Synthesis and Evaluation of Cryptolepine Analogues for Their Potential as New Antimalarial Agents

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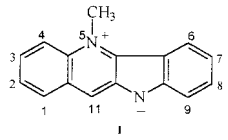
The indoloquinoline alkaloid cryptolepine **1** has potent in vitro antiplasmodial activity, but it is also a DNA intercalator with cytotoxic properties. We have shown that the antiplasmodial mechanism of **1** is likely to be due, at least in part, to a chloroquine-like action that does not depend on intercalation into DNA. A number of substituted analogues of **1** have been prepared that have potent activities against both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* and also have in common with chloroquine the inhibition of β-hematin formation in a cell-free system. Several compounds also displayed activity against *Plasmodium berghei* in mice, the most potent being 2,7-dibromocryptolepine **8**, which suppressed parasitemia by 89% as compared to untreated infected controls at a dose of 12.5 mg kg^{-1} day^{-1} ip. No correlation was observed between in vitro cytotoxicity and the effect of compounds on the melting point of DNA (ΔT_m value) or toxicity in the mouse−malaria model.

Introduction

Cryptolepine (5-methyl,10*H*-indolo[3,2-*b*]quinoline; **1**) is an indoloquinoline alkaloid found in the west African climbing shrub *Cryptolepis sanguinolenta* (family Periplocaceae). A decoction of the roots of this species is used in traditional medicine for the treatment of malaria as well as for a number of other diseases.[1] Previous work has shown that cryptolepine has potent in vitro antiplasmodial activity,[2] but this alkaloid also has cytotoxic properties that are likely due to its abilities to intercalate into DNA and inhibit topoisomerase II as well as DNA synthesis.[3]

In this paper, we report the synthesis of 15 cryptolepine derivatives (of which 11 are novel) to determine whether it is possible to prepare cryptolepine analogues that have reduced abilities to interact with DNA (and hence may be less cytotoxic) but that retain potent antiplasmodial activities. This hypothesis is supported by evidence (presented below) which suggests that the antiplasmodial activity of cryptolepine is due, at least in part, to a chloroquine-like action that does not depend on intercalation into DNA. Chloroquine and related antimalarials appear to act primarily by inhibiting the formation of hemozoin (malaria pigment), which is formed in malaria parasites from hemin, the toxic residue remaining following the digestion of hemoglobin by the parasite.[4] Drugs that have a chloroquine-like mode of action may be detected by testing their ability to prevent the formation of β-hematin (shown to be identical to hemozoin[5]) from hemin in a cell-free system.[6] Peaks at 1660 and 1210 cm^{-1} in the FTIR spectrum of the product confirm the presence of β-hematin (see below). To assess cryptolepine analogues for their potential as leads to selective antimalarial agents, we have determined their antiplasmodial activities against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*. Those compounds displaying potent in vitro antiplasmodial activities have been assessed for in vivo antimalarial activity against *Plasmodium berghei* in mice. Selected compounds have been tested for their abilities to inhibit β-hematin formation. Thermodenaturation techniques have been used to examine the effects of compounds on the melting point of calf thymus DNA as an indication of their potential to interact with DNA, and preliminary cytotoxicity tests have been carried out.



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Chemistry

Cryptolepine (**1**) was isolated from *C. sanguinolenta* as described previously[2] or prepared by methylation of quindoline, which was synthesized using methodology based on that of Holt and Petrow (1947)[7] (Scheme 1). Isatin (**2**a) was condensed with *O*,*N*-acetylindoxyl (**3a)** in the presence of KOH under oxygen-free conditions to give quindoline-11-carboxylic acid (**4a**). The latter was decarboxylated by heating in diphenyl ether to yield quindoline (**5a**), which was then methylated using iodomethane in tetramethylenesulfone[8] (this method was found to be much more efficient than methylation with dimethylsulfate or methyl triflate). The 2-bromo

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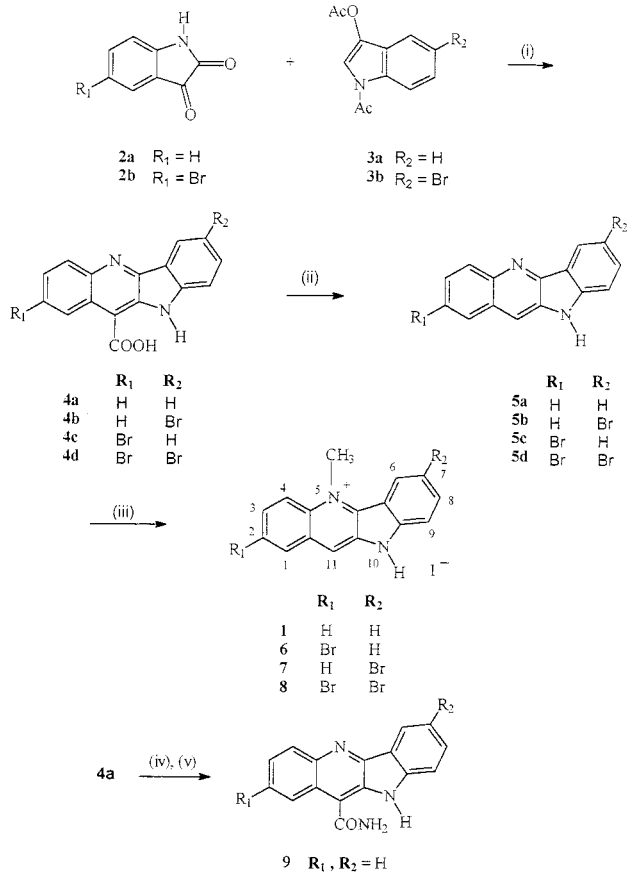
Scheme 1[a]

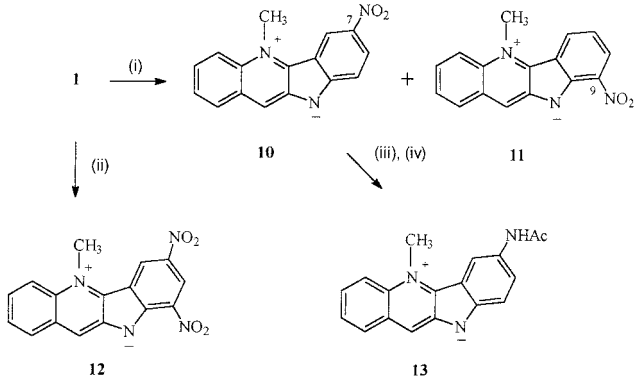


[a] Reagents and conditions: (i) KOH, N_2, 10 days; (ii) Ph_2O, 250 °C; (iii) CH_3I, tetramethylene sulfone, 50 °C; (iv) $SOCl_2$, reflux; (v) HN_4OH.

analogue **6** was prepared similarly using 5-bromoisatin (**2b**) and *O*,*N*-acetylindoxyl (**3a)** as starting materials while the reaction of isatin (**2a**) with 5-bromo-*O*,*N*-acetylindoxyl (**3b**) provided a route to 7-bromocryptolepine (**7**). Similarly, 5-bromoisatin (**2b**) and 5-bromo-*O*,*N*-acetylindoxyl (**3b**) were used as starting materials for the synthesis of 2,7-dibromocryptolepine (**8**). Quindoline-11-amide (**9**) was synthesized by reacting **4a** with thionyl chloride to give the acid chloride, followed by ammoniolysis as previously described[7] (Scheme 1).

