, found 438.0527.

Compound 21. Prepared using a method analogous to General Procedure A, yellow powder (2 mg 18%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.87 (br s, 1H), 11.02 (br s, 1H), 8.45 (t, J = 5.1 Hz, 1H), 7.39–7.31 (m, 4H), 7.06 (d, J = 8.8 Hz, 1H) 6.43 (d, J = 8.8 Hz, 1H), 3.54–3.49 (m, 2H), 2.96 (t, J = 6.9 Hz, 2H); (−)-LRESIMS m/z (rel. int.) 432 (100) [M − H]$^-$; (+)-HRESIMS calcd for $C_{19}H_{13}F_2N_3NaO_5S$ [M + Na]$^+$ 456.0436, found 456.0428.

Compound 22. Prepared using a method analogous to General Procedure A, yellow powder (1 mg, 11%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.86 (br s, 1H), 11.06 (br s, 1H), 8.77 (t, J = 5.3 Hz, 1H), 7.53–7.46 (m, 1H), 7.31 (s, 1H), 7.15 (t, J = 7.8 Hz, 2H), 7.06 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.6 Hz, 1H), 3.53–3.49 (m, 2H), 2.94 (t, J = 7.0 Hz, 2H); (−)-LRESIMS m/z (rel. int.) 432 (100) [M − H]$^-$; (+)-HRESIMS calcd for $C_{19}H_{13}F_2N_3NaO_5S$ [M + Na]$^+$ 456.0436, found 456.0428.

Compound 23. Prepared using a method analogous to General Procedure A, brown powder (3 mg, 21%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.86 (br s, 1H), 11.06 (br s, 1H), 9.47 (t, J = 5.0 Hz, 1H), 7.47 (d, J = 8.6 Hz, 1H), 7.38 (s, 1H), 7.35–7.27 (m, 2H), 7.06 (d, J = 8.4 Hz, 1H), 6.42 (d, J = 8.9 Hz, 1H), 3.66–3.61 (m, 4H), 3.63–3.58 (m, 2H), 3.01 (t, J = 6.4 Hz, 2H), 2.89–2.83 (m, 4H); (+)-LRESIMS m/z (rel. int.) 501 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{23}H_{21}FN_4NaO_6S$ [M + Na]$^+$ 523.1058, found 523.1052.

Compound 24. Prepared using a method analogous to General Procedure A, yellow powder (2 mg, 15%), ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.93 (br s, 1H), 11.00 (br s, 1H), 8.09 (t, J = 5.4 Hz, 1H), 7.31–7.23 (m, 3H), 7.16–7.10 (m, 2H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.44 (s, 2H), 3.35–3.28 (m, 2H), 2.85 (t, J = 7.0 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 430 (100) [M + H]$^+$; (+)-HRESIMS calcd for $C_{20}H_{16}FN_3NaO_5S$ [M + Na]$^+$ 452.0687, found 452.0684.

Compound 25. Prepared using a method analogous to General Procedure A, yellow powder (2 mg, 12%); ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 8.60–8.55 (m, 1H), 7.93–7.85 (m, 2H), 7.53 (t, J = 9.0 Hz, 1H), 7.33 (s, 1H), 7.06 (d, J = 8.2 Hz, 1H), 6.39 (d, J = 8.0 Hz, 1H), 3.56–3.49 (m, 2H), 2.97 (t, J = 6.6 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 506 (100) [M + Na]$^+$; (+)-HRESIMS calcd for $C_{20}H_{13}F_4N_3NaO_5S$ [M + Na]$^+$ 506.0404, found 506.0409.

Compound 26. Prepared according to General Procedure A, yellow-orange powder (8 mg, 37%); ^{1}H NMR (500 MHz, DMSO-d_6) 12.93 (br s, 1H), 11.00 (br s, 1H), 8.48 (t, J = 5.5 Hz, 1H), 7.91 (d, J = 2.1 Hz, 1H), 7.82 (dd, J = 8.7, 2.2 Hz, 1H), 7.31 (s, 1H), 7.21 (d, J = 8.6 Hz, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.91 (s, 3H), 3.50 (app. q, J = 6.5 Hz, 2H), 2.96 (t, J = 7.1 Hz, 2H). (+)-LRESIMS m/z (rel. int.) 462 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{20}H_{16}ClN_3NaO_6S$ $[M + Na]^+$ 484.0341, found 484.0349.

