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1,2,4-Triazino-[5,6*b*]indole derivatives: effects of the trifluoromethyl group on in vitro antimalarial activity

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Abstract—In an attempt to search for new and alternative antimalarial agents, a series of unsubstituted and 6-trifluoromethyl-1,2,4-triazino[5,6*b*]indole and 5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole derivatives were synthesized and their chemical structures confirmed by ^{1}H NMR and ^{13}C NMR, elemental, IR and mass spectrophotometric analyses. The in vitro antimalarial activities of these compounds were evaluated against the chloroquine-sensitive (D10) and the chloroquine-resistant (RSA11) strains of *Plasmodium falciparum*. The 1,2,4-triazino[5,6*b*]indole derivatives (**4**, **6** and **8**) with a trifluoromethyl group at position 6 exhibit increased in vitro activity when compared to the unsubstituted analogues, which are all devoid of activity. The presence of the trifluoromethyl group in the 5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole ring system leads to compounds with diminished antimalarial activity when compared to the corresponding unsubstituted analogues. The compounds associate with ferriprotoporphyrin IX and interact with DNA to more or less the same extent.

1. Introduction

The triazino[5,6*b*]indole derivatives have aroused considerable interest as a result of their broad spectrum of antibacterial, antifungal and antiparasitic activities.[1] The majority of the 5*H*-*as*-triazino[5,6*b*] indoles are active in vitro against a variety of viruses including several strains of rhinovirus.[2] Since they are purine analogues, it is expected that they should exert their pharmacological effects by interfering with the DNA metabolism. Recent hypotheses seem to suggest that the quinoline-type antimalarial drugs coordinate to the malaria parasite's endogenous antimalarial agent, Ferriprotoporphyrin IX (FP), thus disrupting the conversion of haematin to haemozoin (malaria pigment).[3] A free FP as a toxic substance to the malaria parasites, serves as a receptor for the accumulation of antimalarial drugs in the food vacuoles.[4] The parasites which are lacking in haem oxygenase are unable to detoxify the free FP by metabolism, but the malaria parasites have evolved an autocatalytic detoxification process in which FP is oxidized to haematin.[5] In this process, the malarial parasites convert the haematin to a haemazoin, a repeating array of coordinated dimers held together in a crystalline matrix by hydrogen bonding interactions.[6,7] The accumulation of FP allows for a significant concentration of the free haematin and haematin–antimalarial drug complex to remain in the food vacuole and in this way, the ability of the parasite and host red blood cells to maintain cationic gradients is impaired, leading to the death of the parasite.[8–11]

The activities of these antimalarial drugs are a function of both the ability of the drug to accumulate to pharmacologically relevant concentrations at the site of drug action, and the ability of the drugs to interfere with the haemozoin formation.[12] It also possible that haematin–antimalarial drug complex causes parasite death by a multiple mechanism,[13] with evidence suggesting that haem, rather than the haem–drug complex, is responsible for actual lysis of the vacuolar membrane.[9] It is also possible that both the free FP and its complex with chloroquine-type drugs inhibits proteases, which are essential for the degradation of haemoglobin, and thus affect the growth of the parasite.[14] The binding of the quinoline-type antimalarial drugs may also stabilize

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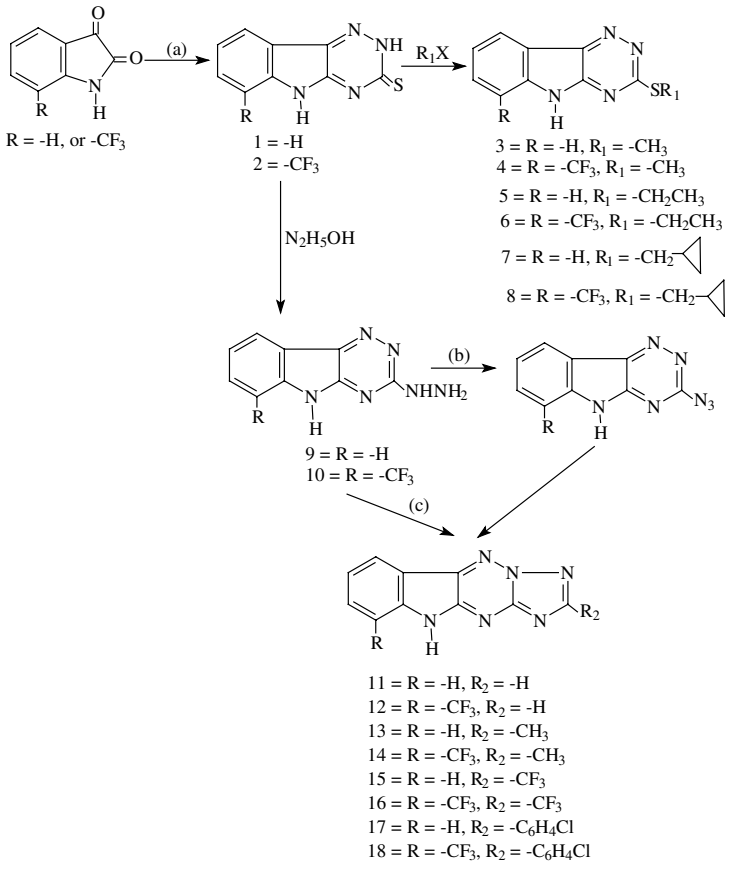
the μ-oxo dimer relative to the monomers, shifting the dimerization equilibrium to the right and reducing the amount of haematin monomers available for incorporation into the growing haemozoin.[15]

