Pergamon

SCIENCE DIRECT®

Tetrahedron 59 (2003) 2289–2296

TETRAHEDRON

Exploring the potential of xanthene derivatives as trypanothione reductase inhibitors and chloroquine potentiating agents

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Received 6 November 2002; revised 14 January 2003; accepted 6 February 2003

Abstract—Xanthene derivatives were synthesized and evaluated for their potential as trypanothione reductase (TryR) inhibitors and chloroquine (CQ) potentiating agents. Some derivatives displayed inhibitory activity against TryR comparable to known tricyclic antidepressants. On the other hand a number of derivatives increased CQ accumulation and potentiating effects in a resistant strain of *Plasmodium falciparum* with one compound also displaying strong intrinsic antimalarial activity.

1. Introduction

The parasitic protozoa *Trypanosoma* and *Leishmania* are responsible for African sleeping sickness in humans (nagana in cattle), Chagas disease and leishmaniasis. These parasites utilize the trypanothione/trypanothione reductase (TryR) system for the maintenance of an intracellular reducing environment.[1,2] This is in marked contrast to many other organisms including humans which utilize the glutathione/glutathione reductase (GR) system.[3,4] This biochemical difference between parasite and mammalian cells suggests trypanothione metabolism as an attractive target for antitrypanosomal and antileishmanial agents where it might be possible to combat trypanosomiasis and leishmaniasis with a single agent. Effective inhibition of TryR should compromise the parasites ability to defend itself against reactive oxygen molecules like hydrogen peroxide and hydroxyl radicals, agents known to destroy DNA and cellular membranes.

On the other hand, *Plasmodium falciparum* malaria continues to be a major health threat throughout the tropical world and, while potential demand for antimalarials are high, drug resistance to *P. falciparum* is a major problem. Previous first line drugs such as chloroquine (CQ) have been rendered completely ineffective in most endemic areas. Due to the safety, effectiveness and low cost of this drug for poor endemic areas, there is a justifiable need to reverse CQ resistance through the understanding of the mechanism of resistance and development of resistance reversing agents which can be co-administered with CQ. Since resistant malaria parasites accumulate less CQ compared to sensitive parasites, chemical agents which increase CQ accumulation in resistant parasites have potential as CQ resistance reversing agents.[5]

Inspection of the chemical structures of agents which increase accumulation and/or reverse CQ resistance reveals the importance of a hydrophobic group and a protonatable nitrogen.[6] Incidentally these are the same chemical features known to be important for potency and selectivity (against the human homologue GR) in several inhibitors of TryR.[7–9] This is exemplified in the antimalarial acridine quinacrine **1**[10] and tricyclic antidepressants promazine **2** and clomipramine **3**, Figure 1.[6,7] A wide variety of structurally diverse drugs (including **2** and **3**) have been described as CQ resistance reversal agents.[6,11–13] Thus it is reasonable to envisage that these tricyclics could be utilized as dual purpose scaffolds for the discovery and development of CQ resistance reversal agents and inhibitors of TryR. A literature search revealed that tricyclics based on the xanthene moiety have not been previously investigated as inhibitors of TryR or CQ potentiating agents. Thus, an investigation into the potential of these compounds for this purpose was undertaken.

In the context of TryR inhibitors and CQ potentiating

Keywords: 9,9-dimethylxanthenes; trypanothione reductase inhibitors; chloroquine potentiating agents; trypanosomiasis; leishmaniasis; malaria.
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doi:10.1016/S0040-4020(03)00240-0

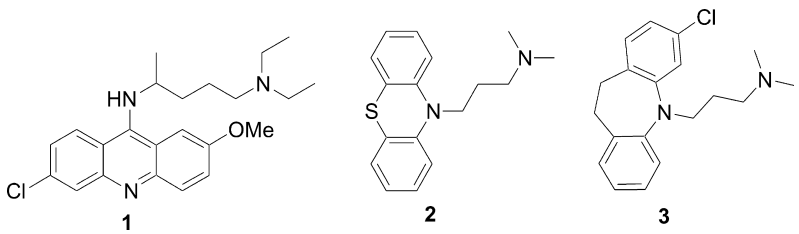
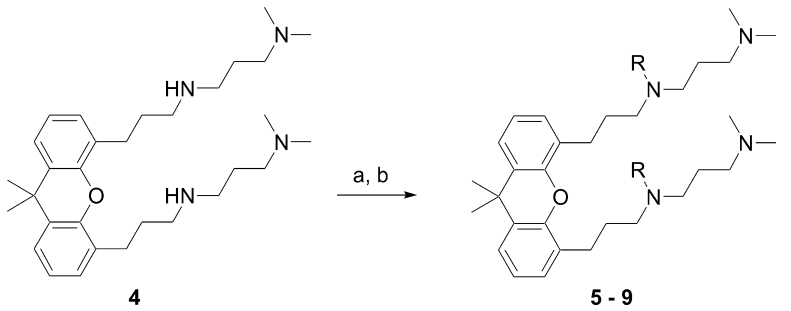


Figure 1. Tricyclic compounds described as TryR inhibitors and/or CQ potentiating agents.