Compound 27. Prepared according to General Procedure A, brown powder (1 mg, 3%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.06 (br s, 1H), 12.93 (br s, 1H), 11.02 (br s, 1H), 8.67 (t, J = 5.4 Hz, 1H), 7.76 (d, J = 8.9 Hz, 1H), 7.32 (s, 1H), 7.06 (d, J = 8.8 Hz, 1H), 6.48–6.38 (m, 3H), 3.77 (s, 3H), 3.54 (app. q, J = 7.0 Hz, 2H), 2.99 (t, J = 7.0 Hz, 2H). (+)-LRESIMS m/z (rel. int.) 444 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{20}H_{17}N_3NaO_7S$ $[M + Na]^+$ 466.0679, found 466.0675.

Compound 28. Prepared according to General Procedure A, yellow powder (11 mg, 51%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.82 (br s, 1H), 11.03 (br s, 1H), 7.80 (t, J = 5.6 Hz, 1H), 7.28 (s, 1H), 7.07–7.04 (m, 2H), 7.05 (d, J = 8.8 Hz, 1H), 6.83–6.80 (m, 2H), 6.41 (d, J = 8.8 Hz, 1H), 3.71 (s, 3H), 3.39–3.32 (m, 2H, *CH_2N– obscured by H_2O*) 2.83 (t, J = 7.0 Hz, 2H), 2.43 (t, J = 7.6 Hz, 2H), 2.03 (t, J = 7.4 Hz, 2H), 1.72 (app. p, J = 7.5 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 470 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{23}H_{23}N_3NaO_6S$ $[M + Na]^+$ 492.1200, found 492.1202.

Compound 29. Prepared according to General Procedure A, brown powder (5 mg, 25%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.93 (br s, 1H), 10.99 (br s, 1H), 8.87 (t, J = 5.9 Hz, 1H), 8.81 (d, J = 8.8 Hz, 1H), 8.52 (d, J = 5.6 Hz, 1H), 8.03 (d, J = 8.3 Hz, 1H), 7.99 (d, J = 5.7 Hz, 1H), 7.81 (ddd, J = 8.2, 6.9, 1.2 Hz, 1H), 7.70 (ddd, J = 8.4, 6.8, 1.4 Hz, 1H), 7.38 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.64 (app. q, J = 6.7 Hz, 2H), 3.05 (t, J = 6.9 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 167.6, 166.1, 151.5, 140.8, 134.0, 136.4, 130.5, 130.2, 128.8, 128.2, 127.7, 127.0, 126.5, 125.3, 124.5, 123.4, 123.0, 114.2, 111.5, 38.6, 25.2. (+)-LRESIMS m/z (rel. int.) 449 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{22}H_{16}N_4NaO_5S$ $[M + Na]^+$ 471.0734, found 471.0732.

Compound 30. Prepared using a method analogous to General Procedure A, yellow powder (5 mg, 43%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.91 (br s, 1H), 11.02 (br s, 1H), 8.20 (t, J = 5.7 Hz, 1H), 8.13 (d, J = 1.0 Hz, 1H), 7.69 (t, J = 1.7 Hz, 1H), 7.30 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.82 (d, J = 1.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 3.46 (app. q, J = 6.6 Hz, 2H), 2.94 (t, J = 7.1 Hz, 2H); (+)-LRESIMS m/z (rel. int.) 388 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{17}H_{13}N_3NaO_6S$ $[M + Na]^+$ 410.0417, found 410.0413.

Compound 31. Prepared using a method analogous to General Procedure A, brown powder (2.5 mg, 20%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.86 (br s, 1H), 11.00 (br s, 1H), 8.00 (app. t, J = 5.2 Hz, 1H), 7.71 (s, 1H), 7.30 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.40 (d, J = 8.7 Hz, 1H), 3.93 (dd, J = 8.5, 4.5 Hz, 1H), 3.37–3.31 (m, 2H), 2.85 (t, J = 7.2 Hz, 2H), 2.24–2.01 (m, 3H), 1.84–1.77 (m, 1H); (+)-LRESIMS m/z (rel. int.) 405 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{17}H_{16}N_4NaO_6S$ $[M + Na]^+$ 427.0683, found 427.0689.