Since the discovery of the non-phototoxic, but highly effective quinolinemethanol antimalarial agent, mefloquine,[16] the trifluoromethyl group has aroused considerable interest as a pharmacophore. Halofantrine also containing a trifluoromethyl group compares favourably with mefloquine, both compounds being effective against multidrug-resistant *Plasmodium falciparum*, including strains, which are highly resistant to chloroquine.[17,18] Although there is no such a large difference in the size between the CF_3 (van der Waal's radius of 2.44 Å) and the CH_3 (2.00 Å) groups, but a very large difference in electronic effects,[19,20] the former has been found to contribute greatly to activity, since fluorocarbons often have different physicochemical properties when compared to the other halocarbons, thus altering the biological properties of the compounds in which they appear.[21] Once introduced, the fluorine, being a sterically demanding atom, with small van der Waals radius, creates a high carbon–fluorine bond energy, which renders substituent relatively resistant to metabolic transformation.[22] When substituted for hydrogen, it is able to alter, quite drastically, such parameters as neighbouring group stability and reactivity, dipole moments and most importantly, the pK_a.[23] Fluorine atom has high ionization potential, high electronegativity, small size and tightly held non-bonding electron pairs in comparison with chlorine.[24]

The emergence of multidrug-resistant strains of *Plasmodia* has created a near-desperate situation, where the need for new and inexpensive antimalarials to circumvent the parasite's resistance mechanism has become vital.[25] Chloroquine resistance is associated with reduced concentrations of the drug from the acid food vacuoles of the parasite due to increased efflux of the drug from the cell.[26] In this paper, in a search for alternative agents to the quinoline antimalarial compounds, we report the synthesis and the exploratory evaluation of the in vitro antimalarial activities of a series of 6-trifluoromethyl-1,2,4-triazino[5,6*b*]- and 5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino-[5,6*b*] indole derivatives, together with their characteristic interactions with ferriprotoporphyrin IX to ascertain their mode of antimalarial action.

2. Chemistry

Isatin or the 6-trifluoromethylisatin prepared earlier[27] was condensed with thiocarbazide in K_2CO_3 solution to form 1,2,4-triazino-[5,6*b*]indole-3-thione (**1**) or 6-trifluoromethyl-1,2,4-triazino-[5,6*b*]indole-3-thione (**2**) (see Scheme 1). Each of one of these products was alkylated with each of methyl chloride, ethylchloride and chlorocyclopropylmethane to form 3-methylthio- (**3**), 3-methylthio-6-trifluoromethyl- (**4**), 3-ethylthio- (**5**), 3-



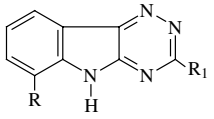
Scheme 1. Reagents: (a) $NHCSNHNH_2$, K_2CO_3; (b) $NaNO_2$/HCl; (c) HCOOH; CF_3COOH; ClC_6H_4COOH in 4% NaOH.

ethylthio-6-trifluoromethyl-(**6**) and 3-cyclopropylmethylthio- (**7**) and 3-cyclopropylmethylthio-6-trifluoromethyl- (**8**) 1,2,4-triazino-[5,6*b*]indole derivatives following a known procedure.[1] Refluxing a mixture of **1** or **2** with hydrazine hydrate in HCl resulted in the formation of 3-hydrazo-1,2,4-triazino-[5,6*b*]indole (**9**) or 3-hydrazo-6-trifluoromethyl-1,2,4-triazino-[5,6*b*]indole (**10**), which on reaction with sodium nitrite resulted in the formation of 3-azido derivatives. In the presence of HCl, formic, acetic, trifluoroacetic or chlorobenzoic acid, the hydrazo and azido derivatives cyclize to form fused tetrazole compounds, 5*H*-1,2,4-triazolo-[1′5′2,3]-1,2,4-triazino-[5,6*b*]indole derivatives (**11** and **12**), 3-methyl- (**13** and **14**), 3-trifluoromethyl- (**15**) and 3,6-bis(trifluoromethyl)- (**16**) and 3-(2-chlorophenyl)- (**17** and **18**) 5*H*-1,2,4-triazolo-[1′5′,2,3]-1,2,4-triazino-[5,6*b*]indole derivatives.

3. Results and discussion

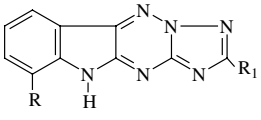
The unsubstituted and the 6-trifluoromethyl-1,2,4-triazino[5,6*b*]indole analogues synthesized were evaluated for antimalarial activity in vitro against the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. From the results which are summarized in Table 1, it is clear that the CF_3 group tends to lead to an increased in vitro antimalarial activity of the 1,2,4-triazino[5,6*b*]indole albeit to a smaller degree in some compounds. Analogues without the trifluoromethyl group, which were evaluated simultaneously, were all devoid of activity even at concentrations as high as 400 μM. The increased activity resulting from the presence of this group could be ascribed to the increased lipophilicity of the compound, as this group is known to be more hydrophobic than even the fluorine atom.[24] In this series, the size of the substituent on the thiol group does not have significant effect on in vitro antimalarial activity. Replacement of a thiol group by a hydrazino group in the trifluoromethyl substituted derivatives leads to compounds with half the potency of those with alkyl groups on the thiol group. On the other hand, the activity of the 6-trifluoromethyl-5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole series (**11**–**18**) (Table 2) is almost identical to those of the 6-trifluoromethyl-1,2,4-triazino[5,6*b*]indole-3-alkylthiols, with the exception of compound **14** containing the 6-CF_3 and the 3-CH_3 groups. The latter exhibits a fourfold improvement in activity against the chloroquine-sensitive strains. However, introduction of a second CF_3 particularly at position 3 (**16**) tends to lead to compounds with diminished antimalarial activity. Further consideration of the structure–activity relationship of compounds in the series without the 6-CF_3 indicates that **13** (R = –H and R_1 = $–CH_3$) is the most active against the chloroquine-sensitive strains followed by **11** (R = –H and R_1 = –H). Compound **13** exhibits activity against the chloroquine-sensitive strains in the same molar range as chloroquine and mefloquine under identical

