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Structure–activity relationship of antiparasitic and cytotoxic indoloquinoline alkaloids, and their tricyclic and bicyclic analogues

Gitte Van Baelen [a], Steven Hostyn [a], Liene Dhooghe [b], Pál Tapolcsányi [c], Péter Mátyus [c], Guy Lemière [a], Roger Dommisse [d], Marcel Kaiser [e], Reto Brun [e], Paul Cos [f], Louis Maes [f], György Hajós [g], Zsuzsanna Riedl [g], Ildikó Nagy [g], Bert U. W. Maes [a], Luc Pieters [b,*]

[a] Laboratory of Organic Synthesis, Department of Chemistry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
[b] Laboratory of Pharmacognosy and Pharmaceutical Analysis, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium
[c] Department of Organic Chemistry, Semmelweis University, H-1092 Budapest, Hőgyes E. u. 7., Hungary
[d] Research Group for Applied NMR, Department of Chemistry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
[e] Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland
[f] Laboratory of Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
[g] Institute of Biomolecular Chemistry, Chemical Research Center, Hungarian Academy of Sciences, Pusztaszeri ut 59, H-1025 Budapest, Hungary

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ABSTRACT

Based on the indoloquinoline alkaloids cryptolepine (**1**), neocryptolepine (**2**), isocryptolepine (**3**) and isoneocryptolepine (**4**), used as lead compounds for new antimalarial agents, a series of tricyclic and bicyclic analogues, including carbolines, azaindoles, pyrroloquinolines and pyrroloisoquinolines was synthesized and biologically evaluated. None of the bicyclic compounds was significantly active against the chloroquine-resistant strain *Plasmodium falciparum* K1, in contrast to the tricyclic derivatives. The tricyclic compound 2-methyl-2*H*-pyrido[3,4-*b*]indole (**9**), or 2-methyl-β-carboline, showed the best in vitro activity, with an IC_{50} value of 0.45 μM against *P. falciparum* K1, without apparent cytotoxicity against L6 cells (SI > 1000). However, this compound was not active in the *Plasmodium berghei* mouse model. Structure–activity relationships are discussed and compared with related naturally occurring compounds.

1. Introduction

Malaria is a tropical parasitic disease caused by protozoa belonging to the genus *Plasmodium*; the most serious infections are due to *Plasmodium falciparum*. In spite of considerable efforts, it still is one of the most important infectious diseases in the world. Malaria occurs not only in the tropics, but also in more temperate regions and it is expected that, due to global climate changes, its prevalence may increase. Because of the increasing resistance against antimalarial drugs, there is a continuing need for new therapeutic agents. Isolation of new lead compounds from plants used in traditional medicine is one of the strategies that can be followed in the search for new drugs.[1] *Cryptolepis sanguinolenta* is a plant used in traditional medicine in West and Central Africa against malaria. Its antiplasmodial activity is attributed to cryptolepine (**1**), which is the major alkaloid (Fig. 1).[2] Cryptolepine is an indolo[3,2-*b*]quinoline or a benzo-δ-carboline. Minor alkaloids, such as neocryptolepine (**2**) or isocryptolepine (**3**), showing isomeric indoloquinoline structures, as well as some alkaloids not belonging to the indoloquinoline-series, have been obtained from the same plant, and for some of these antiplasmodial activity has been reported.[3–6] Moreover, the corresponding benzo-β-carboline **4**, which has not been found in nature yet, and for which the name isoneocryptolepine was adopted, has recently been synthesized,[7,8] as well as the two isomeric indoloisoquinolines **5** and **6**.[9] The indoloquinoline alkaloids have been used as lead compounds for the synthesis of new antiplasmodial substances.[10–12] In addition, some tricyclic δ-carbolines derived from cryptolepine were prepared.[10] In the series of the tricyclic δ-carbolines, it was observed that the hydrochloride of 1-methyl-δ-carboline (**13**) (Fig. 2) exhibited an IC_{50} > 200 μM against L6 (murine myoblast-derived) cells, and an

* Corresponding author. Tel.: +32 3 265 27 15; fax: +32 3 265 27 09.
E-mail address: luc.pieters@ua.ac.be (L. Pieters).

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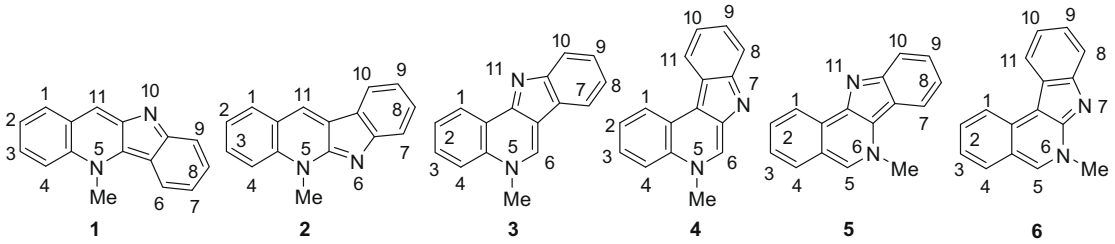


Figure 1. Indoloquinolines and indoloisoquinolines **1**–**6**.

IC_{50} of 1.6 μM against *P. falciparum* (strain K1, chloroquine-resistant), resulting in a selectivity index >100. This compound was found to accumulate in a parasite structure that could correspond to the parasite nucleus, but no further studies of the mechanism of action were carried out. Since then, this lead has not been investigated further. Therefore, in this work we report the synthesis of the tricyclic α-, β- γ- and δ-carbolines, derived from neocryptolepine, isoneocryptolepine, isocryptolepine and cryptolepine, respectively, the corresponding bicyclic azaindoles, some tricyclic pyrroloquinolines and -isoquinolines, their antiparasitic activity and cytotoxicity (Fig. 2). Their biological activities were compared to those of the parent indoloquinoline alkaloids and the isomeric indoloisoquinolines in order to establish structure–activity relationships. Some selected compounds were evaluated in two functional assays related to possible mechanisms of action, that is, inhibition of haemozoin formation and DNA intercalation. The most promising compound was evaluated in vivo for its antiplasmodial activity in infected laboratory animals.

2. Chemistry

The synthesis of 6-methyl-6*H*-indolo[3,2-*c*]isoquinoline[9] (**5**), 6-methyl-6*H*-indolo[2,3-*c*]isoquinoline[9] (**6**), 1-methyl-1*H*-pyrrido[2,3-*b*]indole[13] (**7**), 3*H*-pyrrolo[2,3-*c*]quinoline[14] (**17**), 5-methyl-5*H*-pyrrolo[2,3-*c*]quinoline[14] (**18**), 3*H*-pyrrolo[2,3-*c*]isoquinoline[14] (**19**) and 4-methyl-4*H*-pyrrolo[2,3-*c*]isoquinoline[14] (**20**) was already reported in previous articles of us. 7-Methyl-7*H*-pyrrolo[2,3-*b*]pyridine (**8**), 2-methyl-2*H*-pyrido[3,4-*b*]indole (**9**), 2-methyl-2*H*-pyrido[4,3-*b*]indole (**11**) and 1-methyl-1*H*-pyrido[3,2-*b*]indole (**13**) were prepared by selective methylation of their corresponding free bases following our literature procedure (Schemes 1 and 2).[15] Free bases **27** and **21** were obtained from commercial sources. The base skeleton of **11**, namely 5*H*-pyrido[4,3-*b*]indole (**25**), was synthesized by applying previously reported reaction conditions for α-carboline synthesis (Scheme 1).[13,15] The synthesis of 5*H*-pyrido[3,2-*b*]indole (**26**), the base skeleton of **13,** was already reported by our laboratory.[16] 6-Methyl-1*H*-pyrrolo[2,3-*c*]pyridin-6-ium iodide (**10**), 5-methyl-5*H*-pyrrolo[3,2-*c*]pyridine (**12**) and 4-methyl-4*H*-pyrrolo[3,2-*b*]pyridine (**14**) were obtained by methylation of their respective base skeleton **28**, **29** and **30** (Scheme 2). These base skeletons were synthesized following literature procedures.[17–19] 1-Methyl-1*H*-pyrrolo[2,3-*c*]pyridine (**15**) was synthesized according to a slightly modified literature procedure[17] and 1-methyl-1*H*-pyrrolo[3,2-*b*]pyridine (**16**) could be obtained after deprotonation and subsequently methylation of 1*H*-pyrrolo[3,2-*b*]pyridine (**30**) (Scheme 2).