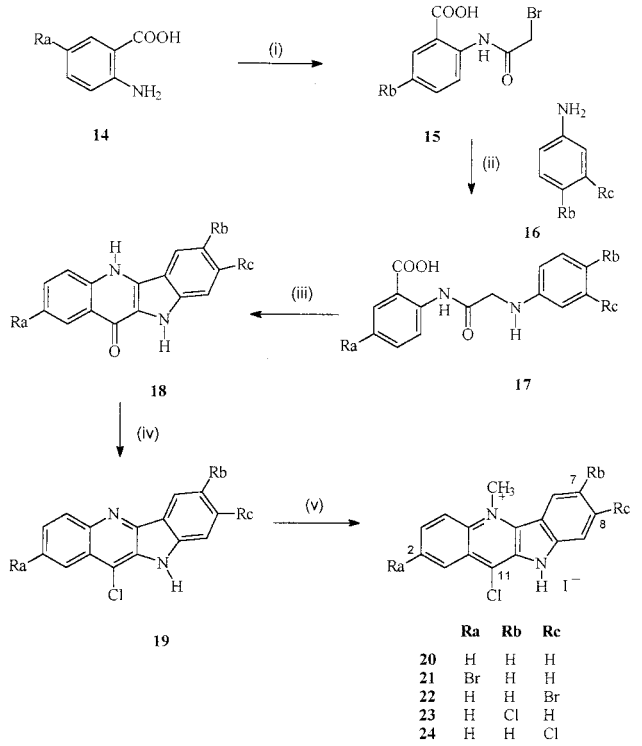
Nitration was used as a convenient method of preparing derivatives directly from **1** (Scheme 2). At room temperature, nitration of **1** with a mixture of nitric and glacial acetic acids (1:1) yielded 7-nitrocryptolepine (**10)** as the major product together with a smaller amount of the 9-nitro- isomer **11,** which were then separated by column chromatography over silica gel eluted with chloroform/methanol. Refluxing the above reaction mixture afforded 7,9-dinitrocryptolepine (**12)** as the sole product. Two meta-coupled doublets in the ^{1}H NMR spectrum of **12** (δ 9.13 and 9.64, J = 2.2 Hz) together with the downfield shift of the singlet due to H-11 (δ 9.55) initially suggested that **12** was the 1,3-dinitro analogue of **1**, but X-ray crystallographic analysis of **12** (data not shown)[9] proved unequivocally that **12** is the 7,9-dinitro analogue of **1**. Reduction of 7-nitrocryptolepine (**10)** (Sn/HCl) gave the reactive and light-sensitive 7-amino analogue that was isolated and immediately acetylated to give 7-*N*-acetylcryptolepine **13** (Scheme 2).

Scheme 2[a]



[a] Reagents and conditions: (i) HNO_3:HOAc (1:1), room temperature, overnight; (ii) HNO_3:HOAc (1:1), reflux; (iii) Sn/HCl; (iv) Ac_2O, reflux.

Scheme 3[a]



[a] Reagents and conditions: (i) bromoacetyl bromide, room temperature; (ii) reflux, 30 h; (iii) polyphosphoric acid, 130 °C, 2 h; (iv) $POCl_3$, 120 °C, 2 h; (v) CH_3I, tetramethylene sulfone, 50 °C, overnight.

The 11-chloro derivative **20** was prepared as previously described by Bierer et al. (1998),[10] and the same route (Scheme 3) was used to synthesize the novel dihalogenated derivatives **21**−**24**. Reaction of an anthranilic acid derivative **14** with bromoacetylbromide gave **15,** which was treated with an aniline derivative **16**. The resulting anthranilic acid derivative **17** was then cyclized using polyphosphoric acid to yield quindolone **18**; treatment with phosphorus oxychloride gave 11-chloroquindoline derivative **19**, which was then methylated with iodomethane in tetramethylenesulfone to give the corresponding cryptolepine analogues, **20**−**24**.

Results and Discussion

The activities of compounds against *P. falciparum* in vitro and against *P. berghei* in vivo and their effects on

Table 1. In Vitro Antiplasmodial and In Vivo Antimalarial Activities of Cryptolepine Derivatives and Chloroquine Diphosphate against Malaria Parasites and Effects on Formation of β-Hematin

	activity vs *P. falciparum* in vitro[b]			activity against *P. berghei* (ANKA) in mice		
compd[a]	chloroquine-resistant strain K1	chloroquine-sensitive strain HB3	inhibition of β-hematin formation	dose schedule (mg kg^{-1} for 4 d)	mean parasitemia (% ± SD)	suppression of parasitemia (%)
1 (as sulfate)	0.44 ± 0.22(9)	0.27 ± 0.06(3)	yes	20[c]	NT[d]	*e*
4a	>100	NT	no	NT	NT	NT
5a	>100	NT	no	NT	NT	NT
6	0.26 ± 0.094(4)	0.45 ± 0.17(3)	yes	25	21.6 ± 1.9	5.9
7 (as hydroiodide)	0.26 ± 0.21(4)	0.19 ± 0.09(3)	yes	20	11.9 ± 2.0	41.5
8	0.049 ± 0.017	0.026 ± 0.005(3)	yes	12.5	1.4 ± 0.4	89.1
9	>100	NT	no	NT	NT	NT
10	0.65 ± 0.28(6)	0.14 ± 0.05(3)	yes	20	6.0 ± 1.2	61.4
11	6.92 ± 1.89(4)	4.14 ± 2.29(3)	ND[f]	20	11.9 ± 2.0	23.5
12	0.65 ± 0.21(6)	0.45 ± 0.22(3)	yes	20	20.3 ± 2.0	−30.2
13	0.52 ± 0.21(5)	0.47 ± 0.16(3)	ND	20	13.3 ± 2.1	14.7
20	0.24 ± 0.1(3)	1.67 ± 1.27(3)	ND	20	NT	*g*
21 (as hydroiodide)	4.75 ± 0.37(3)	NT	ND	NT	NT	NT
22	7.18 ± 3.5(4)	NT	ND	NT	NT	NT
23	7.62 ± 2.7(4)	NT	NT	NT	NT	NT
24	27.0 ± 5.4(3)	NT	ND	NT	NT	NT
chloroquine diphosphate	0.18 ± 0.025(7)	0.023 ± 0.0015(3)	yes	10	1.3 ± 0.5	93.8

[a] Tested as hydrochloride salt unless stated otherwise. [b] IC_{50} μM ± SD(n). n, number of separate determinations. [c] Tested as hydrochloride. [d] NT, not tested. [e] Toxic after second dose. [f] ND, could not be determined as compound has peaks in the IR spectrum close to 1660 cm^{-1}. [g] Toxic after first dose.