Scheme 1. *Reagents and conditions*: (a) 2.1 equiv. of $ArSO_2Cl$ or ArNCO, 2.2 equiv. of PS-Morpholine for $ArSO_2Cl$, CH_2Cl_2, rt, 5–24 h; (b) 4.0 equiv. of PS-tris(2-aminoethyl) amine, CH_2Cl_2, rt, 12 h.

agents, the advantages of the 9,9-dimethylxanthene scaffold are two-fold: first, being aromatic and tricyclic in nature, this moiety bears a resemblance to the aromatic hydrophobic tricyclic moieties found in other tricyclic compounds already reported as competitive inhibitors of TryR, where the tricyclic moiety binds in the hydrophobic pocket involved in the recognition of the spermidine moiety of trypanothione disulfide, the substrate for TryR. The tricyclic nucleus also bears a resemblance to the tricyclic nucleus found in known CQ potentiating agents. Second, the chemically reactive 2,7 and 4,5 positions provide potential multiple sites for introducing chemical diversity. This would ultimately aid analogue synthesis and an exploration of structure–activity relationships within this class of compounds.[6,7] Incorporating a terminal amino group (exemplified by the alkylamino group) into target compounds provides a protonatable nitrogen which has been shown to favor TryR over GR, the most closely related host enzyme.[9] A protonatable nitrogen is also essential for effective CQ potentiating activity.[6,11–13]

2. Results

2.1. Synthesis

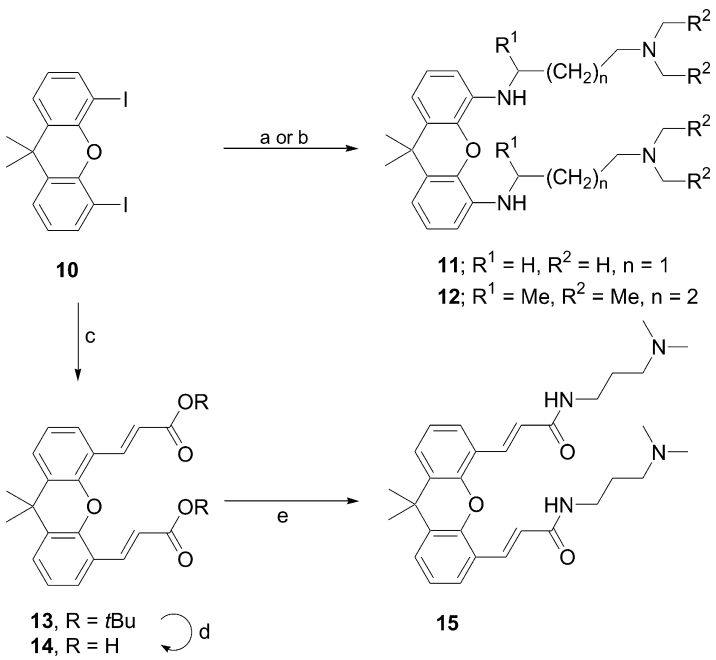
Polyamine **4** was previously synthesized and reported to be a weak inhibitor of TryR.[14] In an effort to improve the activity of this series of compounds, we attempted to utilize **4** as a core bifunctional molecule. The idea of using a core molecule possessing multiple functional groups to synthesize libraries was initially introduced by Rebek Jr.[15,16] For example, a library of 55 compounds can be produced theoretically by using a bi-functional scaffold and 10 building blocks.[15,16] Principally, by condensing a variety of multifunctional core molecules with a variety of building blocks, well-defined libraries of small organic molecules with biological significance can be generated. Thus, polyamine **4** presented us with an attractive core molecule to explore the parallel synthesis of potential arrays of xanthene derivatives with diverse structures with a view to improving the inhibitory potency of this class of compounds.

A solution of polyamine **4** was dispensed into five reaction vials and dissolved in a small amount of CH_2Cl_2. The solutions were then treated with 2.1 equiv. of either aryl sulphonyl chlorides (in the presence of 2.2 equiv. of polymer-supported morpholine) or aryl isocyanates, Scheme 1. TLC analysis showed that after 5 h, the isocyanate reactions were complete, whilst the sulphonyl chloride reactions were slower with a total reaction time of 24 h required for completion at the same room temperature conditions. The sulfonamide solutions were filtered to remove the PS-morpholine. Excess sulphonyl chloride and isocyanate were removed from the mixture by the addition of 4 equiv. of PS-tris(2-aminoethyl) amine. To ensure complete removal, the suspension was shaken for a further 12 h. Filtration gave the sulfonamides and ureas in yields ranging from 50 to 83% (Table 1). ^1H NMR analyses revealed minor impurities and thus, the compounds were subjected to silica gel chromatography in order to obtain pure molecules for biological evaluation.

4,5-Diiodo-9,9-dimethylxanthene[17] **10** provided a starting point in the synthesis of aryl amines **11** and **12** as well as

Table 1. Chemical yields of sulfonamide and urea derivatives

Product	Substitution (R)	Yield (%)
5	$-SO_2PhMe$	50
6	$-SO_2$(2-Naphthalene)	60
7	$-CONHCH_2Ph$	56
8	$-CONHPhCl$	83
9	$-CONHPhF$	62



Scheme 2. *Reagents and conditions*: (a) 2.0 mol% $Pd_2(dba)_3$, 6.0 mol% BINAP, 2.8 equiv. of NaO*t*Bu, 2.5 equiv. of 3-dimethylamino-1-propylamine, PhMe, 100°C, 18 h, 89%; (b) 2.0 mol% $Pd_2(dba)_3$, 6.0 mol% BINAP, 2.8 equiv. of NaO*t*Bu, 2.5 equiv. of 2-amino-5-diethylaminopentane, PhMe, 100°C, 6 h, 60%; (c) 10 mol% $Pd(OAc)_2$, 20 mol% PPh_3, 11.0 equiv. of *tert*-butyl acrylate, 2.0 equiv. of Et_3N, DMF, 75°C, 12 h, 94%; (d) TFA, CH_2Cl_2, 0°C, 20 min, rt, 2 h, 99%; (e) 6.0 equiv. of $(COCl)_2$, CH_2Cl_2, reflux, 3 h; then 5.0 equiv. of $H_2N(CH_2)_3NMe_2$, CH_2Cl_2, 0°C→rt, 1 h, 75%.