Table 1. In vitro IC_{50} (μM) of the 6-trifluoromethyl-1,2,4-triazino-[5,6]indoles (n = 4)



Compound	R	R_1	Melting point (°C)	Chloroquine sensitive	Chloroquine resistant
2	$–CF_3$	=S	262–264	260.00 ± 10	ND
4	$–CF_3$	$–SCH_3$	168–170	35.00 ± 5	14.00 ± 2
6	$–CF_3$	$–SCH_2CH_3$	244–246	44.00 ± 8	20.00 ± 5
8	$–CF_3$	$–CH_2$-cyclopropyl	259–260	62.00 ± 10	20.00 ± 2
10	$–CF_3$	$–NHNH_2$	279–281	187.00 ± 10	ND

Table 2. In vitro IC_{50} (μM) of the 5*H*-1,2,4-triazolo-[1′,5′,2,3]-1,2,4-triazino-[5,6*b*]indoles (n = 4)



Compound	R	R_1	Melting point (°C)	Chloroquine sensitive	Chloroquine resistant
11	–H	–H	372–374	7.10 ± 1.2	48.0 ± 0.05
12	$–CF_3$	–H	236–238	40.00 ± 10	ND
13	–H	$–CH_3$	401–402	36.00 ± 1.3	20.10 ± 1.5
14	$–CF_3$	$–CH_3$	241–244	86.00 ± 1.2	26.00 ± 2.1
15	–H	$–CF_3$	398–400	54.00 ± 10	ND
16	$–CF_3$	$–CF_3$	263–265	142.00 ± 12	ND
17	–H	$–C_6H_4Cl$	376–378	>312.00 ± 32	ND
18	$–CF_3$	$–C_6H_4Cl$	286–288	46.00 ± 8	ND
Chloroquine				29.64 ± 7.06	277.5 ± 40.6
Mefloquine				38.45 ± 2.84	8.12 ± 3.62

ND = not done.

experimental conditions. On the other hand, compound **11** is the most active against the chloroquine-resistant strains of *P. falciparum*. Both $-CF_3$ and $-C_6H_4Cl$ groups at position 3 without CF_3 at position 6 lead to relatively inactive compounds **17** and **18**.

The fact that the *P. falciparum* lactate dehydrogenase (PfLDH) activity can be distinguishable from the host LDH[28] by using the 3-acetyl pyridine adenine dinucleotide analogue of nicotinamide adenine dinucleotide (APAD) has afforded an opportunity for the development of an enzymatic method for the evaluation of antimalarial compounds.[29] The compounds selected for screening against the chloroquine-resistant strain of *P. falciparum* show identical but much higher in vitro activity than against the chloroquine-sensitive strain, with the exception of compound **11** which has a IC_{50} value of 48.0 μM, which is very close to that of chloroquine (IC_{50} of 277.5 μM) against this strain. At position 3, the CF_3 group does not lead to any improvement on the in vitro antimalarial activity than it does when attached at position 6. The 3,6-bis(trifluoromethyl)-5*H*-1,2,4-triazolo-[1′,5′,2,3]-1,2,4-triazino-[5,6*b*]indole (**16**) has a much lower activity against both the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* than the other 6-CF_3 substituted derivatives (Tables 1 and 2). As a result of a direct relationship between the level of drug accumulation in the parasite food vacuole and antimalarial drug potency, but no simple relationship between accumulation and either pK_a or lipophilicity,[5,23,30,31] it could be inferred that the bulkiness of the molecule conferred by the second CF_3 group will affect the relative membrane permeability of the molecule, leading to reduced drug accumulation within the parasite food vacuole. Earlier confirmation[32,33] is that when the substituent in position 6 of the quinoline ring is either decreased in relative electronegativity, or increased in volume, the antimalarial potency is decreased as these structural changes are likely to decrease the affinity for the 2-amino group of the guanine in the DNA molecule or are likely provide steric hindrance to intercalation. Introduction of one or two fluorine atoms into the phenolic moiety of the aminoquinolines results in a significant decrease in drug potency in the chloroquine-sensitive isolates, but no real effect on potency against the chloroquine-resistant isolates in comparison with aminoquinoline.[22]

The results of the spectrophotometric titration of Ferriprotopophyrin IX (FP) with these compounds (Fig. 1), indicate a decrease and slight shift in absorbance of the Soret band of FP, albeit to the same extent by all the compounds. This behaviour is distinctively observed with molecular complex formation between quinoline-type molecules and metalloporphyrins.[23] However, it also appears that the structural features of the compounds have no bearing on the manner in which they associate with FP. Two processes could be involved here: either addition of micromolar concentrations of the drugs induces aggregation of FP, or the changes reflect association of the drugs with FP.[34] It is also not clear whether these associations are through complex formation between the drugs and FP. While large decrease in the absorbance of the Soret band is often an indication of aggregation, equally large decrease can be caused by formation of π–π complexes. For the quinolines to inhibit parasite growth by inhibition of haemazoin formation, it is required that they accumulate and concentrate in the parasitic food vacuoles to levels greater than micromolar levels that are required to inhibit haematin dimerization in vitro.[8] The extent of drug accumulation at the site of haematin dimerization is also a regulator of antimalarial activity and this may be influenced by the physiological properties of the drug and the proton gradient, which exist between the external environment and the intracellular parasite.[35]