Table 2. Cytotoxicity and Effect on DNA Melting Point of Cryptolepine Derivatives

		IC_{50} (μM ± SD) (n = 3)[a]					
		cytotoxicity against A549 cells		cytotoxicity against DLD-1 cells		cytotoxicity against MAC15a cells	
compd	effect on DNA melting point, ΔT_m (°C)	drug exposure time 1 h	drug exposure time 96 h	drug exposure time 1 h	drug exposure time 96 h	drug exposure time 1 h	drug exposure time 96 h
1 (as sulfate)	9	61.4 ± 18.2	0.55 ± 0.051	93 ± 29	1.44 ± 0.0015	67.2 ± 26.3	9.65 ± 1.37
5a	0	NT[b]	NT	NT	NT	NT	NT
6	4	>100	2.07 ± 0.21	>100	2.29 ± 0.67	>100	5.79 ± 0.91
7 (as hydroiodide)	4	NT	NT	NT	NT	66.2 ± 8.02	NT
8	3	NT	NT	NT	NT	NT	6.04 ± 0.49
10	5	35.6 ± 2.2	NT	48.9 ± 7.6	NT	35.4 ± 4.8	NT
11	4	90.2 ± 7.8	NT	>100	NT	>100	NT
12	4	>100	NT	>100	NT	28.5 ± 9.3	NT
13	6	>100	5.04 ± 1.05	>100	20.41 ± 1.1	>100	15.47 ± 4.2
20	0	NT	NT	NT	NT	30.5 ± 6.2	NT
22	9	NT	NT	NT	NT	23.1 ± 2.7	NT
24	9	NT	NT	NT	NT	14.4 ± 2.8	NT

[a] n, number of separate determinations. [b] NT, not tested.

β-hematin formation are shown in Table 1; ΔT_m values (increase in melting point of DNA) and cytotoxic activities are reported in Table 2.

In Vitro Antiplasmodial Activity. Cryptolepine (**1**) was found to have potent in vitro activity (IC_{50} = 0.44 μM) against *P. falciparum* (multidrug resistant strain K1) similar to that published in previous reports.[2,11] However, quindoline (**5a**), quindoline-11-carboxylic acid (**4a**), and its amide **9** were inactive against malaria parasites (strain K1), indicating that the 5-methyl group in **1** is a prerequisite for antiplasmodial activity. The 7-nitro (**10**), 7,9-dinitro (**12**), and 7-*N*-acetyl (**13**) derivatives were of similar potency to **1** against *P. falciparum* while 9-nitrocryptolepine (**11**) was 10-fold less active. Monohalogenated derivatives **6**, **7**, and **20** were slightly more potent than **1** against *P. falciparum* (strain K1). Compound **8** (2,7-dibromocryptolepine) was prepared because both the 2-bromo (**6**) and the 7-bromo (**7**) analogues showed improved antiplasmodial activities as compared with **1**. This strategy was successful as the antiplasmodial activity of **8** was found to be 9-fold greater than that of **1**. However, in contrast, the 2-bromo,11-chloro derivative (**21**) was found to be about 20-fold less active than the 2-bromo (**6**) and 11-chloro (**20**) derivatives. Similarly, the other dihalogenated analogues that were 11-chloro-substituted (**22**−**24**) have little antiplasmodial activity.

Compounds active against *P. falciparum* (strain K1) were also tested against chloroquine-sensitive strain HB3 to determine whether they exhibit cross-resistance with chloroquine. As shown in Table 1, chloroquine was 8-fold less active against chloroquine-resistant strain K1 than against strain HB3. The antiplasmodial activities of seven of the nine compounds tested were similar against both parasite strains (less than a 2-fold variation). One compound, 7-nitrocryptolepine (**10**), was 4-fold less active against strain HB3 while the 11-choro analogue **20** was 7-fold more active against chloroquine-resistant strain K1. These results suggest that cryptolepine and its derivatives with the possible exception of **10** do not show cross-resistance with chloroquine,

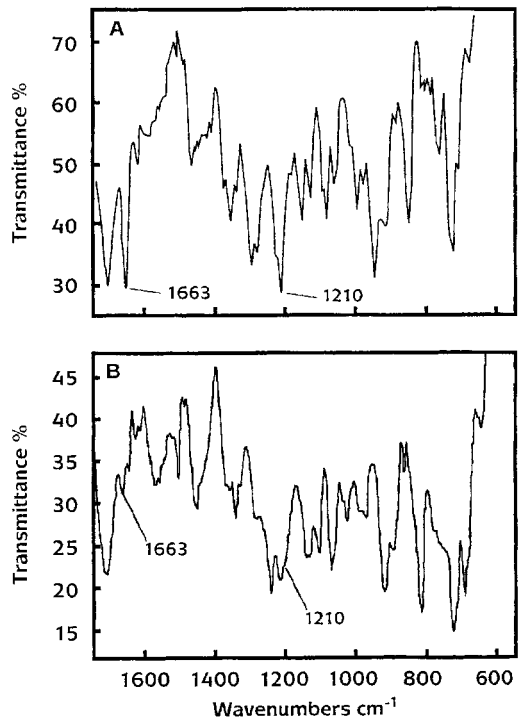


Figure 1. Inhibition of β-hematin formation by **1**. (A) FTIR spectrum of control reaction product showing peaks at 1663 and 1210 cm^{-1} characteristic of β-hematin. (B) As above in the presence of **1**, demonstrating inhibition of β-hematin formation.

although, as discussed below, they share the ability to inhibit β-hematin formation.

In Vivo Antimalarial Activity. Cryptolepine (**1**) was toxic to *P. berghei*-infected mice by ip injection at a dose of 12.5 mg kg^{-1} day^{-1} (Table 1). Toxicity appears to be related to the route of administration as no deaths were reported in previous studies in which subcutaneous (at 113 mg kg^{-1} kg^{-1} day)[11] or oral (50 mg kg^{-1} day^{-1})[2] routes were used. In the former study, no effect on parasitemia was observed, but oral administration of **1** at 50 mg kg^{-1} day^{-1} for 4 days suppressed parasitemia by 80.5%[2].