α,β-unsaturated amide **15**, Scheme 2. Palladium-catalyzed amination[18] of **11** using 3-dimethylamino-1-propylamine and 0.5% Pd at 80°C for 2 h resulted in a 96% conversion of the starting material and isolation of bis- and mono-amination products in a 1:1 ratio. Complete conversion could not be achieved even after an extended reaction time of 48 h. However, use of a slight excess of the amine (2.5 equiv.), increased catalyst loading (2 mol% Pd), a higher temperature (100°C) and a concentration of 2 mL toluene/mmol halide resulted in a complete reaction within 18 h giving the bis-aminated product **11** exclusively in good yield. The same conditions were applied to the synthesis of aryl amine **12** in moderate yield using 2-amino-5-diethylaminopentane as the primary amine. The reaction in this case went to completion within 6 h.

The α,β-unsaturated amide **15** was obtained in three steps from **10**. Palladium-mediated Heck coupling using *tert*-butyl acrylate, gave unsaturated *tert*-butyl ester **13** in excellent yield. Hydrolysis of this ester using trifluoroacetic acid (TFA) followed by conversion of the resulting acid **14** to the corresponding acid chloride and reaction with 3-dimethylamino-1-propylamine gave the unsaturated amide **15** in moderate yield, Scheme 2.

3. Biological evaluation

3.1. Inhibition of TryR

The data in Table 2 shows a comparison of the percentage inhibition of *Trypanosoma cruzi* TryR at 100 μM of the polyamine **4** and the corresponding sulfonamide and urea derivatives.

By derivatizing polyamine **4**, the percentage inhibition is decreased compared to the parent amine. There is no clear advantage of sulfonamides over ureas in this case. The reason for this loss of activity is unclear but it might be postulated that the addition of sulfonamide and urea groups might result in the molecule adopting a different spatial arrangement preventing certain pharmacophores on the molecule interacting with its putative binding site. It could also be argued that the partial loss of the polyamine backbone could be a contributing factor since some of the most potent inhibitors of TryR are polyamines. Due to the obvious weak inhibitory activity of these compounds, it was deemed unnecessary to determine IC_{50} and/or K_i values.

On the other hand, aryl amines **11** and **12** and α,β-unsaturated amide **15** showed higher percentages of inhibition (data not shown) and in this case it was deemed necessary to at least determine IC_{50} values shown in Table 3. The aryl amines showed comparable activity to each other but the amide **15** was superior amongst the new xanthenes. Interestingly, the IC_{50} value of this amide was comparable to that of clomipramine, while values for the aryl amines were comparable to promazine **2** and the dibenzazepine derivative clomipramine (**3**).[20,21] No inhibition of the human homologue (hGR) by **15** was observed at 200 μM, suggesting good selectivity.

Table 2. Percentage inhibition of TryR by selected xanthene derivatives

Compound #	Inhibition at 100 μM (%)
4	63.0
5	13.9
6	26.8
7	21.5
8	11.8
9	11.1

TryR assays were performed as described before.[19]

Table 3. Inhibition of TryR by selected xanthenes and their comparison to known tricyclic inhibitors

Compound #	IC_{50} (μM)
2 (Promazine)	108
3 (Clomipramine)	32.4
11	108±1
12	105±8
15	35.7±1.5

TryR inhibition assays were performed as described before.[19]

Table 4. A comparison of the intrinsic antimalarial activity of selected xanthene derivatives in D10 and K1

Compound	Mean IC_{50} in D10 (μM)	Mean IC_{50} in K1 (μM)
CQ	0.032±0.002	0.239±0.028
4	1.853±0.027	1.844±0.390
5	0.049±0.008	0.059±0.024
6	0.097±0.025	0.169±0.027
11	2.444±0.111	0.209±0.084
12	2.868±0.114	0.229±0.130
15	1.748±0.115	0.392±0.078

The superior activity of amide **15** may be rationalized on the basis of two factors: (i) the more rigid geometry of **15** may be complementary to, and hence well tolerated in, the rigid active site of TryR. Crystallographic studies suggest that the active site of TryR is rigid and undergoes little conformational change upon substrate binding;[22,23] (ii) if **15** were to dock in the active site such that the α,β-unsaturated Michael acceptor unit and the active site Cys-53 are well aligned for conjugate addition, then nucleophilic attack by Cys-53 could be envisaged. TryR is known to be alkylated specifically at Cys-53, an active site nucleophile implicated in an attack on trypanothione disulfide, during enzymatic reduction. This Michael addition mechanism has also been invoked in the case of the natural product lunarine and a related simplified benzofuran analogue inhibitors of TryR.[24,25] An α,β-unsaturated system is also present in these compounds.

3.2. Effect of selected compounds on chloroquine accumulation

It is well accepted that CQ resistant parasites accumulate less drug than their susceptible counterparts.[5] Therefore, the transport and accumulation of CQ into the parasite are not only essential for activity but are also intimately related to the resistance phenotype. Selected xanthene derivatives synthesized were also evaluated for their ability to potentiate the accumulation of tritiated CQ in a CQ resistant (K1) strain of *P. falciparum*.