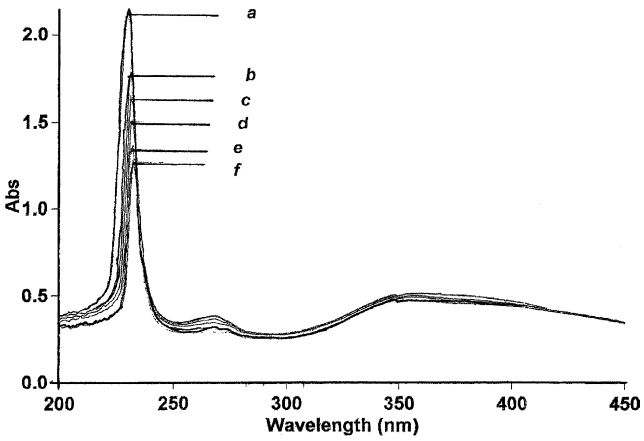


Figure 1. Spectrophotometric titration of Ferriprotoporphyrin IX (FP) (1.3×10–4 M) with compound **14**. In each case a is a curve for FP, and b–f represent the concentrations of **14** at 6.7×10^{-7} M, 1.3×10^{-6} M; 2.2×10^{-6} M; 3.3×10^{-6} M and 4.3×10^{-6} M added. The reference cell was titrated simultaneous with equimolar amounts of compound **14** and the absorbance values corrected for dilution.

The results of the spectrophotometric titration of DNA–ethidium bromide complex with these compounds (Fig. 2) indicate that all the compounds displace ethidium from its complex with DNA. Although the experiments

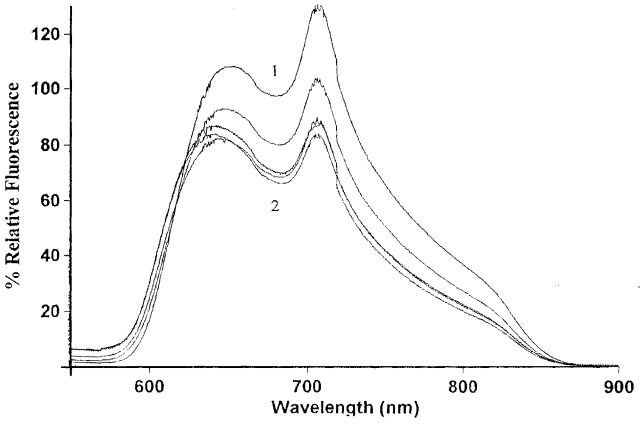


Figure 2. Spectrophotometric titration of DNA–ethidium bromide complex with compound **14**. Similar results were obtained with other compounds. In each case, 1 is the peak for DNA saturated with ethidium bromide, while peak 2 is after addition of increasing concentration of compound **14**.

reported here are not sufficient to establish a binding mechanism for these compounds, the slight spectral shifts obtained on the DNA titration support the intercalation into or formation of a complex with the DNA helix as their mode of binding.[36,37]

4. Conclusion

Although the trifluoromethyl group at position 6 of the 1,2,4-triazino-[5,6*b*]indole derivatives does confer some antimalarial activity on these compounds when compared to the unsubstituted analogues, the activity is not better than that exhibited by the known antimalarial agents such as chloroquine. In the 5*H*-1,2,4-triazolo-[1′,5′,2,3]-1,2,4-triazino-[5,6*b*]indole series, the trifluoromethyl group tends to produce compounds, which are less active than the unsubstituted analogues. An except is 3-methyl-6-trifluoromethyl-5*H*-1,2,4-triazolo-[1′5′,2,3]-1,2,4-triazino-[5,6*b*]indole (**14**), which exhibit antimalarial activity in the same range as 5*H*-1,2,4-triazolo-[1′,5′,2,3]-1,2,4-triazino-[5,6*b*]indole (**11**). The unsubstituted or methyl substituted derivatives in this tetrazolo series exhibit antimalarial activity in the same range as chloroquine and mefloquine against both the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Replacement of the H or CH_3 by a bulkier group such as CF_3 or C_6H_4Cl tends to result in loss of activity. Although the mechanism of action of these compounds is uncertain, they all associate with both Ferriprotoporphyrin IX and DNA. However, further structural investigation of these planar tetracyclic compounds is still needed to confirm the potential toxicity, antimalarial activity and the exact mechanism of action of these DNA intercalators.

5. Experimental

Melting points were determined in open capillary tubes on a Büchi B-545 apparatus and are uncorrected. IR spectra were recorded in KBr on a Perkin–Elmer paragon 1000 FT-IR spectrophotometer. ^{1}H NMR spectra were taken on a GEMINI 200BB spectrometer. Chemical shifts are reported in δ (ppm) relative to internal $(CH_3)_4Si$ DMSO-d_6 unless otherwise noted. Peak multiplicity are designated as follows: s, singlet, d, doublet, t, triplet, q, quartet, br s, broad singlet, m, multiplet. ^{13}C NMR spectra were recorded on the GEMINI 200BB spectrometer at 75 MHz. Chemical ionization mass spectra as *m*/*z* (% relative intensity) values were carried out on a Finnigan spectrometer (Model 8200). TLC was carried out on pre-coated silica gel $60F_{254}$ analytical plates and the resulting chromatograms visualized under UV light (254 nm) to check the purity of all the compounds.