Compound 32. Prepared according to General Procedure D, maroon powder (100 mg, 38%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.90 (br s, 1H), 11.49 (s, 1H), 11.01 (br s, 1H), 8.47 (t, J = 5.8 Hz, 1H), 7.59 (d, J = 7.8 Hz, 1H), 7.41 (d, J = 8.4 Hz, 1H), 7.32 (s, 1H), 7.16 (t, J = 7.6 Hz, 1H), 7.08 (d, J = 1.2 Hz, 1H), 7.05 (d, J = 8.8 Hz, 1H), 7.02 (t, J = 7.3 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.56 (app. q, J = 6.6 Hz, 2H), 3.00 (t, J = 6.8 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.6, 167.5, 161.0, 139.9, 136.3, 131.7, 130.1, 128.9, 127.7, 127.1, 124.6, 123.3, 123.1, 121.4, 119.6, 114.2, 112.2, 111.6, 102.4, 38.7, 25.3; (+)-LRESIMS m/z (rel. int.) 437 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{21}H_{16}N_4NaO_5S$ $[M + Na]^+$ 459.0734, found 459.0729.

Compound 33. Prepared according to General Procedure C, orange powder (8 mg, 68%); ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 7.26 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.38 (d, J = 8.8 Hz, 1H), 5.78 (t, J = 5.8 Hz, 1H), 5.75 (t, J = 5.7 Hz, 1H), 3.25–3.20 (m, 2H), 2.99–2.93 (m, 2H), 2.79 (t, J = 7.0 Hz, 2H), 1.36–1.29 (m, 2H), 1.29–1.20 (m, 2H), 0.86 (t, J = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 167.5, 158.0, 139.9, 130.1, 128.8, 127.7, 124.9, 123.2, 114.2, 111.5, 39.0, 38.9, 32.1, 26.3, 19.5, 13.7; (–)-LRESIMS m/z (rel. int.) 391 (100) $[M - H]^-$; (+)-HRESIMS calcd for $C_{17}H_{20}N_4NaO_5S$ $[M + Na]^+$ 415.1047, found 415.1047.

Compound 34. Prepared according to General Procedure C, orange powder (4 mg, 34%); ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 7.27 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.40 (d, J = 8.8 Hz, 1H), 5.63 (t, J = 5.7 Hz, 1H), 5.58 (s, 1H), 3.22–3.15 (m, 2H), 2.77 (t, J = 7.0 Hz, 2H), 1.20 (s, 9H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 167.5, 157.3, 139.9, 130.1, 128.9, 127.6, 124.9, 123.2, 114.2, 111.5, 48.9, 38.7, 29.3 (3C), 26.32; (–)-LRESIMS m/z (rel. int.) 391 (100) $[M - H]^-$; (–)-HRESIMS calcd for $C_{17}H_{19}N_4O_5S$ $[M - H]^-$ 391.1082, found 391.1084.

Compound 35. Boc-protected thiaplakortone A was prepared in an analogous method to that previously reported in the literature.[12] The NMR and MS data of **35** were identical to the literature values.[12]

Compound 36. Prepared according to General Procedure C, yellow powder (5 mg, 34%); ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 7.27 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.84 (d, J = 1.6 Hz, 1H), 6.74 (dd, J = 8.1, 1.5 Hz, 1H), 6.39 (d, J = 8.8 Hz, 1H), 6.20 (t, J = 5.6 Hz, 1H), 5.93 (t, J = 5.8 Hz, 1H), 4.11 (d, J = 5.9 Hz, 2H), 3.71 (s, 6H), 3.28–3.24 (m, 2H), 2.82 (t, J = 6.9 Hz, 2H); (–)-LRESIMS m/z (rel. int.) 485 (100) $[M - H]^-$; (+)-HRESIMS calcd for $C_{22}H_{22}N_4NaO_7S$ $[M + Na]^+$ 509.1101, found 509.1108.