Figure 2 shows the effect on ^{3}H-CQ accumulation in the presence of 5 μM of selected derivatives in K1 in comparison with verapamil (VPL), the well known modulator in multidrug resistant (MDR) mammalian cancer cells and malarial parasites. When co-administered with CQ, all derivatives with the exception of polyamine **4** showed a 5–6 fold increase in CQ accumulation. In the same assay VPL showed a 3.5-fold increase in CQ accumulation.

3.3. Intrinsic anti-malarial effect

An ideal CQ potentiating agent should possess little or no anti-malarial activity. This property is advantageous if the resistance reversal activity is solely being tested. Thus the intrinsic anti-malarial activities of target compounds were determined for both the sensitive (D10) and resistant (K1) strains, Table 4. A typical potentiating agent should have no effect on the sensitive strain but when co-administered with a CQ potentiating agent, CQ should recover its potent antimalarial activity against a previously resistant strain and completely reverse the resistance of cells to the cytotoxic action of drugs.[26] Most compounds displayed a weak anti-malarial activity against the sensitive strain compared to CQ except sulfonamides **5** and **6** which showed activity comparable and superior to CQ in D10 and K1, respectively. The high potency of **5** is particularly noteworthy. On the other hand the aryl amines **11** and **12** as well as α,β-unsaturated amide **15** were comparable to CQ in K1.

3.4. Chloroquine potentiating effect

The response modification index (R.M.I), calculated by dividing the IC_{50} for the compounds combined with CQ with that of CQ alone, was determined for each compound. This gives a fraction, which represents an indication of the activity of the compound relative to CQ. An R.M.I<1 indicates a resistance reversing or potentiating effect, an

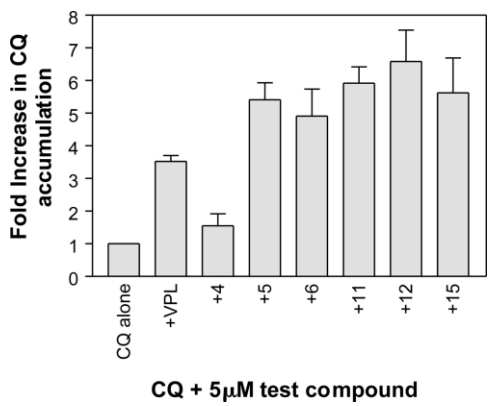


Figure 2. Accumulation of ^{3}H-CQ in K1 in the presence of 5 μM xanthene derivatives and verapamil expressed as fold increase in CQ accumulation.

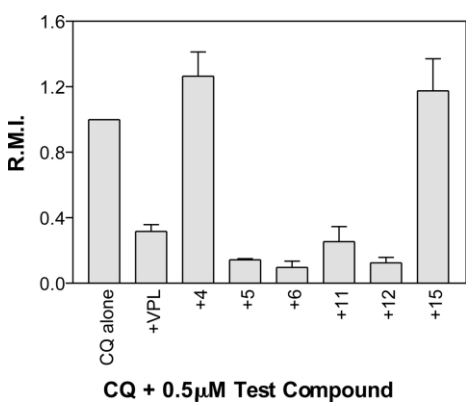


Figure 3. R.M.I for the selected xanthene derivatives in combination with CQ in K1.

R.M.I around 1 shows no activity and an R.M.I>1 indicates an antagonistic effect.

As shown in Figure 3 most derivatives showed a direct relationship between CQ accumulation and potentiation. Polyamine **4** did not show a potentiating affect but rather had a slight antagonistic effect. It is noteworthy that the α,β-unsaturated amide **15** had no reversal effect albeit showing a 5.6-fold increase in CQ accumulation in K1. However, this observation is not surprising and is consistent with earlier observations to the effect that increase in CQ accumulation is not solely responsible for potentiation.[27] This result is also consistent with the behavior of anticancer anthracyclines and *vinca* alkaloids which raise CQ accumulation but do not act as chemosensitizers.

The ability of the best potentiating agents in the series to increase CQ accumulation is quite marked so their antimalarial activity does not interfere with CQ activity. The compounds act more strongly on CQ than does VPL in both accumulation and resistance reversal. They must therefore be having a synergistic effect with CQ as opposed to an antagonistic effect. Although most derivatives showed a reversal activity superior to VPL, the compounds are lethal to resistant parasites at a concentration of 0.5 μM. Because the intrinsic antimalarial activities covered a big range, a concentration of 0.5 μM was chosen for the reversal assays that would enable comparison of the effects of all the compounds. Thus, if the compounds are assayed at a concentration sub-lethal to parasites, they might still show an effect that is comparable to VPL. Strictly speaking the data in Figure 3 are not purely response modification as at least a component of the activity is due to an additive or antimalarial effect.

4. Conclusion

The data reported establishes that molecules based on the 9,9-dimethylxanthene moiety have potential as TryR inhibitors and CQ potentiating agents. It should be noted that although these derivatives possess relatively high antimalarial activity to be ideal chemosensitizers, this is actually an advantage from a therapeutic point of view as compounds showing both antimalarial and CQ potentiating activity would be extremely useful. Work is currently in progress in our laboratories to optimize the potency of these compounds and fully explore structure–activity relationships.