5.1. Syntheses

As the preparation of the unsubstituted 1,2,4-triazino-[5,6*b*]indole thione derivatives has been outlined,[1] and they are all devoid of any antimalarial activity, their synthetic procedure will not be included in this section, except those that have shown promising activity.

5.1.1. Trifluoromethyl-1,2,4-triazino-[5,6*b*]indole-3-thione (2). A mixture of 7-trifluoromethylisatin (21.5 g: 0.1 mol), thiosemicarbazide (9.1 g: 0.1 mol) and K_2CO_3 (56.7 g: 0.15 mol) in 500 mL of water was refluxed with stirring for 3 h. On cooling the mixture was filtered and precipitated by acidification with acetic acid. The solid was washed with water and dried. A sample was recrystallized from DMF. Yield 10.8 g (40%). Mp 262–264 °C. (Found: C, 43.04; H, 1.13; N, 21.89. $C_9H_5N_4F_3S$ requires C, 41.84; H, 1.95; N, 21.71.) ^{1}H NMR: δ = 7.1 (t, H/H, H-5), 7.6 (d, J(H/H) = 6 Hz, H-6), 7.9 (d, J(H/H) = 8 Hz, H-4), 9.6 (s, H/H, NH). ^{13}C NMR: δ = 115.8 (q, $^2J_{CF}$ = 32 Hz), 119.6, 120.8, 122.3, 125.6 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 127.3, 140.6, 147.9, 149.9, 156.2. MS (70 eV, EI): *m*/*z* (%): M^+ 260 (100), 222 (46), 193 (24), 152 (47), 60 (51), 43 (33), 28 (29). IR (KBr): ν = 3430m, 3048s, 1602m, 1515s, 1407m, 1318s, 1182s, 1113s.

5.2. A general procedure for the synthesis of 3-alkylthio-1,2,4-triazino[5,6*b*]indoles

To a solution of **1** (0.01 mol) in aqueous sodium hydroxide (4%), alkyl halide (0.01 mol) was added during 2–5 min with stirring. The mixture was stirred for 1 h and the precipitate filtered and washed with water and dried.

5.2.1. 3-Methylthio-6-trifluoromethyl-1,2,4-triazino-[5,6*b*]indole (4). Yield 46%. Recrystallization solvent, methanol. Mp 168–170 °C. (Found: C, 42.32; H, 2.93; N, 20.34. $C_{10}H_7N_4F_3S$ requires C, 44.10; H, 2.59; N, 20.59.) ^{1}H NMR: δ = 1.6 (s, CH_3), 7.2 (t, H/H, H-5), 7.8 (d, J(H/H) = 6 Hz, H-6), 8.3 (d, J(H/H) = 6 Hz, H-4), 9.8 (br s, NH). ^{13}C NMR: δ = 51.7 (CH_3), 115.6 (q, $^2J_{CF}$ = 32 Hz), 119.4, 122.5, 124.3, 125.7 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 127.4, 142.4, 149.7, 154.7, 163.4. MS (70 eV, EI): *m*/*z* (%): M^+ 272 (100), 256 (19), 241 (18), 220 (33), 177 (20). IR (KBr): ν = 3415s, 3068m, 2926m, 1596s, 1411m, 1319s, 1175s.

5.2.2. 3-Ethylthio-6-trifluoromethyl-1,2,4-triazino-[5,6*b*]-indole (6). Yield 88%. Recrystallization solvent, methanol. Mp 244–246 °C. (Found: C, 45.97; H, 2.98; N, 20.03. $C_{11}H_9N_4F_3S$ requires C, 46.13; H, 3.17; N, 19.58.) ^{1}H NMR: δ = 1.4 (t, (H/H), 3H, CH_3); 2.3 (q, J(H/H) = 7.4 Hz, 2H, CH_2), 7.6 (t, (H/H), H-5), 7.9 (d, J(H/H) = 6 Hz, H-4), 8.6 (d, J(H/H) = 6 Hz, H-6). ^{13}C NMR: δ = 14.5 (CH_3), 49.0 (CH_2), 113.1 (q, $^2J_{CF}$ = 32 Hz), 119.9, 122.3, 124.7, 125.6 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 127.3, 140.0, 147.9, 155.8, 166.9. MS (70 eV, EI): *m*/*z* (%): M^+ 286 (100), 242 (8), 222 (10), 177 (6), 69 (2), 44 (20). IR (KBr): ν = 3410s, 1563s, 1413s, 1320s, 1182s, 1121s.

5.2.3. 3-Cyclopropylmethylthio-6-trifluoromethyl-1,2,4-triazino-[5,6*b*]indole (8). Yield 55%. Recrystallization solvent, DMF. Mp 259–260 °C. (Found: C, 49.45; H, 4.06; N, 18.67. $C_{13}H_{11}N_4F_3S$ requires C, 49.97; H, 3.55; N, 17.95.) ^{1}H NMR: δ = 1.9 (d, J(H/H) = 8.2 Hz,

2H, CH_2), 2.5 (q, 2H, CH_2), 3.4 (m, 4H, CH), 7.3 (t, (H/H) H-5), 7.8 (d, J(H/H) = 6 Hz, H-4), 8.1 (d, J(H/H) = 6 Hz, H-6), 8.3 (s, 1H, NH). ^{13}C NMR: δ = 14.7 (CH_2), 25.6 (CH_2), 53.5 (CH), 112.7 (br q, $^2J_{CF}$ = 32 Hz), 118.7, 122.5, 124.3, 124.8, 125.6 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 137.2, 139.5, 161.0. MS (70 eV, EI): *m*/*z* (%): M^+ 312 (60), 309 (30), 270 (100), 242 (21), 222 (68). IR (KBr): ν = 3460s, 1597s, 1413m, 1320m, 1161s.