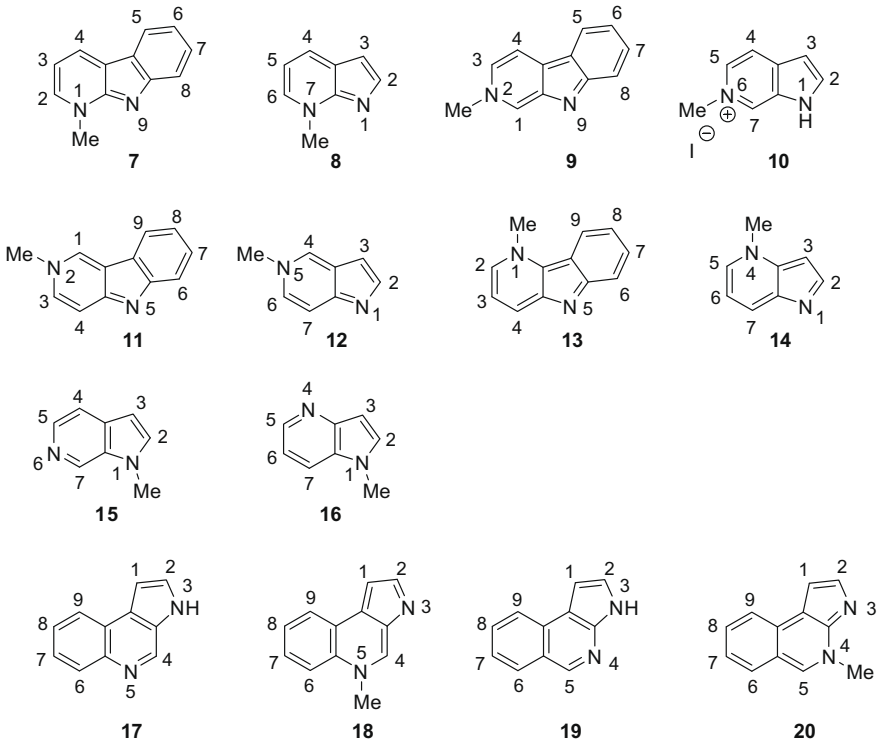
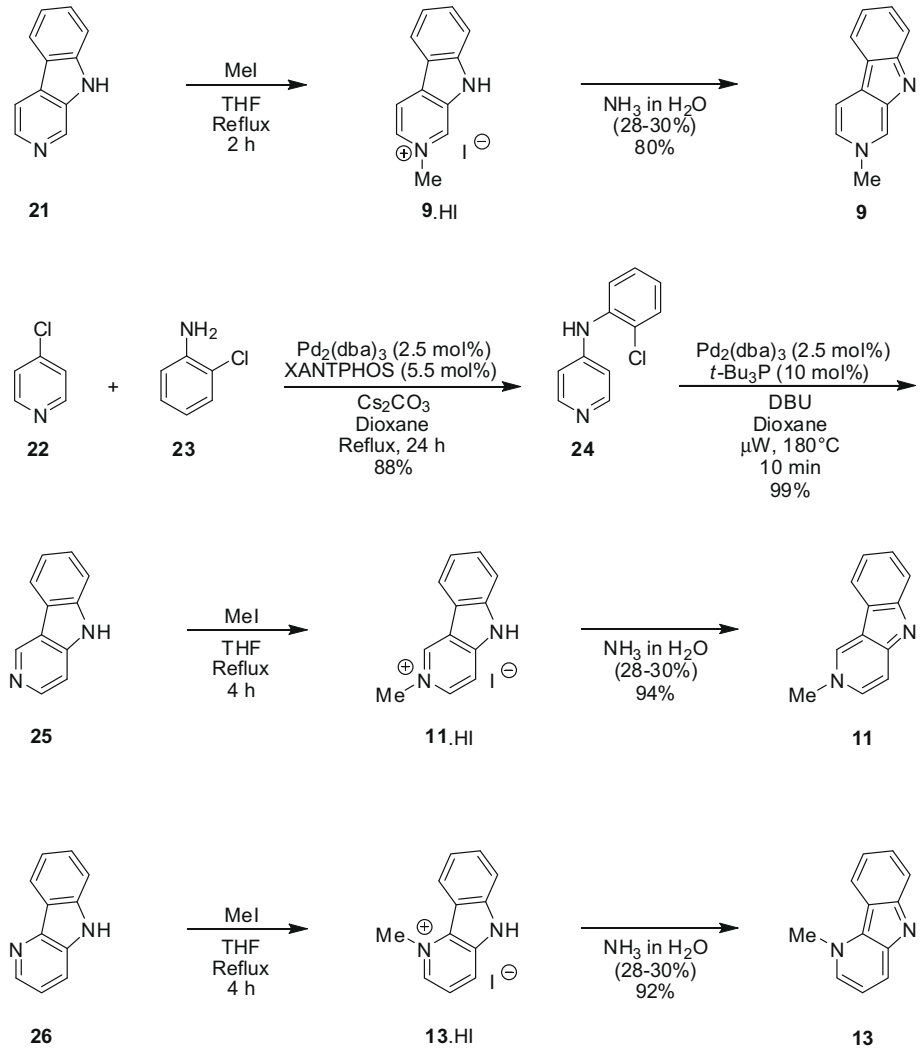


Figure 2. Carbolines, azaindoles, pyrroloquinolines and pyrroloisoquinolines **7**–**20**.