5. Experimental

5.1. General

All chemicals were reagent grade obtained from Sigma-Aldrich and solvents were purified before use. Reactions were monitored by thin layer chromatography on aluminium-backed silica gel 60 F_{254} plates (Merck) and visualized with a combination of ultraviolet light (254 nm) and either anisaldehyde spray (freshly prepared from a 2.5% solution of *p*-methoxybenzaldehyde (20 cm^3) and 18 M sulfuric acid (1 cm^3)) or ceric ammonium sulphate in 8 M sulphuric acid, followed by heating at 200°C. Column chromatography was carried out using Merck Kieselgel 60: 70–230 mesh. Melting points were determined on a Reichert–Jung hot stage apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded on either a Varian VXR-200 at 200 MHz, Varian Mercury 300 MHz or a Varian Unity spectrometer at 400 MHz and are recorded in parts per million (ppm) as measured from tetramethylsilane. Infrared (IR) spectra were recorded on a Satellite FTIR Spectrometer. Mass spectra were recorded on a VG micromass 16F spectrometer and high resolution mass determinations were performed on a Kratos Limited MS9/50 spectrometer.

5.2. Synthesis of urea and sulfonamide derivatives

Arylsulfonyl chloride or aryl isocyanate (0.63 mmol) were added to a solution of amine **4** (0.15 g, 0.30 mmol) in 2 mL dry dichloromethane at room temperature. For sulfonyl chlorides, polymer supported morpholine (0.19 g, 0.66 mmol) was added. The reaction mixture was shaken at room temperature for 5 h (ureas) and 24 h (sulfonamides). The solutions containing polymers were filtered through a sintered funnel and polymer-supported tris(2-aminoethyl) amine (1.2 mmol) added to the filtrate. The suspensions were shaken at room temperature for a further 12 h and polymers removed by filtration. The compounds were purified by silica gel chromatography, eluting with 10% MeOH in CH_2Cl_2. For the urea products, a second elution of 5% NH_3(aq.)–MeOH was required. Yields ranged from 50 to 83% (Table 1).

Sulfonyl chlorides and isocyanates used were toluene sulphonyl chloride, naphthalenesulphonyl chloride, benzyl isocyanate, 4-chlorophenyl isocyanate and 4-fluorophenyl isocyanate.

5.2.1. *N*-(3-Dimethylaminopropyl)-*N*-[3-(5-{3-[(3-dimethylaminopropyl)-4-methyl benzenesulfonylamino]propyl}-9,9-dimethyl-9*H*-xanthen-4-yl)propyl]-4-methyl benzenesulfonamide (5). Colorless syrup. R_f 0.15 (10% MeOH–CH_2Cl_2); IR ($CHCl_3$ film) 1172 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 (dd, *J*=2.0, 8.4 Hz, 4H, aromatic), 7.66 (d, *J*=8.4 Hz, 4H, aromatic), 7.53–7.50 (m, 6H, aromatic), 3.14–2.98 (m, 12H, $-CH_2-N-CH_2-$ and $-CH_2NMe_2$), 2.80–2.70 (m, 4H, Ar–CH_2-), 2.73 (s, 12H, $-N(CH_3)_2$), 2.68 (s, 6H, CH_3 on toluene rings), 2.06 (quintet, *J*=8.4 Hz, 4H, Ar–C–CH_2–C–), 2.01–1.90 (m, 4H, –N–C–CH_2–C–), 1.61 (s, 6H, 9-$(CH_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.3, 143.6, 135.8, 130.2, 129.9, 128.2, 127.2, 125.9, 124.1, 122.9, 55.9, 49.0, 46.1, 43.8, 43.5, 40.6, 32.2, 28.6, 24.8, 21.5; HRMS (FAB) found M^+ 803.4123 $C_{45}H_{62}N_4O_5S_2$ requires *M*, 803.4120.

5.2.2. *N*-(3-Dimethylaminopropyl)-*N*-[3-(5-{3-[(3-dimethylaminopropyl)-2-naphthalenesulfonylamino]-propyl}-9,9-dimethyl-9*H*-xanthen-4-yl)propyl]-2-naphthalenesulfonamide (6). Colorless syrup. R_f 0.20 (10% MeOH–CH_2Cl_2); IR ($CHCl_3$ film) 1172 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 2H, aromatic), 7.86–7.80 (m, 10H, aromatic), 7.62–7.55 (m, 6H, aromatic), 7.30–7.22 (m, 2H, aromatic), 3.16–3.10 (m, 8H, $-CH_2-$

N–CH_2–), 2.82–2.70 (m, 20H, Ar–CH_2– and –CH_2N(CH_3)$_2$), 2.16 (quintet, J=6.9 Hz, 4H, Ar–C–CH_2–C–), 1.95 (quintet, J=7.5 Hz, 4H, –N–C–CH_2–C–), 1.59 (s, 6H, 9-(CH_3)$_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.2, 135.6, 134.7, 132.2, 130.1, 129.3, 128.2, 128.0, 127.8, 127.1, 124.0, 122.9, 122.4, 55.7, 49.0, 46.0, 43.2, 40.0, 32.1, 28.6, 27.2, 24.6; HRMS (FAB) found M^+ 875.3036 $C_{51}H_{62}N_4O_5S_2$ requires M, 875.3041.