Compound 37. A mixture of thiaplakortone A hydrochloride (220 mg, 0.67 mmol), *N*-methoxy-*N*-methyl-1*H*-imidazole-1-carboxamide (400 mg, 2.60 mmol)[15] and TEA (220 μL, 1.57 mmol) in DMF (10 mL) was heated at 50 °C under an atmosphere of nitrogen for 18 h. After solvent removal the residue was purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford urea **37** (67 mg, 26%) as an orange powder; ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 7.28 (s, 1H), 7.11 (app. t, J = 5.5 Hz, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.40 (d, J = 8.8 Hz, 1H), 3.53 (s, 3H), 3.31–3.28 (m, 2H), 2.90 (s, 3H), 2.88 (app. t, J = 6.9 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ

178.5, 167.5, 159.9, 139.9, 130.1, 128.8, 127.7, 124.7, 123.4, 114.2, 111.5, 61.1, 39.4, 35.4, 25.5. (−)-LRESIMS *m/z* (rel. int.) 379 (100) $[M - H]^-$; (+)-HRESIMS calcd for $C_{15}H_{16}N_4NaO_6S$ $[M + Na]^+$ 403.0683, found 403.0679.

Compound 38. Prepared using a method analogous to General Procedure C, orange powder (13 mg, 29%); ^{1}H NMR (500 MHz, DMSO-d_6) *thiazine and indole NH not observed* δ 7.27 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.38 (d, J = 8.7 Hz, 1H), 6.19 (t, J = 5.6 Hz, 1H), 6.15 (t, J = 5.5 Hz, 1H), 4.08 (q, J = 7.1 Hz, 2H), 3.75 (d, J = 6.0 Hz, 2H), 3.24 (app. q, J = 6.6 Hz, 2H), 2.81 (t, J = 7.0 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.4, 171.1, 167.5, 157.8, 139.9, 130.1, 128.8, 127.6, 124.7, 123.2, 114.2, 111.5, 109.5, 60.1, 41.5, 26.1, 14.1 (−)-LRESIMS *m/z* (rel. int.) 421 (100) $[M - H]^-$; (−)-HRESIMS calcd for $C_{17}H_{17}N_4O_7S$ $[M - H]^-$ 421.0823, found 421.0822.

Compound 39. A mixture of thiaplakortone A hydrochloride (30 mg, 91 μmol) and triethylamine (30 μL, 0.21 mmol) in anhydrous DMF (2 mL) was treated with *N*-adamant-2-yl-1*H*-imidazole-1-carboxamide (29 mg, 0.12 mmol)[16] in one portion and stirred at rt for 18 h. The mixture was concentrated *in vacuo* then purified by flash chromatography (silica, 1 : 10 v/v MeOH–DCM elution) to afford compound **39** (16 mg, 37%) as an orange powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.96 (br s, 1H), 10.99 (br s, 1H), 7.28 (s, 1H), 7.05 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 6.03 (d, J = 8.1 Hz, 1H), 5.85 (t, J = 5.8 Hz, 1H), 3.65 (d, J = 8.1 Hz, 1H), 3.28–3.21 (m, 2H), 2.80 (t, J = 7.0 Hz, 2H), 1.86–1.61 (m, 12H), 1.49 (d, J = 12.5 Hz, 2H); (+)-LRESIMS *m/z* (rel. int.) 471 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{23}H_{26}N_4NaO_5S$ $[M + Na]^+$ 493.1516, found 493.1519.

Compound 40. Compound **6** (14 mg, 42 μmol) was freebased by subjection to General Procedure E and then the crude residue was dissolved in anhydrous DMF (1 mL) and treated with *N*-acetylimidazole (7 mg, 63 μmol) and stirred for 18 h. The solvent was removed and the residue purified by reversed-phase HPLC Method 1 which afforded **40** (8 mg, 55%) as an orange powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.95 (d, J = 5.8 Hz, 1H), 7.88 (t, J = 5.7 Hz, 1H), 7.36 (s, 1H), 7.06 (dd, J = 8.9, 5.7 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 3.91 (s, 3H), 3.27–3.23 (m, 2H), 2.81 (t, J = 7.2 Hz, 2H), 1.79 (s, 3H); (−)-LRESIMS *m/z* (rel. int.) 348 (100) $[M - H]^-$; (−)-HRESIMS calcd for $C_{15}H_{14}N_3O_5S$ $[M - H]^-$ 348.0660, found 348.0661.