In this study, **7**, **8**, **10**, and **11** suppressed parasitemia by more than 20%. Although **11** displayed only weak in vitro antiplasmodial activity it was found to have greater in vivo activity (23% suppression of parasitemia) than compounds **6, 12**, and **13,** which all have potent in vitro antiplasmodial activities. Interestingly, mice treated with **12** were found to have a mean parasitemia 30% above that of untreated control animals, while in common with (**1**), the 11-chloro analogue **20** was toxic to mice. Compounds **7** and **10** possess moderate in vivo antimalarial activities (42 and 61%, respectively, at 20 mg kg^{-1} day^{-1}), but the most potent compound, 2,7-dibromocryptolepine (**8**), was found to suppress parasitemia by 89% at a daily dose of 12.5 mg kg^{-1}. The in vivo activity of **8** is comparable to that of chloroquine, and further studies to determine the oral effectiveness this compound and its ability to cure malaria in mice are currently being carried out.

Effect on β-Hematin Formation. Cryptolepine (**1**) prevented the formation of β-hematin (Figure 1), which suggests that its antiplasmodial effect depends (at least in part) on a quinine-like mode of action, although it is possible that its effects on DNA synthesis and inhibition of topoisomerase II may also contribute. This is supported by a recent study using fluorescence microscopy, which suggests that **1** accumulates into parasite structures that may correspond to the parasite nucleus.[12] In contrast, **4**, **5**, and **9**, which are inactive against malaria parasites, failed to inhibit β-hematin formation (Table 1). Several of the derivatives possessing potent antiplasmodial activities (**6**−**8**, **10**, and **12**) like **1** inhibited β-hematin formation, indicating that this property may be important for their action against malaria parasites. It was not possible to determine the effects of **11, 13,** and **20** on β-hematin formation because their IR spectra have peaks close to 1660 cm^{-1}, which may mask the peak of β-hematin that occurs at this wavelength.

Cytotoxicity and Effect on ΔT_m Value. As **1** is known to be toxic to cancer cells,[3] initial tests for cytotoxicity were carried out by exposing cancer cells (A549, DLD-1, and MAC15A) to compounds for 1 h followed by a 96-h incubation period. The results suggested that three compounds **6**, **11**, and **13** may be less toxic than **1** against all three cell lines (Table 2). However, as the IC_{50} values for **1** are close to 100 μM, the highest concentration tested, further experiments using a drug−cell contact time of 96 h were carried out to determine the extent by which **6** and **13** are less toxic than **1**; the latter were selected as they possess good antiplasmodial activities. Against A549 and DLD-1 cells, **6** was found to be about 4- and 2-fold less toxic, respectively, than **1**, but it was about 2-fold more toxic than **1** against MAC15A cells. The best selectivity was seen with **13** against DLD-1 cells (about 14-fold less toxic) and against A549 cells (about 9-fold less toxic), but little difference was observed with MAC15A cells (about 1.6-fold less toxic). However, there appears to be little correlation between the in vitro cytoxicity of these compounds and toxicity in the mouse−malaria model. While **10** and **20** were about twice as toxic to MAC15A cells than **1**, in the antimalarial test only **20** was toxic to mice. Compound **8** was slightly more cytotoxic to cancer cells than **1**, but no apparent toxicity was seen in mice in striking contrast to **1**, which was substantially toxic to mice after two doses, although it must be noted that the daily dose of **1** given to mice (20 mg kg^{-1}) was greater than that of **8** (12.5 mg kg^{-1}).

The effects of compounds on the melting point of calf thymus DNA (ΔT_m values) were measured in an attempt to obtain an indication of their propensities to interact with DNA. The value of 9 °C found for **1** is consistent with its known DNA-intercalating ability[3]. With the exceptions of **22** and **24**, the ΔT_m values of the other compounds tested were all 6 °C or less (Table 2), which may indicate a reduced tendency of the latter to interact with DNA as compared to **1**. However, no correlation was observed between ΔT_m value and cytotoxicity or toxicity in the mouse−malaria model; this is well illustrated with reference to **1** and **20**, which were both toxic to mice but were found to have ΔT_m values of 9 and 0 °C, respectively.

Conclusion

A number of analogues of **1** have been synthesized that have improved antiplasmodial activities as compared to the parent against both chloroquine-sensitive

and chloroquine-resistant strains of *P. falciparum*. With the possible exception of **10**, there was no evidence of cross-resistance with chloroquine even though these compounds (like the parent) appear to have a chloroquine-like mode of action against malaria parasites. Several compounds were found to have some activity against *P. berghei* in mice, the most potent being 2,7-dibromocryptolepine (**8**), which suppressed parasitemia by 89% at a dose of 12.5 mg kg^{-1} day^{-1} with no apparent toxicity to the mice. There appears to be no correlation between the in vitro cytotoxicity and the effect of compounds on the melting point of DNA or toxicity in the mouse−malaria model.

This study has shown that some derivatives of **1**, particularly **8**, are promising leads in the search for new antimalarial agents. In addition, analogues of **1,** which have enhanced cytotoxicity (such as **24**, which was 4−5-fold more active than **1** against MAC15A cells), may be worthy of investigation as antitumor agents. This is supported by recent studies on the cytotoxic properties of compounds related to **1**.[13−15]

Experimental Section

Chemistry. Chemicals were purchased from Sigma-Aldrich Chemical Co. Ltd., Poole, U.K. ^{1}H NMR spectra were acquired on a JEOL GX270 FT NMR spectrometer at 270 MHz. Mass spectra were run on an AEI MS902 spectrometer equipped with an MSS data acquisition system, version 10 (Mass Spectrometer Services, Manchester, U.K.). C, H, and N analyses were carried out by the Chemical and Materials Analysis Unit, University of Newcastle, U.K., on a Carlo Erba 1106 elemental analyzer. FTIR spectra were recorded on a Mattson Galaxy 6020 FTIR spectrometer.