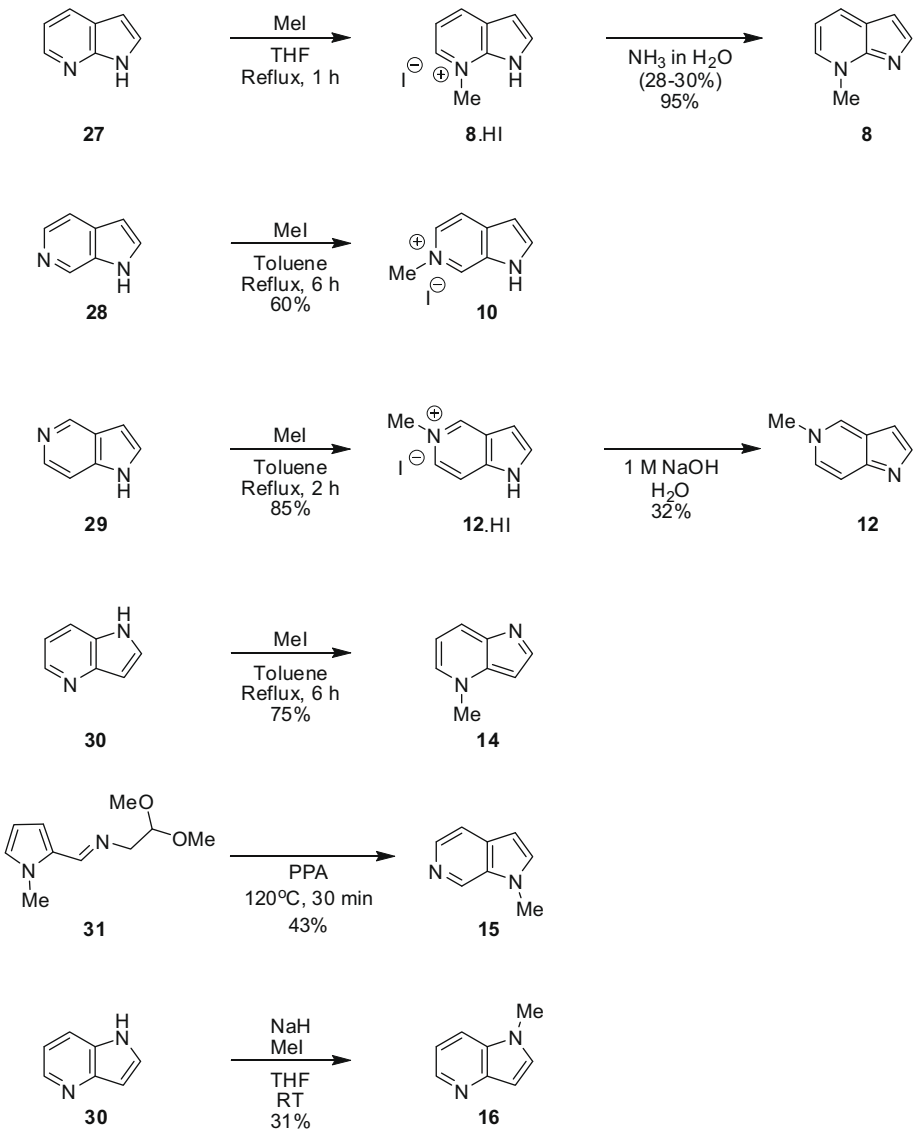
Scheme 1. Synthesis of methylated carbolines **9**, **11** and **13**.

3. Results

Results, expressed as IC_{50} values in μM, of all test compounds in the antiparasitic screen, which included the protozoa *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* (axenically grown amastigote forms), *P. falciparum* (strain K1, chloroquine-resistant), and a cytotoxicity control (L6 cells), are displayed in Table 1. With regard to the antiplasmodial activity, the main focus of this work, a selectivity index (SI) was calculated (cytotoxic IC_{50}/antiplasmodial IC_{50}). Results obtained for the parent indoloquinoline alkaloids neocryptolepine, isoneocryptolepine, isocryptolepine, and cryptolepine and the corresponding indoloisoquinolines **5** and **6**, were taken from our previous work and included for comparison purposes.[8,9] The high activity and selectivity of 1-methyl-δ-carboline, reported before by Arzel et al.[10] was confirmed, both when tested as a base (**13**) or the corresponding hydroiodide salt. In both cases an IC_{50} against *P. falciparum* <0.5 μM was obtained, with an SI > 345. The high selectivity is also obvious from the inactivity against the other parasites. However, stripping the molecule even more to the corresponding N-methylated azaindole **14** resulted in an almost complete loss of antiprotozoal activity and cytotoxicity.

In contrast, in the α-carboline series (neocryptolepine derivatives), 1-methyl-α-carboline, both when tested as a base (**7**) or the corresponding hydroiodide salt, was much less active and cytotoxic than neocryptolepine **2**. The same was true for the corresponding azaindole **8**, with the exception of a moderate activity against *T. cruzi*. In the β-carboline series, based on the synthetic indoloquinoline isoneocryptolepine (**4**), similar results as in the cryptolepine series (δ-carbolines) were obtained. Both 2-methyl-β-carboline (**9**) and its hydroiodide salt showed a high antiplasmodial activity (IC_{50} < 0.5 μM) and selectivity. The selectivity was even better than observed for the δ-carbolines, since the IC_{50} values were comparable, in the absence of cytotoxicity in the concentration range tested, resulting in an SI > 1000 for compound **9**. Against the other parasites no or only marginal activity was observed. Compound **10** was only marginally active. The same tendency, although less pronounced, could be observed in the isocryptolepine (**3**) series (γ-carbolines). The selectivity of the tricyclic analogue **11** and its hydroiodide salt was better than for the parent alkaloid, but the improvement was rather modest compared to the δ- and especially the β-carboline series. Also in the γ-series the corresponding azaindole **12** was only marginally active. The two azaindoles where the pyrrole-*N* was methylated (compounds **15** and **16**) showed no biological activity.

The synthetic indoloisoquinolines **5** and **6** as well as their hydroiodides suffered from the same disadvantage as the indoloquinolines, that is, they were very active against *P. falciparum* (IC_{50} < 1 μM for **5** and even <0.05 μM for **6**), but they were highly cytotoxic and showed a low selectivity.



Scheme 2. Synthesis of methylated azaindoles **8**, **10**, **12** and **14**–**16**.

In addition to the tricyclic carboline analogues of the parent indoloisoquinolines, also some tricyclic pyrroloquinolines and -isoquinolines were prepared. In the pyrroloquinoline series, the tricyclic compound corresponding to isoneocryptolepine was prepared in its non-methylated (**17**) and methylated (**18**) form, and in the pyrroloisoquinoline series the tricyclic analogue of compound **5** in its non-methylated (**19**) and methylated (**20**) form. All compounds showed a similar moderate cytotoxic activity. In general the antiplasmodial activity was more pronounced than the activity against other parasites. The best selectivity index was obtained for compound **18** (SI 50), indicating that also these derivatives lack selectivity.

The most selective compound, which could be considered as the most promising one to be developed as an antimalarial agent, that is, 2-methyl-β-carboline (**9**) and its hydroiodide salt, were selected for further investigation of their mechanism of action and in vivo antiplasmodial activity in infected laboratory animals. At least two mechanisms of action are thought to contribute to the antiplasmodial activity of indoloquinoline alkaloids, that is, inhibition of haemozoin formation, which is a selective mechanism of action against the *Plasmodium* parasite, and DNA interactions, which is a non-selective mechanism usually accompanied by cytotoxicty.[12,20] Therefore, 2-methyl-β-carboline (**9**) as well as its hydroiodide salt were evaluated in the β-haematin inhibitory assay (BHIA)[21], an in vitro assay mimicking the process of haemozoin formation in vivo, and in the DNA–methylgreen assay, where the displacement of the dye methylgreen from a DNA–methylgreen complex is determined as a measure of DNA interactions.[22] In contrast to the parent compounds cryptolepine (**1**), neocryptolepine (**2**), isocryptolepine (**3**) and isoneocryptolepine (**4**), which were active in both assays, compounds **9** and its hydroiodide were not active in the concentration range tested. In the β-haematin inhibitory assay, the IC_{50} value, representing the molar equivalents of test compound, relative to haemin, required to inhibit β-haematin formation by 50%, was >10 and >6, respectively, whereas for cryptolepine **1**, for instance, a value of 1.72 was obtained. Chloroquine, used as a positive control, produced an IC_{50} value of 2.56 in this assay. In the DNA–methylgreen assay, an IC_{50} value of >3800 μM and >2800 μM was obtained for compounds **9** and its hydroiodide, respectively, indicating the absence of DNA interactions. Cryptolepine, an established DNA intercalating agent, showed an IC_{50} of 65 μM in this assay.[8]

In addition, the most active and selective indoloisoquinoline **6** and its hydroiodide were evaluated as well in both functional as-

Table 1
Antiprotozoal activity and cytotoxicity of test compounds (IC_{50} or $IC_{50} \pm SD$, μM).