5.2.3. 3-Benzyl-1-[3-(5-{3-[3-benzyl-1-(3-dimethylaminopropyl)ureido]propyl}-9,9-dimethyl-9*H*-xanthen-4-yl)-propyl-1-(3-dimethylaminopropyl) urea (7). Colorless syrup. R_f 0.39 (8:2:0.1, MeOH/CH_2Cl_2/Et_3N); IR ($CHCl_3$ film) 3392, 1652 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.28 (m, 12H, aromatic), 7.05 (dd, J=1.6, 7.3 Hz, 2H, aromatic), 7.00 (t, J=7.5 Hz, 2H, aromatic), 6.48 (broad s, 2H, CONH), 4.32 (s, 4H, CONHCH_2Ph), 3.37–3.44 (m, 8H, –CH_2–N–CH_2–), 2.77–2.86 (m, 8H, Ar–CH_2– and –CH_2–NMe_2), 2.53 (s, 12H, –N(CH_3)$_2$), 1.96–1.99 (m, 8H, –CH_2–C–N–C–CH_2–), 1.59 (s, 6H, 9-(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.54, 148.26, 140.33, 130.15, 128.70, 128.35, 127.40, 126.80, 123.92, 122.84, 55.30, 53.39, 46.52, 44.70, 43.21, 34.30, 32.15, 28.70, 27.32, 24.27; HRMS (EI) found M^+ 760.5030. $C_{47}H_{64}N_6O_3$ requires M, 760.5036.

5.2.4. 1-(3-Dimethylaminopropyl)-1-[3-(5-{3-[1-(3-dimethylaminopropyl)-3-(4-chlorophenyl)ureido]propyl}-9,9-dimethyl-9*H*-xanthen-4-yl)-propyl]-3-(4-chlorophenyl) urea (8). Colorless syrup. R_f 0.63 (8:2:0.1, MeOH/CH_2Cl_2/Et_3N); IR ($CHCl_3$ film) 3392, 1652 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (broad s, 2H, CONH), 7.33 (d, J=8.8 Hz, 4H, Ar–H on chlorophenyl ring), 7.29 (dd, J=1.6, 7.7 Hz, 2H, aromatic), 7.14 (d, J=8.8 Hz, 4H, Ar–H on chlorophenyl ring), 7.09 (dd, J=1.6, 7.3 Hz, 2H, aromatic), 7.03 (t, J=7.5 Hz, 2H, aromatic), 3.46–3.53 (m, 8H, –CH_2–N–CH_2–), 2.81–2.88 (m, 8H, Ar–CH_2– and CH_2–NMe_2), 2.63 (s, 12H, –N(CH_3)$_2$), 1.98–2.03 (m, 8H, –CH_2–C–N–C–CH_2–), 1.60 (s, 6H, 9-(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.12, 148.23, 138.56, 130.21, 128.60, 128.45, 127.52, 124.06, 122.94, 121.62, 55.29, 46.77, 43.92, 43.54, 34.31, 32.21, 28.64, 27.35, 24.10; HRMS (EI) found M^+ 800.3933. $C_{45}H_{58}N_6O_3Cl_2$ requires M, 800.3940.

5.2.5. 1-(3-Dimethylaminopropyl)-1-[3-(5-{3-[1-(3-dimethylaminopropyl)-3-(4-fluorophenyl)ureido]propyl}-9,9-dimethyl-9*H*-xanthen-4-yl)propyl]-3-(4-fluorophenyl) urea (9). Colorless syrup. R_f 0.54 (8:2:0.1, MeOH/CH_2Cl_2/Et_3N); IR ($CHCl_3$ film) 3392, 1652 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 2H, CONH), 7.32–7.36 (m, 4H, Ar–H on fluorophenyl rings), 7.28 (dd, J=1.6, 7.7 Hz, 2H, aromatic), 7.08 (dd, J=1.6, 7.3 Hz, 2H, aromatic), 7.02 (t, J=7.5 Hz, 2H, aromatic), 6.88 (t, J=8.8 Hz, 4H, Ar–H on fluorophenyl rings), 3.46–3.54 (m, 8H, –CH_2–N–CH_2), 3.00 (t, J=7.0 Hz, 4H, Ar–CH_2–C), 2.82 (t, J=7.3 Hz, 4H, –CH_2N(CH_3)$_2$), 2.70 (s, 12H, –N(CH_3)$_2$), 1.98–2.03 (m, 8H, –CH_2–C–N–C–CH_2–), 1.58 (s, 6H, 9-(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.86, 157.46, 156.17, 148.21, 135.66, 130.21, 128.58, 127.50, 124.08, 122.72, 114.98, 55.54, 46.86, 43.92, 43.26, 34.30, 32.19, 28.71, 27.25, 23.86; HRMS (EI) found M^+ 768.4546. $C_{45}H_{58}F_2N_6O_3$ requires M, 768.4535.

5.3. Synthesis of aryl amines

5.3.1. *N*,*N*-Bis(3-dimethylaminopropyl)-9,9-dimethyl-9*H*-xanthene-4,5-diamine (11). Iodide **10** (1.0 g, 2.17 mmol), 3-dimethylamino-1-propylamine (0.55 g, 0.68 mL, 5.43 mmol), sodium *tert*-butoxide (0.58 g, 6.08 mmol), tris(dibenzylideneacetone) dipalladium (0) (Pd_2dba_3) (39.7 mg, 2 mol%) and (*R*)-(+)-2,2′-bis(diphenylphosphino)1,1′-binaphthyl (BINAP) (81.1 mg, 6 mol%) were combined in dry toluene (5 mL) under nitrogen. The suspension was heated to 100°C and stirred at this temperature for 18 h. The reaction mixture was cooled to room temperature and diethyl ether (20 mL) added. After stirring for 1 h, the solution was filtered through Celite and the filtrate concentrated. The resulting dark oil was purified by silica gel column chromatography, by first eluting with 20% MeOH/CH_2Cl_2 and switching to 10% NH_3(aq.)–MeOH to give the aryl amine as a brown oil (0.79 g, 89%). R_f 0.30 (5% NH_3(aq.)–MeOH); IR ($CHCl_3$ film) 3663, 3588 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 (t, J=7.8 Hz, 2H, aromatic), 6.73 (dd, J=1.5, 8.1 Hz, 2H, aromatic), 6.52 (dd, J=1.2, 7.5 Hz, 2H, aromatic), 3.36 (t, J=6.6 Hz, 4H, Ar–N–CH_2), 5.10 (broad s, 2H, NH), 2.57 (t, J=6.6 Hz, 4H, C–C$H_2$$NMe_2$), 2.36 (s, 12H, N$Me_2$), 1.89 (t, J=6.3 Hz, 4H, –C–CH_2–C–NMe_2), 1.59 (s, 6H, 9-Me_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.9, 136.7, 129.5, 123.0, 113.1, 107.8, 57.3, 45.0, 41.6, 34.1, 32.0, 26.1; HRMS (EI) found M^+ 410.3048. $C_{25}H_{38}N_4O$ requires M, 410.3045.