General Method for Preparation of 2-Bromoquindoline (5b) and 7-Bromoquindoline (5c). *O,N*-Acetylindoxyl (**3a**) (5 g, 23.0 mmol) for **5b** or 5-bromo-*O,N*-acetylindoxyl (**3b**)-(6.84 g, 23.0 mmol) for **5c** and water (50 mL) were stirred under nitrogen at room temperature. A solution of 5-bromoisatin (**2b**) (5.73 g, 25.3 mmol) for **5b** or isatin (**2a**) (4.41 g, 25.3 mmol) for **5c** and KOH (26 g 0.46 M) in water (50 mL) was slowly added. Stirring was continued for 10 days at room temperature and then additional water (20 mL) was added, and the mixture was heated to 70 °C while air was bubbled through it for 20 min. The mixture was filtered through Celite, which was then washed with hot water (60 °C), and an equal volume of ethanol was added to the filtrate followed by acidification to pH 1 with concentrated HCl. The product (bromoquindoline-11-carboxylic acid) **4b** or **4c** was collected, washed with 1:1 ethanol:water, and dried. Decarboxylation was carried out by refluxing the derivative (7.3 g, 21.3 mmol) and diphenyl ether (50 mL) for 6 h with stirring. After the mixture was cooled, petroleum ether (55 mL) was added, and the precipitate was collected and dried, dissolved in methanol (300 mL), and filtered. Concentration of the filtrate gave the brominated quindoline derivatives **5b** and **5c** that were used without further purification.

2-Bromocryptolepine Hydrochloride (6). 2-Bromoquindoline (**5b**) (0.116 g, 0.34 mmol), dimethylsulfate (0.265 g, 3.4 mmol), and chloroform (20 mL) were stirred under reflux for 10 days; the course of the reaction was monitored using TLC over silica gel G with chloroform:methanol:concentrated ammonia (9:1:0.1). Following evaporation of the solvent, NaOH solution (10%, 20 mL) was added to the residue, and the mixture was extracted with chloroform, washed with saturated NaCl solution, and dried over anhydrous Na_2SO_4. The dried product was column-chromatographed over silica gel eluted with chloroform:methanol:ammonia (9:1:0.1), converted to the hydrochloride salt with concentrated HCl, and crystallized from chloroform:methanol (3:1) to give **6** as a yellow-brown solid; overall yield 13%. (Note: This yield may be increased significantly if methylation is carried out using iodomethane and tetramethylenesulfone as described below for the preparation of **7**.) ^{1}H NMR of free base (CD_3OD, δ): 5.11 (3H, s, NCH_3), 7.62 (1H, t, J = 8.0 Hz, 7 or 8-*H*), 7.94 (1H, d, J = 8.8 Hz, 6-*H*), 8.05 (1H, t, J = 8 Hz, 7 or 8-*H*), 8.24 (1H, dd, J = 9.5, 2.2 Hz, 3-*H*), 8.62 (1H, d, J = 9.5 Hz, 4-*H*), 8.72 (1H, d, J = 2.2 Hz, 1-*H*), 8.79 (1H, d, J = 8.4 Hz, 9-*H*), 9.26 (1H, s, 11-*H*). MS (EI, *m*/*z*, relative intensity, %) 312 (97), 310 (100) [M^+ + H], 297 (7), 295 (7), 230 (18), 154 (18), 156 (18), 115 (24). Anal. ($C_{16}H_{11}N_2Br$·HCl·H_2O) H, N; C: calcd, 52.5; found 52.0.

7-Bromocryptolepine Hydroiodide (7). 7-Bromoquindoline (**5b**) (0.06 g, 0.18 mmol), tetramethylenesulfone (2 mL), and iodomethane (0.06 g, 23.4 mmol) were stirred overnight at 50 °C in a sealed container. After the solution was cooled, ether (8 mL) and a few drops of methanol were added, and the resulting precipitate was washed twice with ethyl acetate (2 × 5 mL) and crystallized from chloroform:methanol (3:1) to give **7** as orange-brown needles; overall yield 38%. ^{1}H NMR of free base (CD_3OD, δ): 4.70 (3H, s, NCH_3), 7.54 (1H, dd, J = 9.2, 1.8 Hz, 8-*H*), 7.64 (1H, t, J = 7.3 Hz, 2 or 3-*H*), 7.71 (1H, d, J = 9.2 Hz, 9-*H*), 7.86 (1H, t, J = 7.9 Hz, 2 or 3-*H*), 8.11 (1H, d, J = 8.8 Hz, 1 or 4-*H*), 8.20 (1H, d, J = 8.1 Hz, 1 or 4-*H*), 8.30 (1H, d. J = 1.8 Hz, 6-*H*), 8.80 (1H, s, 11-*H*). MS (EI, *m*/*z*, relative intensity, %) 312 (100), 310 (100) [M^+ + H], 296 (26), 294 (24), 216 (18), 154 (15), 115 (13), 89 (28). Anal. ($C_{16}H_{11}N_2Br$·HI) C, H, N.

2,7-Dibromocryptolepine Hydrochloride (8). The title compound was prepared from 5-bromo-*O,N*-acetylindoxyl (**3b**)-(6.84 g, 23 mmol) and 5-bromoisatin (**2b**) (5.73 g, 25.3 mmol) as starting materials using the same methodology as for **6** except that the reaction mixture for the first step was refluxed for 4 h instead of stirring at room temperature. Recrystallization from chloroform:acetone (3:1) gave **8** as an orange-yellow solid; overall yield 23%. ^{1}H NMR of free base ($CDCl_3$, δ): 4.72 (3H, s, NCH_3), 7.53 (1H, dd, J = 9.2, 1.8 Hz, 3-*H* or 8-*H*), 7.66 (1H, d, J = 9.2 Hz, 4-*H* or 9-*H*), 7.91 (1H, dd, J = 9.2, 2.2 Hz, 3-*H* or 8-*H*), 8.0 (1H, d, J = 9.2 Hz, 4-*H* or 9-*H*), 8.19 (1H, d, J = 1.8 Hz, 1-*H* or 6-*H*), 8.27 (1H, d, J = 2.2 Hz, 1-*H* or 6-*H*), 8.74 (1H, s, 11-*H*). MS (EI, *m*/*z*, relative intensity, %) 392 (46), 390 (92), 388 (38) [M^+], 378 (42), 376 (100), 374 (42), 297 (14), 295 (14), 216 (16), 215 (18). Anal. ($C_{16}H_{10}N_2Br_2$·1.5HCl) C, H, N.

7-Nitro- (10) and 9-Nitrocryptolepine Hydrochloride (11). Cryptolepine (**1**) as sulfate (1 g, 3 mmol) was dissolved in 40 mL of nitric acid (69%):glacial acetic acid (1:1) and stirred at room temperature for 24−48 h. The reaction mixture was cooled on ice, basified with strong NaOH solution, and filtered, and the precipitate was washed with ice cold water. The dried product was column-chromatographed over silica gel under positive pressure eluted with chloroform containing increasing amounts of methanol (1−20%) to yield fractions containing **10** (less polar) and **11** (more polar). The total yield of nitrated products was 56%. These were converted to their hydrochloride salts with concentrated HCl and crystallized from choroform: methanol (3:1).