Compound no.	*Trypanosoma brucei rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani* (axenic amastigotes)	*Plasmodium falciparum* (K1) ($n \geqslant 3$)	Cytotoxicity (L6 cells) ($n \geqslant 3$)	Selectivity index (SI) (cytotox./*Plasm.*)
α-Series						
(**2**·HCl)	2.23 ± 0.82[a]	2.01 ± 1.30[a]	49.5 ± 3.7[a]	2.61 ± 0.67	3.24 ± 0.04	1.2
7	21.3	89.2	>160	14.7 ± 2.7	299.6 ± 115.7	20.4
(**7**·HI)	34.0	64.5	>95	13.0 ± 2.0	235.7 ± 68.7	18.1
	361.5	4.5	>225	>37	>680	–
β-Series						
(**4**)·HCl	6.48 ± 0.67[a]	21.0 ± 0.2[a]	75.4 ± 2.4[a]	0.23 ± 0.04	4.32 ± 0.04	18.8
9	93.7	>160	92.1	0.45 ± 0.02	>490	>1089
(**9**·HI)	155.0	>95	52.3	0.34 ± 0.04	>290	>853
10	>680	>225	>225	19.0 ± 3.6	>680	>35
17	25.5 ± 8.3	81.0 ± 30.4	182.7 ± 96.0	6.4 ± 4.2	173.2 ± 20.1	27
18	31.5 ± 18.4	76.8 ± 15.3	169.1 ± 35.0	3.2 ± 1.4	159.9 ± 19.7	50
γ-Series						
(**3**·HCl)	0.52 ± 0.11[a]	1.27 ± 0.78[a]	39.1 ± 11.5[a]	0.78 ± 0.30	1.19 ± 0.26	1.5
11	138.1	>160	>160	2.1 ± 0.1	390.2 ± 126.8	185.8
(**11**·HI)	109.3	>95	>95	2.4 ± 0.3	>290	>120
12	541.8	>225	>225	13.4 ± 0.8	>680	>51
(**12**·HI)	346.0	>115	>115	17.6 ± 2.7	>340	>19
δ-Series						
(**1**·HCl)	0.60 ± 0.07[a]	0.22 ± 0.07[a]	2.68 ± 0.89[a]	0.12 ± 0.02	1.12 ± 0.07	9.3
13	138.4	>160	>160	0.48 ± 0.05	178.8 ± 74.0	372.5
(**13**·HI)	86.1	>95	>95	0.38 ± 0.02	132.5 ± 62.9	348.7
14	600.6	>225	>225	29.3 ± 7.7	>680	>23.2
15	624.2	>225	>225	>37	>680	–
16	368.3	>225	>225	>37	>680	–
5	0.72	1.66	15.5	0.64 ± 0.09	1.48 ± 0.83	2.3
(**5**·HI)	0.55	1.53	16.8	0.57 ± 0.03	2.2 ± 1.1	3.9
6	3.33	20.7	4.8	0.040 ± 0.007	1.3 ± 1.0	32.5
(**6**·HI)	2.59	11.9	6.6	0.032 ± 0.004	1.5 ± 0.4	46.9
19	43.5 ± 16.4	69.6 ± 14.4	30.3 ± 14.2	23.2 ± 1.9	118.7 ± 35.2	5
20	8.4 ± 3.5	40.9 ± 15.9	97.5 ± 43.6	7.8 ± 3.9	152.8 ± 9.9	20
Melarsoprol	0.010 ± 0.03				8.6 ± 2.1	
Benznidazole		1.25 ± 0.28			>90	
Miltefosine			0.26 ± 0.08		58.4 ± 19.1	
Chloroquine				0.24 ± 0.04	43.1 ± 13.3	
Podophyllotoxin					0.012 ± 0.002	

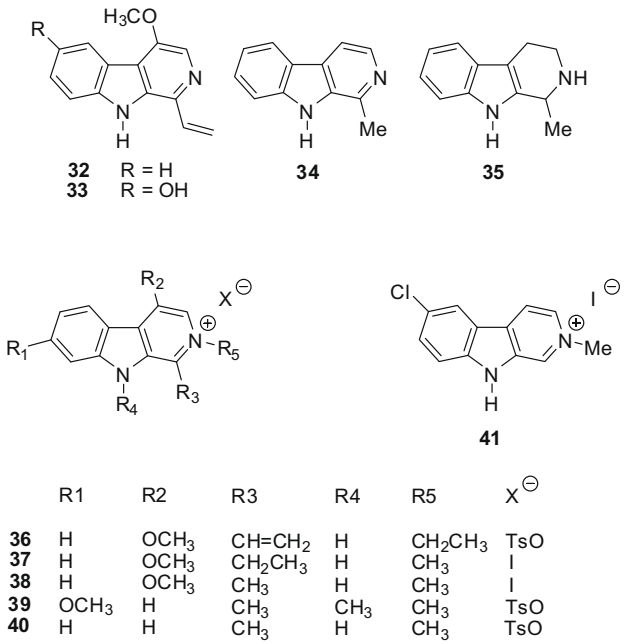
[a] $n = 3$.[8]

says. The IC_{50} values in the β-haematin inhibitory assay were 2.76 and 3.32 molar equivalents, respectively, indicating the potential of these compounds to inhibit haemozoin formation in vivo. In the DNA–methylgreen assay, IC_{50} values of 81 μM and 87 μM, respectively, were obtained, indicating DNA intercalating properties similarly to cryptolepine.

Compound **9** (HI salt) was evaluated in the *Plasmodium berghei* ANKA mouse model for in vivo antiplasmodial potential after intraperitoneal dosing at 50 mg/kg for 5 consecutive days. Chloroquine was used as reference treatment (10 mg/kg, IP for 5 days). Untreated infected controls developed severe malaria with most animals showing severe clinical signs on day 4. Treatment with chloroquine resulted in 100% survival until day 7 with low parasitaemia at day 4 post infection. Unfortunately compound **9**, when tested as its hydroiodide salt, failed to show activity. Survival time was not significantly increased compared to uninfected control animals.

4. Discussion

From the results displayed in Table 1, it appears that stripping the indoloquinoline skeleton to a carboline only results in a more selective antiplasmodial action in case of a β- or a δ-carboline (**9** and **13**, respectively). Further simplification to an azaindole only results in inactive compounds. The synthetic indoloisoquinolines **5** and **6** show the same properties as the indoloquinolines, that is, a high antiplasmodial activity accompanied by a high cytotoxicity. Whereas the β-carboline skeleton, biogenetically derived from tryptophan, is widely distributed in nature, α-, γ- and δ-carboline derivatives are relatively rare. To the best of our knowledge, no tricyclic δ-carbolines have been reported in nature; only benzo-δ-carbolines such as cryptolepine (**1**) are known. β-Carboline alkaloids display a whole range of biological activities and compounds with various structures show antiplasmodial properties. Two simple β-carboline derivatives, that is, 4-methoxy-1-vinyl-9*H*-β-carboline (**32**) and 4-methoxy-1-vinyl-9*H*-β-carbolin-6-ol (**33**), were identified as antiplasmodial components of *Picrasma javanica*, a Thai medicinal plant (Fig. 3). IC_{50} values against various *P. falciparum* isolates were about 2.4 μg/mL (10.7 μM) and 3.3 μg/mL (13.7 μM), respectively.[23] Harman (**34**) and tetrahydroharman (**35**), isolated from *Guiera senegalensis*, a plant used in traditional medicine in Mali, showed antiplasmodial activity against a chloroquine-sensitive (D6) (IC_{50} 2.2 μg/mL (12.1 μM) and 3.9 (20.1 μM) μg/mL, respectively), as well as a chloroquine-resistant strain (W2) (IC_{50} 1.3 μg/mL (7.1 μM) and 1.4 μg/mL (7.5 μM), respectively) of *P. falciparum*. Cytotoxicity was evaluated on THP1 cells (human monocytes) and IC_{50} values of 22 μg/mL (121 μM) and 75 μg/mL (403 μM) were obtained, indicating some selectivity against *Plasmodium*.[24] In a more systematic study a series of synthetic and natural β-carboline compounds and their salts were evaluated for their antiplasmodial and cytotoxic activity.[25] It was found that quaternary carbolinium cations showed a higher antiplasmodial activity than neutral β-carbolines. A good correlation was observed between the presence of a π-delocalized lipophilic