5.3.2. *N*,*N*-Bis(4-diethylamino-1-methylbutyl)-9,9-dimethyl-9*H*-xanthene-4,5-diamine (12). Iodide **10** (1.0 g, 2.17 mmol), 2-amino-5-diethylaminopentane (0.86 g, 5.43 mmol), sodium *tert*-butoxide (0.58 g, 6.08 mmol), tris(dibenzylideneacetone) dipalladium (0) (Pd_2dba_3) (39.7 mg, 2 mol%) and (*R*)-(+)-2,2′-bis(diphenylphosphino)1,1′-binaphthyl (BINAP) (81.1 mg, 6 mol%) were combined in dry toluene (5 mL) under nitrogen. The solution was heated to 100°C and stirred for 6 h. After cooling to ambient temperature, the reaction mixture was diluted with excess diethyl ether and filtered through Celite. The filtrate was concentrated and purified on silica gel chromatography eluting with 20% MeOH/CH_2Cl_2 then switching to 5% NH_3(aq.)/MeOH to give the bis-amine as a dark brown oil (0.68 g, 60%). R_f 0.45 (5% NH_3(aq.)–MeOH); IR ($CHCl_3$ film) 3650, 3567 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.93 (t, J=6.0 Hz, 2H, aromatic), 6.72 (dd, J=1.2, 6.0 Hz, 2H, aromatic), 6.54 (dd, J=1.2, 6.0 Hz, 2H, aromatic), 3.57 (sextet, J=5.1 Hz, 2H, –CH), 3.01–3.06 (m, 12H, –CH_2N(C$H_2$$CH_3$)$_3$); 1.69–1.92 (m, 8H, –CHCH_2CH_2–C), 1.54 (s, 6H, 9-Me_2), 1.28–124 (m, 18H, CH_3–CH–+–N(CH_2CH_3)$_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.1, 135.8, 130.9, 123.4, 113.2, 109.5, 51.6, 50.6, 48.6, 46.5, 34.7, 30.9, 30.3, 21.2, 8.4; HRMS (EI) found M^+ 522.4287. $C_{33}H_{54}N_4O$ requires M, 522.4297.

5.4. Synthesis of unsaturated amides

5.4.1. 3-[5-(2-*tert*-Butoxycarbonylvinyl)-9,9-dimethyl-9*H*-xanthen-4-yl]acrylic acid *tert* butyl ester (13). Iodide **10** (4.0 g, 8.66 mmol), palladium acetate (0.2 g, 10 mol%) and triphenylphosphine (0.46 g, 20 mol%) were combined in 75 mL anhydrous DMF in a dry 250 mL round bottom

flask.[9] *tert*-Butyl acrylate (12.3 g, 14.0 mL, 96.0 mmol) and 2.5 mL triethylamine was added. The reaction mixture was heated to 75°C and stirred for 12 h. After cooling to ambient temperature, the reaction was quenched by adding 100 mL distilled water followed by 100 mL diethyl ether. The mixture was filtered through Celite and flushed with diethyl ether. The filtrate was concentrated and purified by chromatography (SiO_2, 10% EtOAc/hexane) to give the ester as a cream colored solid (3.75 g, 94%). Mp 147–149°C; R_f 0.38 (30% EtOAc/hexane); IR ($CHCl_3$ film) 2988, 1691, 1639 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, J=16.0 Hz, 2H, –CH=C–), 7.43–7.49 (m, 4H, aromatic), 7.10 (t, J=7.2 Hz, 2H, aromatic), 6.43 (d, J=16.0 Hz, 2H, –C=CH), 1.62 (s, 6H, 9-$(CH_3)_2$), 1.58 (s, 18H, –$C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8, 148.4, 137.0, 130.9, 127.3, 125.4, 123.4, 123.3, 122.1, 80.4, 34.3, 31.7, 28.1; HRMS (EI) found M^+ 462.2415. $C_{29}H_{34}O_5$ requires M, 462.2406.

5.4.2. 3-[5-(2-Carboxyvinyl)-9,9-dimethyl-9*H*-xanthen-4-yl] acrylic acid (14). Ester **13** was dissolved in 2 mL dichloromethane and cooled to 0°C. Trifluoro acetic acid (1 mL) was added slowly to the cooled solution and stirred at 0°C for 20 min. Thereafter, the reaction was allowed to warm to room temperature and stirred for 2 h. The product precipitated and the solvent was removed by evaporation. The resulting white powder was subjected to high vacuum to remove excess TFA to give the acid as a white powder (0.3 g, 99%). Mp 312–314°C; R_f 0.72 (5% NH_3(aq.)–MeOH); IR (nujol mull) 3862, 2928, 1676 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.07 (d, J=16.2 Hz, 2H, –CH=C–), 7.71 (dd, J=1.2, 7.8 Hz, 2H, aromatic), 7.64 (dd, J=1.5, 7.8 Hz, 2H, aromatic), 7.20 (t, J=7.8 Hz, 2H, aromatic), 6.58 (d, J=15.9 Hz, 2H, –C=CH–), 1.60 (s, 6H, 9-Me_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.2, 147.5, 136.7, 130.7, 128.4, 125.4, 123.8, 122.2, 121.2, 33.9, 31.8; HRMS (EI) found M^+ 350.1156. $C_{21}H_{18}O_5$ requires M, 350.1154.