10. Yellow solid, yield 45%. ^{1}H NMR of free base ($CDCl_3$, δ): 4.99 (3H, s, NCH_3), 7.78 (1H, t, J = 7.2 Hz, 7-*H*), 7.86 (1H, d, J = 9.5 Hz, 9-*H*), 8.01 (1H, t, J = 7.3 Hz, 8-*H*), 8.28 (1H, d, J = 9.5 Hz, 1-*H*), 8.33 (1H, d, J = 8.4 Hz, 6-*H*), 8.44 (1H, d, J = 9.5 Hz, 2-*H*), 9.04 (1H, s, 11-*H*), 9.38 (1H, s, 4-*H*). MS (EI, *m*/*z*, relative intensity. %) 277 (16) [M^+], 263 (100), 247 (32), 232 (36), 217 (59), 190 (19), 111 (20). Anal. ($C_{16}H_{11}N_3$·HCl) C, H, N.

11. Yellow solid, yield 11%. ^{1}HMR of base (270 MHz, CD_3OD, δ): 5.1 (3H, s, NCH_3), 7.54 (1H, t, J = 8.8 Hz, 7-*H*), 7.93 (1H, t, J = 7.7 Hz, 8-*H*), 8.19 (1H, t, J = 7.5 Hz, 3-*H*), 8.48 (1H, d, J = 8.4 Hz, 6-*H*), 8.67 (1H, d, J = 9.2 Hz, 9-*H*), 8.75 (1H, d, J = 8.1 Hz, 4-*H*), 9.11 (1H, d, J = 8.1 Hz, 2-*H*), 9.21 (1H, s, 11-*H*). MS (EI, *m*/*z*, relative intensity, %) 277 (9) [M^+], 263 (26), 232 (100), 217 (44), 190 (18), 111 (8). Anal. ($C_{16}H_{11}N_3$·HCl) C, H, N.

7,9-Dinitrocryptolepine Hydrochloride (12). Cryptolepine (**1**) as sulfate (0.5 g, 1.5 mmol) was refluxed for 30 min in 30 mL of nitric acid (69%):glacial acetic acid (1:1). The product was isolated and purified as described above for **10**

and **11**. Recrystallization from chloroform:methanol (3:1) gave **12** as orange-red crystals; yield 69%. ^{1}H NMR of free base (DMSO-d_6, δ): 4.71 (3H, s, NCH_3), 7.99 (1H, t, J = 8.1 Hz, 7-*H*), 8.69 (1H, t, J = 7.4 Hz, 8-*H*), 8.72 (1H, d, J = 7.3 Hz, 9-*H*), 8.43 (1H, d, J = 7.3 Hz, 6-*H*), 9.13 (1H, d, J = 2.2 Hz, 4-*H*), 9.55 (1H, s, 11-*H*), 9.64 (1H, d, J = 2.2 Hz, 2-*H*). MS (EI, *m*/*z*, relative intensity, %) 322 (38) [M$^+$], 308 (100), 292 (14), 262 (35), 230 (22), 216 (56), 188 (10). Anal. ($C_{16}H_{10}N_4O_4$·HCl) C, H, N.

7-*N*-Acetylcryptolepine Hydrochloride (13). Compound **10** (0.443 g, 1.6 mmol) was dissolved in 30 mL of methanol: concentrated HCl (95:5) in the presence of granulated tin (0.2 g, 1.7 mmol) in a nitrogen atmosphere protected from light and was stirred for 3 h. The reaction mixture was basified with saturated NaOH solution, filtered, and extracted with chloroform. The concentrated chloroform extract was column-chromatographed over alumina gradient eluted with chloroform followed by chloroform containing increasing amounts of methanol (1−10%) to yield a blue fraction containing 7-aminocryptolepine, which is photosensitive. Acetic anhydride (3 mL) was added to the fraction, which was then refluxed for 15 min, cooled, basified with saturated NaOH, and extracted with chloroform:methanol (3:1). Concentrated HCl was added to the extract, which was then dried to give **13**. Crystallization from chloroform:methanol (3:1) gave **13** as red-brown needles; yield 51%. ^{1}H NMR of hydrochloride (DMSO-d_6, δ): 2.15 (3H, s, CONCH_3), 5.01 (3H, s, NCH_3), 7.84 (1H, d, J = 9.2 Hz, 1-*H*), 7.95 (1H, t, J = 8.0 Hz, 7-*H*), 8.04 (1H, d, J = 8.8 Hz, 6-*H*), 8.18 (1H, t, J = 7.9 Hz, 8-*H*), 8.58 (1H, d, J = 8.4 Hz, 9-*H*), 8.73 (1H, d, J = 9.2 Hz, 2-*H*), 9.15 (1H, s, 4 or 11-*H*), 9.30 (1H, s, 4 or 11-*H*), 10.5 (1H, s, 3-N*H*), 13.0 (1H, s, 10-N*H*). MS (EI, *m*/*z*, relative intensity, %) 289 (6) [M$^+$], 246 (8), 232 (3), 159 (2), 149 (4), 60 (32), 44 (100). Anal. ($C_{18}H_{15}N_3O$.HCl·$CHCl_3$) C, H, N.

General Method for Preparation of 11-Chloro-Substituted Derivatives 20−24. *Step 1*. Anthranilic acid or a substituted anthranilic acid derivative, **14** (96.6 mmol), dimethylformamide (35 mL), and dioxane (35 mL) were placed in a sealed flask, which was cooled to 0 °C, and then bromoacetylbromide (19.5 g, 96.6 mmol) was slowly added so that the temperature did not rise above 1 °C. At the end of the addition, the temperature was maintained at 0 °C for a further 10 min, and then the mixture was stirred overnight at room temperature. The contents of the flask were poured into water (300 mL), and the resulting precipitate **15** was filtered, washed with neutral water (3 × 15 mL), and then dried.

Step 2. Aniline or a substituted aniline derivative, **16** (0.28 M), and the crude acid **15** (90 mmol) were stirred and heated under reflux at 120 °C for 30 h. After the cooling process, the reaction mixture was poured onto ice and water (800 mL), and sufficient KOH solution (5%) was added to dissolve the precipitate. The pH was checked, and if necessary, more KOH solution was added to raise the pH to 11. The mixture was extracted with dichloromethane, and the aqueous phase was then acidified to pH 3 with hydrobromic acid solution (5%). The product **17** was collected in the form of a precipitate or as an oil that solidified on standing overnight at room temperature.