	R1	R2	R3	R4	R5	X
36	H	OCH_3	$CH{=}CH_2$	H	CH_2CH_3	TsO
37	H	OCH_3	CH_2CH_3	H	CH_3	I
38	H	OCH_3	CH_3	H	CH_3	I
39	OCH_3	H	CH_3	CH_3	CH_3	TsO
40	H	H	CH_3	H	CH_3	TsO

Figure 3. Naturally occurring β-carboline derivatives with antiplasmodial activity **32**–**41**.

cationic structure and antiplasmodial activity. Compounds **36**–**39** showed IC_{50} values against a chloroquine-sensitive *P. falciparum* strain (FCR-3) below 1 μM, while their IC_{50} values on mouse mammary tumour FM3A cells were >10 μM, resulting in selectivities >32. For compound **40**, which is in fact the naturally occurring alkaloid melinonine F, an IC_{50} value against this *P. falciparum* strain of 2.0 μM was reported, with a selectivity index of 18. Wright et al. had already reported activity of melinonine F (**40**) against *P. falciparum* strain K1 (chloroquine-resistant) (IC_{50} 5.1 μM), as well as for normelinonine F, the latter one being identical to our synthetic compound **9**.[26] For normelinonine F an IC_{50} of 13.6 ± 9.9 μM was reported, which is an order of magnitude less active than in our case. Nostocarboline (**41**) is a chloro-substituted quaternary β-carbolinium alkaloid isolated from the cyanobacterium *Nostoc* 78-12A. It showed a pronounced activity against *P. falciparum* strain K1 (IC_{50} 194 nM), while being inactive against other parasites tested, and it was only weakly cytotoxic against rat myoblast L6 cells (IC_{50} > 0.1 mM).[27]

Since highly active and selective compounds such as **9** were not active in the two functional assays used to establish their mode of action, based on previous experiments on the indoloquinoline alkaloids, the question remains how they act on the parasite. A similar observation has been made for cryptolepine derivatives such as 2,7-dibromocryptolepine showing a high antiplasmodial activity. A detailed investigation of the mode of action of this compound, involving a quantitative measurement of the inhibition of β-haematin formation and calculation of the accumulation ratio of a given compound in the acid food vacuole based on its pK_a value, suggested that its antiplasmodial activity may involve other mechanisms of action in addition to inhibition of the haem detoxification process.[28]

5. Conclusion

The original indoloquinoline leads cryptolepine (**1**), neocryptolepine (**2**), isocryptolepine (**3**) and isoneocryptolepine (**4**), as well as the corresponding indoloisoquinolines **5** and **6**, all suffer from a low selectivity against *P. falciparum* K1, because of pronounced cytotoxicity. The bicyclic analogues discussed above lost their antiplasmodial as well as their cytotoxic properties. In the tricyclic series the most promising compound was 2-methyl-2*H*-pyrido[3,4-*b*]indole (**9**), or 2-methyl-β-carboline, showing an IC_{50} value of 0.45 μM against *P. falciparum* K1, without apparent cytotoxicity against L6 cells (SI > 1000). However, this compound failed to show activity in the *P. berghei* mouse model.

6. Experimental

6.1. Synthesis of carbolines and azaindoles

6.1.1. General experimental methods

All melting points were determined on a Büchi apparatus and are uncorrected. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer or on a Varian INOVA-300 in the solvent indicated with TMS as an internal standard. All coupling constants are given in hertz and chemical shifts are given in parts per million. The assignment of the ^1H NMR signals of all products is based on 2D NMR techniques (COSY, NOESY, HMQC, HSQC and HMBC). For mass spectrometric analysis, samples were dissolved in CH_3OH containing 0.1% formic acid and diluted to a concentration of approximately 10^{-5} mol/L. Accurate mass data were acquired on a Q-TOF 2 (Micromass) mass spectrometer equipped with a standard electrospray ionisation (ESI) interface. Aliquots of 1 μL were introduced in the mass spectrometer at a flow rate of 5 μL/min MeOH (0.1% formic acid), using the CapLC HPLC system (Waters, Millford). The cone voltage (approx. 35 V) and capillary voltage (approx. 3.3 kV) were optimized on one compound and used for all others. For the determination of the accurate mass of the molecular ion $[M+H]^+$, a solution of polyethylene glycol 300 in CH_3OH/H_2O with 1 mmol ammonium acetate, was added just before the mass spectrometer (at a rate of 1 μL/min) to the mobile phase. The calculated masses of PEG $[M+H]^+$ and $[M+NH_4]^+$ ions were used as lock mass. The elemental analysis has been carried out with an Elementar Vario EL III apparatus. Microwave heating was carried out with a single-mode Discover (CEM) unit. The temperature was monitored with an infrared sensor. Experiments were performed in sealed reaction vessels with an Al crimp cap with septum. β-Carboline (**21**), THF (extra dry over molecular sieve, water <50 ppm, stab.), 2-chloroaniline (**23**), $Pd_2(dba)_3$ and CH_3I were purchased from Acros. 1*H*-Pyrrolo[2,3-*b*]pyridine (**27**), 4-chloropyridine hydrochloride, DBU, P(*t*-Bu)$_3$ (1 M in toluene), polyphosphoric acid (PPA), XANTPHOS, $PdCl_2(PPh_3)_2$ and Cs_2CO_3 (99%) were obtained from Sigma–Aldrich. Toluene was obtained from Molar Chemicals and NaH (60% dispersion) from Merck. Flash column chromatography was performed on Kieselgel 60 (ROCC, 0.040–0.063 mm).

6.1.2. 4-Chloropyridine (22)

An ice-cooled 10% aq NaOH solution (6 mL) was slowly added to an ice-cooled solution of 4-chloropyridine hydrochloride (1.500 g, 10.000 mmol) in water (10 mL). After extraction with petroleum ether (3 × 30 mL), the organic phase was dried over $MgSO_4$, filtered and evaporated to dryness to obtain 4-chloropyridine (**22**) in 50% (0.568 g, 5.000 mmol) yield as a colourless liquid. It was immediately used in the next step.

6.1.3. *N*-(2-Chlorophenyl)pyridin-4-amine (24)

A round-bottomed flask was charged with $Pd_2(dba)_3$ (0.069 g, 0.075 mmol, 2.5 mol %) and XANTPHOS [9,9-dimethyl-4,5-bis(diphenylphosphino)-9*H*-xanthene] (0.096 g, 0.165 mmol, 5.5 mol %) followed by dry dioxane (12 mL) (freshly distilled). The mixture was flushed with N_2 for 10 min. Meanwhile, in another round-bottomed flask 4-chloropyridine (**22**) (0.341 g, 3.000 mmol), 2-chloroaniline (**23**) (0.459 g, 3.600 mmol) and

Cs_2CO_3 (2.932 g, 9.000 mmol) were weighed. To this mixture, the Pd-catalyst was added and the flask was flushed with N_2 for 5 min. The resulting mixture was heated at reflux (oil bath temperature: 110 °C) for 24 h under magnetic stirring. After cooling down to room temperature dichloromethane (25 mL) was added and the suspension was filtered over a pad of Celite® and rinsed with CH_2Cl_2 (125 mL). The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel using CH_2Cl_2/MeOH (95:5) as the eluent yielding **24** in 88% (0.540 g, 2.640 mmol).