Compound 41. Boc-protected thiaplakortone A (**35**) (30 mg, 76 μmol) was alkylated with 2-iodopropane (11 μL, 0.11 mmol) to afford the indole-*N*-isopropyl-Boc-protected compound (15 mg, 45%) using a method analogous to that of General Procedure G; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.93 (br s, 1H), 7.56 (s, 1H), 7.07 (d, J = 8.8 Hz, 1H), 6.87 (t, J = 5.5 Hz, 1H), 6.40 (d, J = 8.8 Hz, 1H), 5.20 (hept, J = 6.6 Hz, 1H), 3.21–3.17 (m, 2H), 2.82 (t, J = 7.1 Hz, 2H), 1.43 (d, J = 6.7 Hz, 6H), 1.37 (s, 9H). The above material (15 mg, 0.04 mmol) was then deprotected using General procedure F to afford a residue that was then freebased according to General Procedure E. The residue was then dissolved in anhydrous DMF and treated with *N*-acetylimidazole (9 mg, 0.08 mmol) and stirred for 18 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (amino-bonded silica, 1 : 10 v/v MeOH–DCM elution) to afford acetamide **41** (7 mg, 43%) as an orange powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.93 (br s, 1H), 7.88 (app. t, J = 5.7 Hz, 1H), 7.59 (s, 1H), 7.07 (dd, J = 8.7, 1.0 Hz, 1H), 6.42 (d, J = 8.7 Hz, 1H), 5.20 (m, 1H), 3.28–3.24 (m, 2H), 2.83 (app. t, J = 7.3 Hz, 2H), 1.78 (s, 3H), 1.43 (d, J = 6.7 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.1, 169.0, 167.5, 140.4, 130.3, 129.0, 125.4, 124.0, 123.8, 113.6, 111.4, 49.9, 38.3, 25.5, 22.6, 22.4; (+)-LRESIMS *m/z* (rel. int.) 378 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{17}H_{19}N_3NaO_5S$ $[M + Na]^+$ 400.0938, found 400.0935.

Compound 42. Prepared according to General Procedure G from iodocyclopentane and **8** as an orange powder (4 mg, 26%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.94 (br s, 1H), 7.86 (t, J = 5.5 Hz, 1H), 7.52 (s, 1H), 7.07 (d, J = 8.8 Hz, 1H), 6.40 (d, J = 8.8 Hz, 1H), 5.33–5.26 (m, 1H), 3.28–3.25 (m, 2H), 2.83 (t, J = 7.1 Hz, 2H), 2.16–2.08 (m, 2H), 1.87–1.79 (m, 4H), 1.77 (s, 3H), 1.72–1.63 (m, 2H); (+)-LRESIMS *m/z* (rel. int.) 404 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{19}H_{21}N_3NaO_5S$ $[M + Na]^+$ 426.1094, found 426.1094.

Compound 43. Prepared according to General Procedure G from 3-(bromomethyl)-3-methyloxetane and **8** as an orange powder (5 mg, 26%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.98 (br s, 1H), 7.86 (t, J = 5.5 Hz, 1H), 7.42 (s, 1H), 7.06 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 4.54 (s, 2H), 4.50 (d, J = 6.1 Hz, 2H), 4.17 (d, J = 6.1 Hz, 2H), 3.29–3.25 (m, 2H), 2.84 (t, J = 7.0 Hz, 2H), 1.77 (s, 3H), 1.24 (s, 3H). (+)-LRESIMS *m/z* (rel. int.) 420 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{19}H_{21}N_3NaO_6S$ $[M + Na]^+$ 442.1043, found 442.1044.

Compound 44. Boc-protected thiaplakortone A (**35**) (145 mg, 0.37 mmol) was alkylated with benzyl bromide (69 mg, 0.46 mmol) to afford the indole-*N*-benzyl-Boc-protected compound (47 mg, 26%) using a method analogous to that of General Procedure G; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.96 (br s, 1H), 7.50 (s, 1H), 7.35–7.26 (m, 3H), 7.24 (d, J = 7.0 Hz, 2H), 7.03 (d, J = 8.8 Hz, 1H), 6.83 (t, J = 5.2 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 5.54 (s, 2H), 3.17 (m, 2H), 2.82 (t, J = 7.1 Hz, 2H), 1.34 (s, 9H). A portion of the above material (22 mg, 46 μmol) was then deprotected using General procedure F to afford a residue that was then freebased according to General Procedure E. The residue was then dissolved in anhydrous DMF (5 mL) and treated with *N*-acetylimidazole (10 mg, 91 μmol) and stirred for 18 h. The solvent was removed *in vacuo* and the residue purified by flash chromatography (amino-bonded silica, 1 : 10 v/v MeOH–DCM elution) to afford acetamide **44** (9 mg, 47%) as an orange powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.98 (s, 1H), 7.86 (t, J = 5.4 Hz, 1H), 7.52 (s, 1H), 7.33 (t, J = 7.3 Hz, 2H), 7.28 (t, J = 7.2 Hz, 1H), 7.24 (d, J = 7.2 Hz, 2H), 7.04 (d, J = 8.8 Hz, 1H), 6.42 (d, J = 8.8 Hz, 1H), 5.54 (s, 2H), 3.27 (dd, J = 13.0, 6.8 Hz, 2H), 2.83 (t, J = 7.1 Hz, 2H), 1.75 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.9, 179.0, 177.6, 145.0, 146.8, 143.5, 140.1, 138.6 (2C), 137.7, 137.0, 135.7 (2C), 134.1, 134.0, 124.0, 121.5, 61.5, 48.2, 35.2, 32.5. (+)-LRESIMS *m/z* (rel. int.) 426 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{21}H_{19}N_3NaO_5S$ $[M + Na]^+$ 448.0968, found 448.0933.