Step 3. The crude product from above **17** (15.8 mmol) and polyphosphoric acid (150 g) were stirred at 130 °C for 2 h, and then the mixture was poured into ice/water (1000 mL) and neutralized with saturated KOH solution. The mixture was then extracted with ethyl acetate (3 × 250 mL), washed with saturated NaCl solution and water, and dried (Na_2SO_4). Following evaporation of the solvent, the dried product **18** was column-chromatographed over silica gel eluted with ethyl acetate:methanol (5:1).

Step 4. The latter product **18** (23.8 mmol) and phosphorus oxychloride (60 mL) were stirred under reflux at 120 °C for 2 h. After the reaction mixture was cooled, it was poured onto ice/water (150 mL) and then neutralized with saturated KOH solution, taking care to prevent the temperature rising above 40 °C. The product was extracted into ethyl acetate (3 × 200 mL), washed with saturated NaCl solution and water, and then dried (Na_2SO_4). Evaporation of the solvent gave the crude product **19**, which was chromatographed over silica gel twice; the eluent was ethyl acetate:hexane (1:6) followed by chloroform:methanol (9.5:0.5).

Step 5. Finally, methylation of **19** was carried out as described above as in the preparation of **7**. Compounds **22**−**24** were converted to their hydrochloride salts by the addition of dilute NH_4OH (10%) and extraction with chloroform followed by the addition of concentrated HCl. The hydrochloride salts were recrystallized from chloroform:methanol (3:1).

2-Bromo-11-chlorocryptolepine Hydroiodide (21). Prepared as above using 5-bromoanthranilic acid (20.1 g, 96.6 mmol) and aniline (26.1 g, 0.28 M) as starting materials. Crude yields were 70%, 38%, 51%, 70%, and 74% for steps 1−5, respectively; overall yield of **21** obtained as a yellow-brown solid, 7%. ^{1}H NMR of free base (DMSO-d_6, δ): 4.96 (3H, s, NCH_3), 7.55 (1H, t, J = 8.0 Hz, 7-*H*), 7.90 (1H, d, J = 8.4 Hz, 6-*H*), 7.99 (1H, t, J = 7.0 Hz, 8-*H*), 8.31 (1H, dd, J = 9.5, 2.2 Hz, 3-*H*), 8.62 (1H, d, J = 2.2 Hz, 1-*H*), 8.76 (1H, d, J = 9.52, 4-*H*), 8.79 (1H, d, J = 8.43, 8-*H*). MS (EI, *m*/*z*, relative intensity, %) 346 (100), 344 (76) [M$^+$], 332 (78), 330 (60), 250 (10), 215 (36), 173 (12). Anal. ($C_{16}H_{10}N_2BrCl$·HI) C, H, N.

8-Bromo-11-chlorocryptolepine Hydrochloride (22). Prepared from anthranilic acid (13.2 g, 96.6 mmol) and 3-bromoaniline (48.4 g 0.28 M). Crude yields, 74%, 75%, 30%, 66%, and 50% for steps 1−5, respectively; overall yield of **22** obtained as a yellow solid, 6%. ^{1}H NMR of free base (DMSO-d_6, δ): 4.60 (3H, s, NCH_3), 6.94 (1H, dd, J = 8.8, 1.8 Hz, 1 or 4-*H*), 7.70 (1H, t, J = 7.3 Hz, 2 or 3-*H*), 7.73 (1H, d, J = 1.1 Hz, 9-*H*), 7.78 (1H, d, J = 9.2 Hz, 1, 4 or 6-*H*), 7.91 (1H, t, J = 7.3 Hz, 2 or 3-*H*), 8.10 (1H, d, J = 9.2 Hz, 1, 4 or 6-*H*), 8.45, (1H, dd, J = 9.0, 1.1 Hz, 7-*H*). MS (EI, *m*/*z*, relative intensity, %) 346 (2), 344 (2) [M$^+$], 328 (98), 326 (100) [M − CH_3], 313 (35), 311 (37), 285 (8), 283 (8), 204 (7), 164 (11), 163 (11), 150 (12), 149 (12). Anal. ($C_{16}H_{10}N_2BrCl$·HCl) C, H, N.

7,11-Dichlorocryptolepine Hydrochloride (23). Prepared from anthranilic acid (13.2 g, 96.6 mmol) and 4-chloroaniline (36 g, 0.28 M). Crude yields, 74%, 43%, 48%, 88%, and 47% for steps 1−5, respectively; overall yield of **23** obtained as a yellow solid, 6%. ^{1}H NMR of free base (DMSO-d_6, δ): 5.08 (3H, s, NCH_3), 7.87 (1H, d, J = 8.9 Hz, 9-*H*), 7.99 (1H, dd, J = 8.9, 1.8 Hz, 8-*H*), 8.09 (1H, t, J = 7.7 Hz, 2 or 3*H*), 8.29 (1H, t, J = 8.1 Hz, 2 or 3-*H*), 8.75 (1H, d, J = 9.5 Hz, 1 or 4-*H*), 8.82 (1H d, J = 10.1 Hz, 1 or 4-*H*), 8.82 (1H, d, J = 1.7 Hz, 6-*H*). MS (EI, *m*/*z*) 302, 300, [M$^+$], 285, 287, 250, 215, 149, 125. Anal. ($C_{16}H_{10}N_2Cl_2$·HCl·H_2O) N; C: calcd, 54.2; found, 52.9; H: calcd, 3.66; found, 3.21.

8,11-Dichlorocryptolepine Hydrochloride (24). Prepared from anthranilic acid (13.2 g, 96.6 mmol) and 3-chloroaniline (36 g, 0.28 M). Crude yields, 74%, 69%, 32%, 41%, and 48% for steps 1−5, respectively; overall yield of **24**, 3% obtained as a yellow solid. ^{1}H NMR of free base (CD_3OD, δ): 5.08 (3H, s, NCH_3), 7.87 (1H, d, J = 9.0 Hz, 6-*H*), 7.99 (1H, dd, J = 8.9, 1.8 Hz, 7-*H*), 8.09 (1H, t, J = 7.7 Hz, 2 or 3-*H*), 8.29 (1H, t, J = 7.4 Hz, 2 or 3-*H*), 8.75 (1H, d, J = 9.5 Hz, 1 or 4-*H*), 8.79 (1H, d, J = 10.1 Hz, 1 or 4-*H*), 8.82 (1H, d, J = 1.7, 9-*H*). MS (EI, *m*/*z*, relative intensity, %) 302 (64), 300 (100) [M$^+$], 286 (15), 149 (46). Anal. ($C_{16}H_{10}N_2Cl_2$·HCl·H_2O) C, H, N.