White solid; mp 123 °C. δ_H ($CDCl_3$): 8.35 (d, J = 6.3 Hz, 2H, H-2), 7.45 (dd, J = 8.1, 1.5 Hz, 1H, H-6′), 7.44 (dd, J = 8.0, 1.5 Hz, 1H, H-3′), 7.25 (ddd, J = 8.1, 7.4, 1.5 Hz, 1H, H-5′), 7.03 (ddd, J = 8.0, 7.4, 1.5 Hz, 1H, H-4′), 6.88 (d, J = 6.3 Hz, 2H, H-3), 6.37 (br s, 1H, NH); δ_C ($CDCl_3$): 150.7, 149.5, 136.8, 130.3, 127.5, 125.6, 124.3, 121.0, 110.5; HRMS (ESI) for $C_{11}H_{10}ClN_2$ $[M+H]^+$: calcd: 205.0533, found: 205.0527.

6.1.4. 5*H*-Pyrido[4,3-*b*]indole (25)

A 10-mL microwave vial was charged with *N*-(2-chlorophenyl)pyridin-4-amine (**24**) (0.123 g, 0.600 mmol), DBU (0.137 g, 0.900 mmol) and 1.0 mL of a stock solution of catalyst* (5 mol % Pd, 10 mol % ligand, solvent = dioxane) and the mixture was stirred and flushed with argon for 1 min. Next, the vial was sealed with an Al crimp cap with a septum and heated at 180 °C in a CEM Discover microwave apparatus. The set power was 300 W and the total heating time was 10 min. After the reaction vial had cooled down to room temperature using a propelled air flow, it was opened and the reaction mixture transferred into a round-bottomed flask using CH_2Cl_2 (50 mL). The solvent was evaporated and the crude product purified via flash column chromatography on silica gel using CH_2Cl_2/7 N ammonia in MeOH (98:2) as the eluent yielding **25** in 99% (0.100 g, 0.594 mmol).

White solid; mp 224 °C (lit. mp 225 °C).[29] The characterization data are identical to those reported in the literature.[30] For comparison, we report here our ^{1}H NMR data: δ_H (DMSO-d_6): 11.72 (br s, 1H, NH), 9.36 (s, 1H, H-1), 8.45 (d, J = 5.7 Hz, 1H, H-3), 8.24 (ddd, J = 7.8, 1.2, 0.8 Hz, 1H, H-9), 7.59 (ddd, J = 8.2, 1.0, 0.8 Hz, 1H, H-6), 7.49 (dd, J = 5.7, 1.0 Hz, 1H, H-4), 7.48 (ddd, J = 8.2, 7.1, 1.2 Hz, 1H, H-7), 7.28 (ddd, J = 7.8, 7.1, 1.0 Hz, 1H, H-8).

*The stock solution of catalyst (5 mol % Pd/1 mL dry dioxane) was prepared by charging a flask with $Pd_2(dba)_3$ (0.137 g, 0.150 mmol) followed by adding dry, freshly distilled dioxane (9.4 mL) and P(*t*-Bu)$_3$ (1 M in toluene) (0.6 mL, 0.6 mmol). The solution was subsequently stirred for 15 min under argon atmosphere.

6.1.5. General methylation procedure for the synthesis of 8, 9, 11, 13

6.1.5.1. 7-Methyl-7*H*-pyrrolo[2,3-*b*]pyridine (8). In a round-bottomed flask 1*H*-pyrrolo[2,3-*b*]pyridine (**27**) (0.059 g, 0.500 mmol), dry THF (5.0 mL) and CH_3I (0.5 mL) were heated at reflux under N_2 atmosphere (oil bath temperature: 80 °C) for 1 h with magnetic stirring. Then the solvent was evaporated to dryness under reduced pressure and the crude product was mixed with silica gel and purified via flash column chromatography on silica gel using CH_2Cl_2/MeOH (90:10) as the eluent yielding 7-methyl-1*H*-pyrrolo[2,3-*b*]pyridine-7-ium iodide (**8**·HI). To obtain the free base, **8**·HI was brought in a mixture of CH_2Cl_2 (100 mL) and 28–30% ammonia in water (100 mL). The organic phase was separated and the aqueous phase was subsequently extracted with CH_2Cl_2 (4 × 50 mL). The combined organic phase was dried over $MgSO_4$, filtered and evaporated to dryness to yield **8** in 95% (0.063 g, 0.477 mmol).

Yellow liquid, δ_H ($CDCl_3$): 8.05 (d, J = 7.4 Hz, 1H, H-4), 7.89 (d, J = 2.6 Hz, 1H, H-2), 7.47 (d, J = 6.2 Hz, 1H, H-6), 6.76 (dd, J = 7.4, 6.2 Hz, 1H, H-5), 6.66 (d, J = 2.6 Hz, 1H, H-3), 4.21 (s, 3H, CH_3); δ_C ($CDCl_3$): 148.8, 145.0, 130.4, 130.0, 129.7, 108.8, 101.2, 40.0; HRMS (ESI) for $C_8H_9N_2$ $[M+H]^+$: calcd 133.0760, found 133.0757.

6.1.5.2. 2-Methyl-2*H*-pyrido[3,4-*b*]indole (9). β-Carboline (**21**) (0.084 g; 0.500 mmol), dry THF (7.5 mL) and CH_3I (3.0 mL). Reaction time = 2 h. Eluent: CH_2Cl_2/MeOH (90:10); yield: 80% (0.073 g, 0.401 mmol). Bright yellow/orange powder; mp 191–192 °C (decomp.) (lit. mp 213–215 °C).[31] δ_H ($CDCl_3$): 8.49 (d, J = 1.4 Hz, 1H, H-1), 8.16 (dt, J = 8.1, 1.0 Hz, 1H, H-5 or H-8), 8.06 (d, J = 6.2 Hz, 1H, H-4), 7.92 (dt, J = 8.5, 0.8 Hz, 1H, H-8 or H-5), 7.57 (ddd, J = 8.5, 6.8, 1.2 Hz, 1H, H-6 or H-7), 7.35 (dd, J = 6.3, 1.7 Hz, 1H, H-3), 7.17 (ddd, J = 8.1, 6.8, 0.9 Hz, 1H, H-7 or H-6), 4.17 (s, 3H, N–CH_3); (lit. δ_H ($CDCl_3$): 8.62 (s, 1H, H-1), 8.16 (t, J = 7 Hz, 2H, H-3 and H-4), 7.90 (d, J = 8 Hz, 1H, H-8), 7.64 (t, J = 8 Hz, 1H, H-6 or H-7), 7.50 (d, J = 8 Hz, 1H, H-5), 7.23 (t, J = 8 Hz, 1H, H-7 or H-6), 4.34 (s, 3H, N–CH_3))[31]; δ_C ($CDCl_3$): 158.8, 146.0, 132.7, 130.8, 129.0, 122.6, 122.1, 121.3, 119.5, 118.1, 115.2, 46.9; HRMS (ESI) for $C_{12}H_{11}N_2$ $[M+H]^+$: calcd 183.0922, found 183.0914.

6.1.5.3. 2-Methyl-2*H*-pyrido[4,3-*b*]indole (11). 5*H*-Pyrido[4,3-*b*]indole (**25**) (0.084 g; 0.500 mmol), dry THF (2.5 mL) and CH_3I (0.25 mL). Reaction time = 4 h. Eluent: CH_2Cl_2/MeOH (90:10); yield: 94% (0.086 g, 0.472 mmol). White-yellowish solid, mp 205–206 °C (lit. mp 172–173 °C).[32] The characterization data are identical to those reported in the literature.[32] For comparison, we report here our ^{1}H NMR data: δ_H (DMSO-d_6): 9.05 (d, J = 1.7 Hz, 1H, H-1), 8.10 (dd, J = 7.8, 1.3 Hz, 1H, H-9), 7.92 (dd, J = 7.2, 1.7 Hz, 1H, H-3), 7.63 (dd, J = 8.2, 0.9 Hz, 1H, H-6), 7.40 (ddd, J = 8.2, 7.0, 1.3 Hz, 1H, H-7), 7.39 (d, J = 7.2 Hz, 1H, H-4), 7.13 (ddd, J = 7.8, 7.0, 0.9 Hz, 1H, H-8), 4.11 (s, 3H, N–CH_3).