5.2.4. 3-Hydrazino-6-trifluoromethyl-1,2,4-triazino[5,6*b*]-indole (10). A mixture of 6-trifluoromethyl-1,2,4-triazino[5,6*b*]indole-3-thione (2.70 g: 0.01 mol) and 10 mL of hydrazine hydrate (98%) was refluxed for 4 h. On cooling a light yellow solid separated and was washed with water, methanol and recrystallized from DMF. Yield 1.40 g (52%). Mp 279–281 °C. (Found: C, 44.97; H, 2.75; N, 30.86. $C_{10}H_7N_6F_3$ requires 44.76; H, 2.63; N, 31.35.) ^{1}H NMR: δ = 2.5 (t, 3H, NH), 3.3 (d, J(H/H) = 8 Hz, 2H, NH_2), 7.4 (t, (H/H), H-5), 7.8 (d, J(H/H) = 6 Hz, H-4), 8.4 (d, J(H/H) = 6 Hz, H-6), 8.8 (s, 1H, NH). ^{13}C NMR: δ = 113.6 (q, $^2J_{CF}$ = 32 Hz), 117.2, 119.8, 122.4, 124.3, 124.6, 125.8 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 127.4, 139.8, 147.8, 168.5. MS (70 eV, EI): *m*/*z* (%): M^+ 268 (100), 204 (34), 177 (35), 44 (11), 28 (8). IR (KBr): ν = 3270s, 1612s, 1518s, 1402m, 1310s, 1176s, 1110s.

5.3. General procedure for the synthesis of the 5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole derivatives

2-Hydrazino-1,2,4-triazino[5,6*b*]indole derivative (**9** or **10**) (0.01 mol) was refluxed in 5 mL of the respective acid for 2 h. The solution was precipitated by addition of water, the product filtered and dried.

5.3.1. 5*H*-1,2,4-Triazolo-[2,3*b*]-1,2,4-triazino-[5,6*b*]indole (11). Yield 78%. Recrystallization solvent, DMF. Mp 372–374 °C. (Found: 56.97; H, 3.07; N, 39.83. $C_{10}H_6N_6$ requires C, 57.12; H, 2.88; N, 40.00.) ^{1}H NMR: δ = 7.3 (t, (H/H), H-5), 7.4 (d, J(H/H) = 6 Hz, H-4), 7.7 (t, (H/H), H-6), 8.1 (d, J(H/H) = 6 Hz, H-7), 9.5 (s, 1H, H-1), 12.2 (br s, 1H, NH). ^{13}C NMR: δ = 112.5, 116.0, 122.4, 133.6, 136.5, 145.8, 151.0, 155.3, 158.3, 168.1. MS (70 eV, EI): *m*/*z* (%): M^+ 210 (100), 155 (58), 128 (79), 103 (91), 76 (63), 50 (26), 44 (34), 28 (20). IR (KBr): ν = 2634w, 1611s, 1452m.

5.3.2. 6-Trifluoromethyl-5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole (12). Yield 58%. Recrystallization solvent, ethanol. Mp 236–238 °C. (Found: 47.56; H, 1.75; N, 30.43. $C_{11}H_5N_6F_3$ requires C, 47.47; H, 1.81; N, 30.22.) ^{1}H NMR: δ = 7.5 (t, (H/H), H-5); 8.0 (d, J(H/H) = 6 Hz, H-4), 8.5 (d, J(H/H) = 6 Hz, H-6), 9.6 (s, 1H, H-1), 12.6 (br s, 1H, NH). ^{13}C NMR: δ = 115.8 (q, $^2J_{CF}$ = 32 Hz), 117.2, 120.9, 126.1 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 128.7, 135.6, 147.8, 148.1, 153.4, 162.1. MS (70 eV, EI): *m*/*z* (%): M^+ 278 (100), 258 (52), 223 (26), 203 (56), 177 (34), 170 (26), 151 (91), 75 (25), 57 (6), 29 (16). IR (KBr): ν = 3105m, 1710m, 1619s, 1533m, 1317s, 1124s, 740m.

5.3.3. 3-Methyl-5*H*-1,2,4-triazolo-[2,3*b*]-1,2,4-triazino-[5,6*b*]indole (13). Yield 76%. Recrystallization solvent, DMF. Mp 401–402 °C. (Found: 59.47; H, 4.03; N, 36.86. $C_{11}H_8N_6$ requires C, 58.90; H, 3,60; N, 37.50.) ^{1}H NMR: δ = 2.7 (s, CH_3), 7.3 (t, (H/H), H-5), 7.4 (d, J(H/H) = 6 Hz, H-4), 7.7 (t, (H/H), H-6), 8.1 (d, J(H/H) = 6 Hz, H-7), 12.1 (br s, NH). ^{13}C NMR: δ = 13.5 (CH_3), 115.6, 118.8, 121.5, 125.6, 131.5, 140.1, 146.7, 148.1, 156.1, 158. 0. MS (70 eV, EI): *m*/*z* (%): M^+ 224 (100), 155 (46), 143 (3), 128 (71), 103 (88), 101 (22), 84 (8), 76 (53), 57 (22), 50 (24), 44 (66), 28 (34). IR (KBr): ν = 3054m, 2943m, 1612s, 1521s, 1463m, 1379m, 1297m, 1081m, 745m.