Antiplasmodial Assay. *P. falciparum* strain K1 was kindly supplied by Professor D. C. Warhurst (London School of Hygiene and Tropical Medicine) and *P. falciparum* strain HB3 was generously provided by Dr L. C. Ranford-Cartwright (Division of Infection and Immunity, University of Glasgow). Malaria parasites were maintained in human A$^+$ erythrocytes suspended in RPMI 1640 medium supplemented with A$^+$ serum and D-glucose according to the methods of Trager and Jensen (1976)[16] and Fairlamb et al. (1985).[17] Cultures containing predominantly early ring stages were used for testing. Compounds were dissolved or micronized in DMSO and further diluted with RPMI 1640 medium (the final DMSO concentration did not exceed 0.5% which did not affect parasite growth). Twofold serial dilutions were made in 96-well microtiter plates

in duplicate, and infected erythrocytes were added to give a final volume of 100 μL with 2.5% hematocrit and 1% parasitemia. Chloroquine diphosphate was used as a positive control, and uninfected and infected erythrocytes without compounds were included in each test. Plates were placed into a modular incubator gassed with 93% nitrogen, 3% oxygen, and 4% carbon dioxide and incubated at 37 °C for 48 h. Parasite growth was assessed by measuring lactate dehydrogenase activity as described by Makler et al. (1993).[18] The reagent used contained the following in each milliliter: acetylpyridine adenine dinucleotide (APAD), 0.74 mg; lithium lactate, 19.2 mg; diaphorase, 0.1 mg; Triton X-100, 2 μL; nitroblue tetrazolium, 1 mg; and phenazine ethosulfate, 0.5 mg. Fifty microliters of this reagent was added to each well and mixed, and plates were incubated for 15 min at 37 °C. Optical densities were read at 550 nm using a Dynatech Laboratories MRX microplate reader, and percent inhibition of growth was calculated by comparison with control values. IC_{50} values were determined using linear regression analysis (Microsoft Excel). A minimum of three separate determinations was carried out for each compound.

In Vivo Antimalarial Test. This was carried out using Peters' 4-day suppressive test[19] against *P. berghei* infection in mice. Female BALB/C mice, weight 18−20 g, were inoculated with *P. berghei* (ANKA); each mouse received 1×10^7 infected erythrocytes by iv injection. Drugs were administered to mice by ip injection in 0.2 mL of inoculum daily for four consecutive days. Control and test groups all contained 5 mice. On day 5 of the test a blood smear was taken, and the animals were killed. The percent suppression of parasitemia was calculated for each dose level by comparing the parasitemias present in infected controls with those of test animals. Chloroquine diphosphate was used as a positive control.

Inhibition of β-Hematin Formation. The methodology used was adapted from that of Egan et al. (1994).[6] Hemin (7.5 mg) was dissolved in 1.25 mL of 0.1 M NaOH, and then 0.125 mL of 1 M HCl was added. The 3 mol equiv of the compound under test (with respect to hemin) was then added followed by 1.15 mL of 9.78 M acetate buffer at pH 5.0, preincubated at 60 °C. The pH was adjusted to 5.0 by the addition of glacial acetic acid, and the mixture was then stirred for 30 min at 60 °C. After being cooled on ice, the precipitate was filtered using a 5-μm membrane filter and washed with water. After being dried under vaccuum for 24 h, the FTIR spectrum of the product was recorded. A control in which no compound was added was also carried out. The formation of β-hematin was detected by the presence of peaks at 1660 and 1210 cm^{-1}.

Thermal Denaturation Studies. These were performed as previously described[20] using a Perkin-Elmer Lambda 5 UV−Vis spectrophotometer linked to a Perkin-Elmer Peltier-Temperature Controller 6 unit that was programmed to produce the following conditions: preheat cell to 50 °C from room temperature over 20 min; heat to 110 °C at 1 °C/min taking absorbance readings at 260 nm every min; hold at 110 °C for 1 min; cool to 25 °C over 30 min. Approximately 20 mg of double-stranded calf thymus DNA (type 1, highly polymerized) was dissolved in 50 mL of buffer containing Tris-HCl (0.008 M) and NaCl (0.05 M) adjusted to pH 7.4 and diluted to give a DNA stock solution containing 2.88 mM Tris-HCl and 18 mM NaCl. A working buffer containing 2.88×10^{-3} M Tris-HCl and 0.018 M NaCl was prepared and then used for all subsequent dilutions. The DNA stock solution (3.3 mL) was further diluted to 10 mL with the working buffer. The absorbance was then determined at 260 nm, and the concentration of DNA was expressed according to the phosphate group concentration, using a molar absorptivity ϵ value of 6600 cm^{-1} M^{-1} at 260 nm.[20] Drug−DNA solutions were prepared from the DNA stock solution and from drug solutions at 10 mM in DMSO. Drug−DNA ratios of 1:10 were used, and absorbances were determined against blanks containing drugs diluted in the working buffer.

Cytotoxicity Test. The cell lines used in this study were A549 (human nonsmall cell lung carcinoma) and DLD-1 (human colon carcinoma) obtained from the European Collection of Animal Cell Cultures (ECACC) and MAC15A (murine adenocarcinoma of the colon) available in our laboratory. All cells were routinely maintained as monolayer cultures in RPMI 1640 culture medium supplemented with foetal calf serum (10%), sodium pyruvate (1 mM), L-glutamine (2 mM), and penicillin/streptomycin (50 IU mL^{-1}/50 μg mL^{-1} and buffered with HEPES (25 mM). Chemosensitivity was assessed using the MTT assay.[21] Briefly, 2×10^3 cells were inoculated into each well of a 96-well plate and incubated overnight at 37 °C in an humidified atmosphere containing 5% CO_2. All drugs were dissolved in DMSO and diluted in culture medium to give a broad range of drug concentrations; the maximum DMSO concentration in any well was 0.1%. Medium was removed from each well and replaced with drug solutions (8 wells per drug concentration). For experiments with a 1-h drug exposure, medium was removed after 1 h, and the cells were washed twice with Hanks balanced salt solution. RPMI 1640 medium was added (200 μL/well) and cells were incubated at 37 °C for a further 4 days before cell survival was determined using the MTT assay. Culture medium was replaced with fresh medium (180 μL) prior to the addition of 20 μL of MTT solution (0.5 mg mL^{-1}). Following 24-h incubation at 37 °C, medium plus MTT was removed from each well, and the formazan crystals were dissolved in DMSO (150 μL/well). Absorbances of the resulting solutions were read at 550 nm, and cell survival was calculated as the absorbance of treated cells divided by the absorbance of the control (RPMI medium plus 0.1% DMSO) wells. Results were expressed in terms of IC_{50} values (i.e., concentration of drug required to kill 50% of cells), and all experiments were performed in triplicate.

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