Compound 45. Prepared according to General Procedure G from 4-chlorobenzyl bromide and **8**, as an orange powder (7 mg, 39%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.96 (s, 1H), 7.86 (t, J = 5.5 Hz, 1H), 7.52 (s, 1H), 7.39 (d, J = 8.5 Hz, 2H), 7.25 (d, J = 8.3 Hz, 2H), 7.03 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 8.8 Hz, 1H), 5.53 (s, 2H), 3.28–3.20 (m, 2H), 2.83 (t, J = 7.1 Hz, 2H), 1.75 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 177.9, 169.0, 167.7, 140.0, 135.8, 133.4, 132.3, 130.2, 129.0 (2C), 128.5 (2C), 125.7, 124.2, 124.0, 114.0, 111.5, 50.8, 38.3, 25.2, 22.5; (+)-LRESIMS m/z (rel. int.) 460 (100) $[M + H]^+$; (+)-HRESIMS calcd for $C_{21}H_{18}ClN_3NaO_5S$ $[M + Na]^+$ 482.0548, found 482.0551.

X-ray structure determination of thiaplakortone A (1) and *n*-propyl amide analogue (9)

Unique data sets for compounds **1** as the dihydrate and **9** with solvated DMSO were measured at 223 and 200 K respectively on an Oxford-Diffraction GEMINI S Ultra CCD diffractometer (Mo-K_α radiation, graphite monochromation) utilising CrysAlis software.[27] The structures were solved by direct methods and refined by full matrix least squares refinement on F^2. Anisotropic thermal parameters were refined for non-hydrogen atoms; (x, y, z, U_{iso})$_H$ were included and constrained at estimated values. Conventional residuals at convergence are quoted; statistical weights were employed. Computation used, SIR-97,[28] SHELX97,[29] TeXsan,[30] ORTEP-3[31] and PLATON[32] programs and software systems.

Full .cif depositions reside with the Cambridge Crystallographic Data Centre, CCDC Nos. 998930 (**1**) and 998931 (**9**).

In vitro P. falciparum growth inhibition assays

The *in vitro* growth inhibitory activity of compounds was tested against *P. falciparum* lines 3D7, Dd2, and C2B using the [^{3}H]-hypoxanthine incorporation assay. Chloroquine (positive control) was prepared as a 10 mM stock solution in PBS. All other compounds were prepared as 20 mM stock solutions in DMSO. Serial dilutions of compounds/controls were prepared in hypoxanthine-free culture media, followed by addition of [^{3}H]-hypoxanthine (0.5 μCi per well) and asynchronous cultures at 1% parasitemia and 1% final haematocrit. The antimalarial drugs chloroquine or atovaquone were included in assays as internal controls. Following incubation for 48 h, the amount of [^{3}H]-hypoxanthine incorporated into parasites was determined by harvesting cultures onto glass fiber filter mats and counting using a Perkin Elmer/Wallac Trilux 1450 MicroBeta scintillation counter. Percentage inhibition of growth compared to matched DMSO controls (0.5%) was determined. IC_{50} values were determined using log-linear interpolation of inhibition curves and are presented as mean (± SD) of three independent assays, each carried out in triplicate wells. Chloroquine [IC_{50} = 0.109 ± 0.060 μM (Dd2); IC_{50} = 0.012 ± 0.004 μM (3D7)] and atovaquone [IC_{50} = 0.0002 ± 0.0001 μM (3D7) IC_{50} = 5.750 ± 0.778 μM (C2B)] served as positive controls.