5.3.4. 3-Methyl-6-trifluoromethyl-5*H*-1,2,4-triazolo[1′,5′, 2,3]-1,2,4-triazino[5,6*b*]indole (14). Yield 47%. Recrystallization solvent, methanol. Mp 241–244 °C. (Found: 48.74; H, 2.98; N, 29.08. $C_{12}H_8N_6F_3$ requires C, 49.13; H, 2.75; N, 28.67). ^{1}H NMR: δ = 2.8 (s, 1H, CH_3), 7.5 (t, (H/H), H-5), 8.0 (d, J(H/H) = 6 Hz, H-4), 8.4 (d, J(H/H) = 6 Hz, H-6), 12.6 (br s, 1H, NH). ^{13}C NMR: δ = 9.8 (CH_3), 114.3 (q, $^2J_{CF}$ = 30 Hz), 118.4, 120.5, 121.8, 125.1 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 129.6, 141.8, 144.3, 148.3, 155.6, 165.1. MS (70 eV, EI): *m*/*z* (%): M^+ 292 (100), 151 (8), 141 (2), 125 (2), 57 (7), 44 (17), 28 (11). IR (KBr): ν = 3372m, 1618s, 1512s, 1456m, 1315s, 1117s, 753m.

5.3.5. 3-Trifluoromethyl-5*H*-1,2,4-triazolo-[2,3*b*]-1,2,4-triazino-[5,6*b*]indole (15). Yield 92%. Recrystallization solvent, DMF. Mp 398–400 °C. (Found: C, 46.97; H, 1.91; N, 31.21. $C_{11}H_5N_6F_3$ requires C, 47.47; H, 1.81; N, 30.22.) ^{1}H NMR: δ = 7.3 (t, (H/H), H-5), 7.5 (d, J(H/H) = 6 Hz, H-4), 7.8 (t, (H/H), H-6), 8.3 (d, J(H/H) = 6 Hz, H-7), 12.6 (br s, NH). ^{13}C NMR: δ = 115.9, 117.2, 118.3, 121.8, 128.3 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 130.8, 140.3, 147.2, 148.7, 155.1 (q, $^2J_{CF}$ = 30 Hz), 156.2. MS (70 eV, EI): *m*/*z* (%): M^+ 278 (100), 200 (54), 155 (34), 143 (8), 128 (68), 103 (94), 76 (52). IR (KBr): ν = 3079m, 2979m, 1677m, 1616s, 1385m, 1195s, 751m.

5.3.6. 3,6-Bis(trifluoromethyl)-5*H*-1,2,4-triazolo[1,5′,2,3]-1,2,4-triazino[5,6*b*]indole (16). Yield 34%. Recrystallization solvent, ethanol. Mp 263–265 °C. (Found: C, 40.87; H, 0.97; N, 24.67. $C_{12}H_4N_6F_6$ requires C, 41.61; H, 1.17; N, 24.28.) ^{1}H NMR: δ = 7.5 (t, (H/H), H-5), 7.9 (d, J(H/H) = 6 Hz, H-4), 8.4 (d, J(H/H) = 6 Hz, H-6). ^{13}C NMR: δ = 115,1 (q, $^2J_{CF}$ = 30 Hz), 118.4, 121.3, 122.8, 126.9 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 128.6 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 131.5, 140.2, 148.2, 148.3, 155.9 (q, $^2J_{CF}$ = 30 Hz), 157.6. MS (70 eV, EI): *m*/*z* (%): [M−1] 345 (3), 286 (4), 268 (11), 253 (12), 205 (14), 177 (9), 128 (5), 97 (8), 57 (22), 44 (100). IR (KBr): ν = 3233m, 2925m, 1679s, 1655s, 1406m, 1322m, 1195s.

5.3.7. 3-(2-Chlorophenyl)-5*H*-1,2,4-triazolo-[2,3*b*]-1,2,4-triazino-[5,6*b*]indole (17). Yield 55%. Recrystallization solvent, DMF. Mp 376–378 °C. (Found: C, 61.07; H, 2.27; N, 25.89. $C_{16}H_9N_6Cl$ requires C, 59.89; H, 2.83; N, 26.22.) ^{1}H NMR: δ = 7.2 (m, (H/H) H-arom), 7.3 (t, (H/H), H-5), 7.4 (d, J(H/H) = 6 Hz, H-4), 7.70 (t,

(H/H) H-6), 8.1 (d, J(H/H) = 6 Hz, H-7), 12.2 (s, 1H, NH). ^{13}C NMR: δ = 112.4, 117.9; 122.1, 123.1, 127.9, 130.0, 131.8, 132.1, 133.6, 136.5, 140.7, 145.8, 155.3, 156.5, 168.3, 198.1. MS (70 eV, EI): m/z (%): M^+ 320 (50), 209 (100), 155 (60), 112 (50), 101 (80), 85 (20), 45 (25). IR (KBr): ν = 2625m, 1610s, 1520s, 1204m, 1151w, 753m.