5.4.3. *N*-(3-Dimethylaminopropyl)-3-{5-[2-(3-dimethylamino propylcarbamoyl)vinyl]-9,9-dimethyl-9*H*-xanthen-4-yl} acrylamide (15). Acrylic acid **14** (0.2 g, 0.57 mmol) was suspended in dry CH_2Cl_2 (5 mL) under nitrogen. Oxalyl chloride (0.30 mL, 3.42 mmol) and a catalytic amount of *N*,*N*-dimethylformamide were added. The solution was refluxed while stirring for 3 h. After cooling to room temperature, the solvent is removed by evaporation and the yellow residue re-dissolved in dry dichloromethane (10 mL) and cooled to 0°C. 3-Dimethylamino-1-propylamine (0.36 mL, 2.85 mmol) was added slowly while stirring. The reaction was warmed to room temperature for 1 h and the solvent evaporated. Purification by silica gel chromatography, eluting with 20% MeOH–CH_2Cl_2 then switching to 5% NH_3(aq.)–MeOH, gave the amide as yellow oil (0.22 g, 75%). R_f 0.34 (5% NH_3(aq.)–MeOH); IR ($CHCl_3$ film) 3391, 2976, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, J=15.6 Hz, 2H, –CH=C–), 7.96 (s, 2H, –CO–NH–), 7.39 (ddd, J=1.6, 7.6, 16.8 Hz, 4H, aromatic), 7.08 (t, J=7.6 Hz, 2H, aromatic), 6.73 (d, J=15.6 Hz, 2H, –C=CH–), 3.47 (q, J=6.8 Hz, 4H, –CO–N–CH_2–), 2.41 (t, J=7.2 Hz, 4H, –CH_2NMe_2), 2.24 (s, 12H, –$N(CH_3)_2$), 1.79 (quintet, J=6.8 Hz, 4H, –C–CH_2–C–), 1.61 (s, 6H, 9-Me_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.3, 149.0, 136.6, 130.5, 128.9, 127.2, 123.7, 123.3, 123.2, 58.2, 45.4, 39.1, 34.0, 32.5, 26.7; HRMS (EI) found M^+ 518.3278. $C_{31}H_{42}N_4O_3$ requires M, 518.3256.

6. Procedures for biological assays

6.1. Inhibition of TryR and GR

For TryR, the standard assay mixture (1 mL) contained TryR (1 mU), 40 mM HEPES (pH 7.5), 1 mM EDTA, 50 μM NADPH, 100 μM trypanothione disulfide and varying concentrations of inhibitor. Inhibitor stock solutions were made up using co-solvent mixtures of assay buffer and DMSO; final assay mixtures contained 2% DMSO. Enzyme mixtures were pre-incubated with NADPH for 5 min at 27°C prior to initiating the reaction by the addition of substrate. Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH oxidation. GR assays were carried out in a similar manner using a buffer system composed of 40 mM HEPES (pH 7.5), 1 mM EDTA, 50 μM NADPH, 100 μM trypanothione disulfide 7.5), 80 mM KCl and 1 mM EDTA, pH 7.5. Due to interfering absorbance at 340 nm and poor compound solubility, it was not possible to determine IC_{50} values for GR.

6.2. Tritiated chloroquine accumulation

Synchronized parasitized erythrocytes in the trophozoite growth stage (1% haematocrit, 5% parasitaemia) were exposed to 1 nM [^{3}H]CQ (7.0 Ci/mmol; Amersham) in a 1.5 mL microcentrifuge tube. Appropriate controls were established for the solvent. The tubes were then incubated at 37°C in a water bath for 1 h. For the combination studies, the parasites were first incubated for 15 min at 37°C in the presence of a fixed concentration of the chemosensitizer before the radioactive CQ were added. After incubation, 100 μl of dibutylphthalate was added to the parasitized erythrocytes. The parasites were then centrifuged and the supernatant was aspirated (Beckman Microfuge E). The micro centrifuge tube tip containing the parasitized erythrocytes was then cut off and placed in a scintillation vial with 2 mL of scintillation fluid (Quicksafe A, Zinsser Analytic) and shaken overnight. The radioactivity within the vials was counted in a Packard Tri-Carb 4640 liquid scintillation spectrophotometer.

6.3. Parasite lactate dehydogenase assay

The IC_{50} of the parasites in the presence and absence of chemosensitizers was measured using a modified method of Makler et al.[28] The parasites were maintained at 1% haematocrit and 2% parasitaemia for 48 h along with the particular drug to be tested. For the combination studies, the parasites were incubated with serially diluted CQ in the presence of a fixed concentration of the chemosensitizers. The Malstat™ (Flow Inc.) reagent was used as an indicator of parasite viability.

Acknowledgements

We would like to thank The Wellcome Trust for an

International Research Development Fellowship award to K. C. (grant number 052075/Z/97). Further financial support from the National Research Foundation of South Africa (K. C.) is gratefully acknowledged. A. H. F. is supported by the Wellcome Trust.

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