6.1.5.4. 1-Methyl-1*H*-pyrido[3,2-*b*]indole (13). 5*H*-Pyrido[3,2-*b*]indole (**26**) (0.084 g; 0.500 mmol), dry THF (2.5 mL) and CH_3I (0.25 mL). Reaction time = 4 h. Eluent: CH_2Cl_2/MeOH (90:10); yield: 92% (0.084 g, 0.461 mmol). Orange solid, mp 202–204 °C (lit. mp 202–206 °C).[10] The characterization data are identical to those reported in the literature.[10] For comparison, we report here our ^{1}H NMR data: δ_H (DMSO-d_6): 8.33–8.27 (m, 3H, H-2, H-4 and H-9), 7.67 (dd, J = 8.5, 1.0 Hz, 1H, H-6), 7.53 (dd, J = 8.3, 5.8 Hz, 1H, H-3), 7.45 (ddd, J = 8.5, 6.8, 1.2 Hz, 1H, H-7), 7.07 (ddd, J = 8.2, 6.8, 1.0 Hz, 1H, H-8), 4.71 (s, 3H, N–CH_3).

6.1.6. General methylation procedure for the preparation of 10 and 14

6.1.6.1. 6-Methyl-1*H*-pyrrolo[2,3-*c*]pyridin-6-ium iodide (10). In a round-bottomed flask 1*H*-pyrrolo[2,3-*c*]pyridine (**28**) (0.132 g, 1.000 mmol), toluene (15.0 mL) and CH_3I (6.0 mL) were heated at reflux under N_2 atmosphere (oil bath temperature: 120 °C) for 6 h with magnetic stirring. Then the precipitated material was filtered off. The crude product was mixed with silica gel and purified via column chromatography on silica gel using $CHCl_3$/MeOH/TEA (80:19:1) as the eluent yielding 6-methyl-1*H*-pyrrolo[2,3-*c*]pyridin-6-ium iodide (**10**) in 60% (0.156 g, 0.600 mmol).

Yellow solid, mp 135–138 °C. δ_H ($CDCl_3$): 9.74 (s, 1H, H-7), 8.02 (d, J = 2.8 Hz, 1H, H-2), 8.00 (dd, J = 6.8, 1.4 Hz, 1H, H-5), 7.92 (dd, J = 6.8, 0.7 Hz, 1H, H-4), 6.78 (dd, J = 2.8, 0.7 Hz, 1H, H-3), 4.47 (s, 3H, N–CH_3); δ_C ($CDCl_3$): 138.7, 136.1, 132.2, 131.9, 131.6, 118.0, 103.9, 47.9; Anal. Calcd for $C_8H_9IN_2$ (260.07): C, 36.95; H, 3.49; N, 10.77. Found: C, 36.67; H, 3.35; N, 10.86.

6.1.6.2. 4-Methyl-4*H*-pyrrolo[3,2-*b*]pyridine (14). 1*H*-Pyrrolo-[3,2-*b*]pyridine (**30**) (0.132 g, 1.000 mmol), toluene (15 mL) and CH_3I (6.0 mL). Reaction time = 6 h. Eluent: $CHCl_3$/MeOH/TEA (80/19/1); yield: 75% (0.099 g, 0.749 mmol). Brown oil, δ_H ($CDCl_3$): 8.28 (d, J = 1.8 Hz, 1H, H-2), 8.18 (dd J = 7.8 Hz, 1H, H-7), 7.58 (d,

J = 6.2 Hz, 1H, H-5), 6.95 (dd, J = 7.8, 6.2 Hz, 1H, H-6), 6.44 (d, J = 1.8 Hz, 1H, H-3), 4.15 (s, 3H, N–CH_3); δ_C ($CDCl_3$): 152.3, 144.6, 141.2, 130.2, 127.7, 109.8, 91.8, 42.8; Anal. Calcd for $C_8H_8N_2$ (132.16): C, 72.70; H, 6.10; N, 21.20. Found: C, 72.45; H, 6.19; N, 21.10.

6.1.7. 5-Methyl-5*H*-pyrrolo[3,2-*c*]pyridine (12)

In a round-bottomed flask 1*H*-pyrrolo[3,2-*c*]pyridine (**29**) (0.354 g, 3,000 mmol), toluene (7.5 mL) and CH_3I (3.0 mL) were heated at reflux under N_2 atmosphere (oil bath temperature: 120 °C) for 2 h under magnetic stirring. Then the precipitated material was filtered off and rinsed well with toluene (50 mL) and diethyl ether (30 mL) and dried under vacuum at 80 °C affording 5-methyl-1*H*-pyrrolo[3,2-*c*]pyridine-5-ium iodide (**12**·HI) in 85% (0.663 g, 2.549 mmol) yield.

Beige solid, mp 127–131 °C. δ_H (DMSO-d_6): 12.75 (s, 1H, N–H), 9.31 (s, 1H, H-4), 8.42 (dd, 1H, J = 6.8, 1.2 Hz, H-6), 7.98 (d, 1H, J = 6.8 Hz, H-7), 7.96 (d, 1H, J = 3.3 Hz, H-2), 7.03 (dd, J = 3.3, 0.9 Hz, H-3), 4.34 (s, 3H, N–CH_3); δ_C (DMSO-d_6): 140.5, 139.6, 134.9, 133.0, 124.5, 109.5, 103.8, 46.7; Anal. Calcd for $C_8H_9IN_2$ (260.07): C, 36.95; H, 3.49; N, 10.77. Found: C, 37.21; H, 3.18; N, 10.70.

To obtain the free base, 5-methyl-1*H*-pyrrolo[3,2-*c*]pyridine-5-ium iodide (**12**·HI) (0.550 g, 2.115 mmol) was brought in a mixture of CH_2Cl_2 (20 mL) and 1 M NaOH solution in water (20 mL). The organic phase was separated and the aqueous phase was subsequently extracted with CH_2Cl_2 (4 × 20 mL). The combined organic phase was dried over $MgSO_4$, filtered and evaporated to dryness to obtain the title compound **12** in 32% (0.089 g, 0.673 mmol) yield. Brownish oil, δ_H (DMSO-d_6): 8.69 (s, 1H, H-4), 7.77 (s, 1H, H-2), 7.71 (dd, 1H, J = 6.7, 1.6 Hz, H-6), 7.47 (d, 1H, J = 6.8 Hz, H-7), 6.66 (s, 1H, H-3), 4.11 (s, 3H, N–CH_3).

6.1.8. 1-Methyl-1*H*-pyrrolo[2,3-*c*]pyridine (15)

A mixture of 2,2-dimethoxy-*N*-[(1*E*)-(1-methyl-1*H*-pyrrol-2-yl)methylene]ethanamine (**31**) (0.980 g, 5.000 mmol) and polyphosphoric acid (15 g) was heated to 115–120 °C (oil bath temperature) for 30 min. The reaction mixture was cooled down and poured onto ice water (150 mL). A solution was formed and the pH was adjusted to 7.5 by adding solid sodium hydroxide and the mixture was subsequently extracted with EtOAc (3 × 30 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The product was isolated by column chromatography on silica gel using $CHCl_3$/MeOH (100:1) as the eluent to give **15** in 43% (0.287 g, 2.172 mmol) yield.