5.3.8. 3-(2-Chlorophenyl)-6-trifluoromethyl-5*H*-1,2,4-triazolo[1′,5′,2,3]-1,2,4-triazino[5,6*b*]indole (18). Yield 48%. Recrystallization solvent, DMF. Mp 286–288 °C. (Found: C, 52.76; H, 1.93; N, 22.56. $C_{17}H_8N_6F_3Cl$ requires C, 52.50; H, 2.08; N, 21.63.) ^{1}H NMR: δ = 7.3 (t, (H/H), 1H arom), 7.5 (t, (H/H), H-5), 8.1 (d, J(H/H) = 6 Hz, H-4), 8.3 (d, J(H/H) = 6 Hz, H-6), 10.3 (br s, NH). ^{13}C NMR: δ = 116.3 (q, $^2J_{CF}$ = 30 Hz), 121.2, 122.6, 126.6 (br q, $^1J_{CF}$ = 300 Hz, CF_3), 127.9, 128.0, 130.2, 131.3, 131.8, 132.1, 135.6, 148.2, 155.3, 157.5, 169.6. MS (70 eV, EI): m/z (%): M^+ 388 (5), 357 (22), 333 (70), 278 (87), 268 (42), 177 (24), 151 (58), 139 (30), 111 (31), 97 (44), 85 (49), 69 (51), 57 (100), 45 (41). IR (KBr): ν = 3248w, 2921m, 1612s, 1315m, 1195s.

5.4. Bioassay methods

5.4.1. In vitro antimalarial screening. The antimalarial activity of these newly synthesized compounds were determined by the measurement of the lactate dehydrogenase activity (LDH)[29] on the chloroquine-sensitive (D10) and chloroquine-resistant (RSA 11) strains of *P. falciparum*. The strains were obtained from the Department of Pharmacology, University of Cape Town, RSA. Sensitivity assays were initiated by adjusting the initial parasitaemia to 1–2% with normal type A human red blood cell (2% haematocrit) suspended in complete tissue culture medium (RPMI 1640 containing 25 μM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane sulfonate]-(HEPES) buffer, 200 μg/ml of gentamycin, 27 μM sodium bicarbonate and 10% normal type A heat-inactivated human serum). The suspension was dispersed in triplicate at 200 μL/well into 96-well flat-bottom microtitre plates, leaving one row for positive control (no drug but containing parasitized red blood cells) and one row for background control (no parasites but only red blood cells). Twenty-five litres of each drug solution containing 0.01–100 μg/ml of the drugs was added to each well except the positive control row. The cultures were placed in a humidified incubator at 37 °C with a gas-controlled environment of 3% O_2, 6% CO_2 and 91% N_2 for 48 h. After the incubation period of 48 h, 10 μL of the parasite culture, 25 μL of nitroblue tetrazolium (NBT) (0.24 mM)/phenazine ethosulfate (PES) (0.033 mM) mixture and 100 μL of APAD were mixed in respective wells in another clean 96-well plate. The plate was left in the dark for 0.5–1 h. As APADH is formed, the NBT is reduced to a formazan product that is blue, which can be detected visually, and measured spectrophotometrically at 620 nm using the Anthos Labtec HT2 model 1.06 spectrophotometer (Anthos Labotec Instruments, Salzburg, Austria). Each well of a test microlitre plate was automatically measured at 30 s intervals, and the individual data points were stored and plotted. From the results of four determinations the plots of % parasite survival versus concentrations were prepared and the IC_{50} values calculated from which the IC_{50} values were read.

5.4.2. Interaction of the compounds with Ferriprotoporphyrin IX. Spectroscopic changes associated with the interaction of the compounds with ferriprotoporphyrin IX was performed following a previous procedure.[34] Stock solutions were prepared by dissolving 6–8 mg of accurately weighed Ferriprotoporphyrin IX (Sigma–Aldrich Chemie, Steinheim, Germany) in 10 mL AR grade DMSO (Sigma–Aldrich Chemie). These stock solutions were stored in the dark. Aqueous-DMSO (40% v/v) solutions of FP were prepared daily by mixing 20 μL of the FP stock solution with 4 mL DMSO and 1 mL 0.2 M HEPES buffer (pH 7.4) and making up to 10 mL with deionized water. Solutions of the compounds of interest were prepared by dissolving them in 0.02 M HEPES and 40% DMSO to obtain final concentrations of about 2 mM. The ferriprotoporphyrin IX–compounds interactions were monitored by spectrophotometric titration of both sample and reference solutions in a thermostated cell holders using a Cary 100 Conc UV/vis Spectrophotometer (Varian Australia (Pty) Ltd, Mulgrave Victoria, Australia) at 25 °C and measuring the absorbance of the Soret band at 230 nm. A reference cell was titrated simultaneously with the compounds. The compounds seem to give similar reaction with Ferriprotoporphyrin IX as shown by Figure 1 for compound **14**.

5.4.3. Interaction of the compounds with thymus calf DNA. The ability of the compounds to displace ethidium from DNA was monitored by recording the changes in fluorescence according to the literature procedure.[38] In this procedure, ethidium bromide (Sigma–Aldrich Chemie, Steinheim, Germany) was added to 3 mL buffered solution (2 mM HEPES, 8 mM NaCl and 0.05 mM EDTA, pH 7) in a 1 cm pathlength cuvette. DNA (calf thymus, Sigma–Aldrich Chemie, Steinheim, Germany) was added so that the scale reading was increased 7-fold. Each of the test compounds was added in microlitre portions via a microsyringe and the change in fluorescence recorded after each addition. All experiments were performed at 25 °C. The titration was performed in duplicate and the IC_{50} values, representing the concentration of the compounds needed to decrease the DNA-bound ethidium fluorescence by 50% were determined and recorded with Figure 2 representing a typical titration results of compound **14** (identical results were obtained with all other compounds).

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