In vitro cytotoxicity assay

Cytotoxicity against a mammalian cell line (neonatal foreskin fibroblast cells; NFF) was assessed by culturing cells in RPMI media (Life Technologies) supplemented with 10% heat inactivated foetal calf serum (CSL Biosciences) and 1% streptomycin (Life Technologies). Chloroquine was prepared as a 10 mM stock solution in PBS. All other compounds were prepared as 20 mM stock solutions in DMSO. Cells were seeded into wells of 96-well tissue culture plates (3000 cells per well) and cultured for 24 h at 37 °C in 5% CO_2 before being treated with a dilution series of each compound/control. After 72 h, medium was removed and plates washed in phosphate buffered saline pH 7.4 (PBS), before fixing with denatured alcohol. Fixative was removed and washed from cells before the addition of sulforhodamine B (0.4%; Sigma 50 μL). After staining for 1 h, plates were washed three times with 1% acetic acid, then 100 μL of 10 mM Tris base (unbuffered, pH > 9) was added to each well. Plates were read at 564 nm in an ELISA micro-plate reader. Percentage inhibition of growth as compared to matched DMSO controls (0.5%) was determined and IC_{50} values calculated using log-linear interpolation of inhibition curves. Data are presented as mean ± SD of three independent assays, each carried out in triplicate wells. The antimalarial drug chloroquine was included as an internal control in each assay, and was shown to display an IC_{50} of 46 ± 14 μM.

Physicochemical and *in vitro* metabolic stability assays

In silico parameters were calculated using JChem for excel (ChemAxon, Budapest). Kinetic solubility in 0.01 M HCl (pH 2) and phosphate buffer (pH 6.5) was determined by serial dilution of a concentrated stock solution prepared in DMSO and the solubility range was determined by nephelometry. Log D was estimated at pH 7.4 using a chromatographic method as described previously.[23]

Metabolic stability was assessed *in vitro* by incubating with mouse liver microsomes (Xenotech, Lenexa, KS) at 37 °C using substrate concentrations of 1 μM and a microsomal protein concentration of 0.4 mg mL^{-1}. Addition of an NADPH-regenerating buffer system (containing 1 mg mL^{-1} NADP, 1 mg mL^{-1} glucose-6-phosphate, 1 U mL^{-1} glucose-6-phosphate dehydrogenase and 0.67 mg mL^{-1} $MgCl_2$) was used to initiate the metabolic reactions, and samples were quenched at various time points over 60 min by the addition of chilled MeCN. Control samples (containing no NADPH) were included to monitor for potential degradation in the absence of cofactor. Samples were analysed by LC-MS on a Waters/Micromass Xevo G2 QTOF or a Waters/Micromass Quattro Ultima PT Triple Quadrupole MS, each coupled to a Waters Acquity uPLC (Milford, MA) under positive electrospray ionisation. Chromatography was conducted using a Supelco Ascentis Express reverse phase C_8 or C_{18} column (50 × 2.1 mm, 2.7 μm) (Sigma-Aldrich, St Louis, MO), equipped with a Phenomenex Security-Guard column hosting a Luna C_8 cartridge (Torrance, CA) and both were maintained at a temperature of 40 °C. The mobile phase consisted of MeCN and H_2O (containing 0.05% formic acid) mixed using a binary gradient at a flow rate of 0.4 mL min^{-1}. The first-order rate constant for substrate depletion was used to calculate the *in vitro* intrinsic clearance.

In vivo amide and urea analogue exposure in mice

All animal studies were conducted using established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. The systemic exposure of the amide **8** and the urea **33** analogues was assessed in non-fasted, noninfected female Swiss Outbred mice following administration of 32 mg kg^{-1} by oral gavage (0.2 mL per mouse) or subcutaneous injection (0.2 mL per mouse) under the skin in the abdomen. Formulations for both routes comprised 10% v/v EtOH, 10% v/v Tween 80 (Sigma Chemical Co., St Louis, MO), and 80% v/v MilliQ H_2O. Blood samples were taken over 8 h with two samples per mouse; one *via* submandibular bleed (~120 μL, conscious sampling) and the other *via* terminal cardiac puncture (0.6 mL under isofluorane anesthesia) after which animals were euthanised by cervical dislocation. Blood samples were transferred to tubes containing heparin and a stabilisation cocktail (complete protease inhibitor cocktail (Roche), potassium fluoride, and EDTA) to minimise the potential for *ex vivo* degradation.