Brown oil, δ_H ($CDCl_3$): 8.73 (s, 1H, H-7), 8.23 (d, J = 5.4 Hz, 1H, H-5), 7.50 (dd, J = 5.4, 1.0 Hz, 1H, H-4), 7.15 (d, J = 3.0 Hz, 1H, H-2), 6.46 (dd, J = 3.0, 1.0 Hz, 1H, H-3), 3.85 (s, 3H, N–CH_3); δ_C ($CDCl_3$): 138.2; 133.6; 133.1; 132.5; 132.4; 115.0; 100.4; 32.9.; Anal. Calcd for $C_8H_8N_2$ (132.16): C, 72.70; H, 6.10; N, 21.20. Found: C, 72.43; H, 6.12; N, 21.18.

6.1.9. 1-Methyl-1*H*-pyrrolo[3,2-*b*]pyridine (16)

A mixture of 1*H*-pyrrolo[3,2-*b*]pyridine (**30**) (0.132 g, 1.000 mmol), THF (10 mL) and NaH (0.150 g, 60% dispersion) was stirred at room temperature for 20 min. Then, CH_3I (119 μL) was added and the reaction mixture was stirred for 24 h. Subsequently, water (15 mL) was added and an extraction with dichloromethane (3 × 10 mL) was performed. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was subjected to preparative TLC on silica gel using $CHCl_3$/MeOH (10:1) as the eluent to give **16** in 31% (0.041 g, 0.310 mmol) yield.

Brown oil, δ_H ($CDCl_3$): 8.46 (d, J = 6.0 Hz, 1H, H-5), 7.65 (d, J = 8.4 Hz, 1H, H-7), 7.28 (d, J = 3.2 Hz, 1H, H-2), 7.13 (dd, J = 8.4, 6.0 Hz, 1H, H-6), 6.69 (d, J = 3.2 Hz, 1H, H-3), 3.82 (s, 3H, N–CH_3); δ_C ($CDCl_3$): 146.6; 142.8; 132.0; 129.5; 116.3; 116.1; 101.9; 32.8; Anal. Calcd for $C_8H_8N_2$ (132.16): C, 72.70; H, 6.10; N, 21.20. Found: C, 72.75; H, 6.05; N, 21.14.

6.2. Functional assays

6.2.1. β-Haematin inhibitory activity (BHIA) assay

The experimental procedure was based on the protocol published by Parapini et al.[21] The microassay was performed in a 96-well V-bottom microplate. When testing water-soluble compounds, 50 μL of an 8 mM haemin solution in DMSO was added to 50 μL of an aqueous solution of test compound. For poorly soluble compounds, 25 μL of a 16 mM haemin solution in DMSO was added to 25 μL of the test compound dissolved in DMSO; 50 μL of water was added afterwards in order to keep the DMSO concentration at a constant level of 25%. As a control 50 μL water was used instead of the test solution. Next, 100 μL of an 8 M acetate buffer (pH 5) was added to initiate β-haematin formation, and the plate was incubated at 37 °C for 18 h. After 15 min of centrifugation at 3000 rpm, the soluble fraction was removed. Then 200 μL of DMSO was added to the wells in order to remove all remaining haematin. The plate was centrifugated again 15 min at 3000 rpm and all supernatant was removed. The residual pellet of pure β-haematin remaining in the well was dissolved in 200 μL of 0.1 M NaOH solution. Next, 75 μL from each well was transferred into a U-bottom microplate and a serial fourfold dilution in 0.1 M NaOH was made. The absorbance was measured at 414 nm using a microtiter plate reader. IC_{50} values were calculated, representing the molar equivalents (M_{eq}) of test compound, relative to haemin, required to inhibit β-haematin formation by 50%.

6.2.2. DNA–methylgreen assay

The DNA–methylgreen assay is a colorimetric microassay to detect agents that interact with DNA. Compounds displacing the methylgreen from a DNA–methylgreen complex cause a loss of colour that can be detected by measuring the absorbance at 620 nm. The experimental procedure was based on the protocol published by Burres et al.[22] The DNA–methylgreen reagent was prepared by suspending 20 mg of DNA–methylgreen in 100 mL of 0.05 M Tris–HCl buffer (pH 7.5) containing 7.5 mM $MgSO_4$, and stirred at 37 °C for 24 h. The samples were transferred to a 96-well microtiter plate and the solvent was removed under vacuum. Next, 200 μL of the DNA–methylgreen reagent was added to the test compounds. The absorbance at 620 nm was measured at the beginning and after 24 h of incubation at 25 °C. The IC_{50} value, corresponding to the concentration in which 50% of methylgreen is displaced from the complex, was calculated by comparing the initial and the final absorbance.

6.3. Antiparasitic activity and cytotoxicity

Antiplasmodial activity was determined against the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine), using a modified [^{3}H]-hypoxanthine incorporation assay as described before.[33] Chloroquine was used as positive controls. Activity against *T. b. rhodesiense*, *T. cruzi*, *L. donovani*, and cytotoxicity against rat skeletal myoblasts (L-6 cells) were evaluated as described before.[33] Melarsoprol was used as positive control against *T. b. rhodesiense*, benznidazole against *T. cruzi*, miltefosine against *L. donovani*, and podophyllotoxin for cytotoxicity. Results are expressed as IC_{50} in μM, and for the antiplasmodial activity and cytotoxicity the standard deviation (SD) is provided ($n \geqslant 3$).

6.4. In vivo activity

Swiss mice (female—BW ~25 g; Janvier France), were allocated randomly to 5 groups of 4 animals each. Drinking water and food

were available *ad libitum* throughout the experiment. The weight of the individual animals did not differ too much from the group mean.

P. berghei (ANKA-strain) was maintained in the laboratory by weekly mechanical subpassage in Swiss mice. The infection inoculum was prepared by taking heparinized blood collected from a clinically ill donor mouse (approximately 20% parasitaemia) and diluted in PBS to obtain an infection inoculum of 0.15 mL with about 4×10^8 infected erythrocytes. The infection inoculum was given intra-peritoneally.

Chloroquine (5×10 mg/kg—daily administration in 0.1 mL IP) was used as positive control. All formulations were made in PEG200. The first dosing with the test compound (5×50 mg/kg—daily administration in 0.1 mL IP) was given approx. 2 h after the artificial infection. Vehicle-treated animals received $5 \times$ PEG200—0.1 mL IP.

A drop of blood was obtained from the tail vein (by cutting a small piece of the tail top) on days 4, 7, 11, 14 (end of trail). Smears were prepared and immediately processed for Giemsa staining and microscopic reading.

The animals were observed for the occurrence/presence of clinical or adverse effects during the two-week course of the experiment. In case of very severe clinical signs, either due to toxicity or malaria, the animals were euthanized for animal welfare reasons.

Parasitaemia was determined on days 4, 7, 11 and 14 on surviving animals. Percentage reduction as compared to infected control animals (UIC) was used as a measure for drug activity. Parasitaemia was microscopically evaluated by counting a minimum of 500 (5 fields). The difference between the mean value of the control group (taken as 100%) and those of the experimental groups was expressed as percent and reduction of parasitaemia was calculated using the following formula:

$$\text{Activity} = 100 - \left(\frac{\text{mean parasitaemia treated}}{\text{mean parasitaemia control}} \times 100\right)$$

This reduction in parasitaemia is a measure for drug activity.

In addition the body weight of the mice was monitored (d0–d4–d10–d14) and the mean survival time (MST) determined to check for both drug activity and toxicity.

The untreated infected controls (UIC) developed severe malaria, as shown by the levels of parasitaemia (38.28%) at day 4 post infection and the presence of severe clinical signs from day 4 (poor appearance, tremor, somnolence, body weight loss). Four out of five animals died before day 7 post infection. Chloroquine showed very high activity at day 4 pi, as reflected by the low parasitaemia (5.24% parasitaemia and 86.3% suppression as compared to UIC) and the overall good health status during the dosing period. As expected, total cure could not be obtained and most animals developed higher levels of parasitaemia and showed poor health from day 9 post infection onwards with 4 survivors at the end of the experiment (day 14).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.057.

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