Following collection, blood samples were centrifuged and plasma stored at −20 °C prior to analysis. Plasma samples were assayed by first thawing, spiking with internal standard (diazepam) and precipitating plasma proteins with the addition of MeCN (2-fold volume ratio), centrifuging and injecting the supernatant onto an LCMS system (Micromass Xevo TQ triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC). The column was a Supelco Ascentis Express RP Amide column (2.7 μm, 50 mm × 2.1 mm i.d.) equipped with a Phenomenex Security Guard column with Synergy Polar packing material, and both columns were maintained at 40 °C. The mobile phase consisted of MeCN and H_2O, both containing 0.05% v/v formic acid, and delivered using a linear gradient over 3.3 min followed by re-equilibration to the starting conditions. The flow rate was 0.4 mL min^{-1}, and the injection volume was 3 μL. LCMS analysis was conducted in positive mode electrospray ionisation and elution of the analytes monitored in MRM mode. Concentrations were determined by comparison to a set of calibration standards prepared in blank mouse plasma. Accuracy was within ± 10%, and precision (% RSD) was less than 11%. The limit of quantitation was 0.5 ng mL^{-1} for **8** and 1 ng mL^{-1} for **33**.

Pharmacokinetic parameters were determined using non-compartmental analysis (WinNonlin version 5.2, Pharsight, Mountain View, CA) and included the maximum plasma concentration (C_{max}), the time to reach the maximum concentration (T_{max}), the apparent terminal elimination half-life (where it could be determined) and the area under the plasma concentration *versus* time profile from 0 to 8 h (AUC_{0-8h}).

In vivo tolerability assessment in mice

Animal studies were approved by the Army Malaria Institute Animal Ethics Committee (AEC no. 05/13) in accord with the Australian Code of Practice for the care and Use of Animals for Scientific Purposes. Tolerability assessment of amide and urea analogues were carried out in groups of three healthy mice administered different doses of compound. The mice were male and female Outbred ARC Swiss mice (Animal Resource Centre, Western Australia) aged between 6 and 7 weeks, with a mean weight of 28 ± 3 g. The animals were observed for physical distress twice daily. Physical adverse events that were monitored included reduced activity and movement, tremour, panting, behaviour changes, reduced appetite, extreme pallor, ruffled coat and weight loss. If any animal exhibited physical adverse events that appeared highly stressful they were euthanised. All compounds were dissolved in 10% EtOH/10% Tween 80/80% distilled H_2O and administered subcutaneously to mice twice daily at about 6 h apart for 4 consecutive days.

In vivo efficacy assessment in the rodent-*P. berghei* model

The *in vivo* efficacy of amide and urea analogues were determined using a modified Peters 4 day test.[33] This test measures the suppressive activity of blood schizontocides over 4 days at a high-tolerated dose that does not cause physical stress in healthy mice. Briefly, female ARC mice (groups of six mice, age 6–7 weeks with a mean weight of 28 ± 3 g) were inoculated intraperitoneally with 20 × 10^6 *P. berghei* (ANKA strain) infected erythrocytes. The mice were then treated subcutaneously or orally at about 1 h and 6 h after parasite inoculation (D0) and then twice daily at about 6 h apart for 3 consecutive days with either the analogue and drug-free vehicle (control). Chloroquine (positive control) was given daily for 4 days. The analogues were dissolved in 10% EtOH/10% Tween 80/80% distilled H_2O and chloroquine was dissolved in H_2O. The drug-free vehicle was 10% EtOH/10% Tween 80/80% distilled H_2O. Thin blood smears were made on D + 1, D + 2, D + 3, and D + 4 and stained with Giemsa. The degree of infection (parasitemia expressed as percentage of infected erythrocytes) was determined microscopically. Blood smears were read independently by two microscopists with results averaged.

Conflict of Interest

The authors declare no competing financial interest. The opinions expressed herein are those of the authors' and do not necessarily reflect those of the Australian Defence Force, Joint Health Command or any extant policy.

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