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Recent developments in naturally derived antimalarials: cryptolepine analogues

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Abstract

Increasing resistance of *Plasmodium falciparum* to commonly used antimalarial drugs has made the need for new agents increasingly urgent. In this paper, the potential of cryptolepine, an alkaloid from the West African shrub *Cryptolepis sanguinolenta*, as a lead towards new antimalarial agents is discussed. Several cryptolepine analogues have been synthesized that have promising in-vitro and in-vivo antimalarial activity. Studies on the antimalarial modes of action of these analogues indicate that they may have different or additional modes of action to the parent compound. Elucidation of the mode of action may facilitate the development of more potent antimalarial cryptolepine analogues.

Introduction

Malaria continues to cause considerable mortality and morbidity, especially in Africa, and it has been estimated that mortality from malaria has doubled in the past 20 years (Trape et al 2002). A major reason for this is the increasing resistance of *Plasmodium falciparum* to chloroquine as well as to other antimalarial drugs. The isolation of artemisinin from the herb *Artemisia annua* in China in 1971 heralded a new era in antimalarial drug development as artemisinin and semi-synthetic derivatives such as artemether and (sodium) artesunate are rapidly effective against parasites resistant to other antimalarials, and as they have gametocyticidal activity they are also able to reduce transmission to the mosquito vector (Wright & Warhurst 2002). Hopes that artemisinins will have a major impact on malaria have, however, been tempered by a recent study in which a number of clinical isolates of *P. falciparum* from malaria patients showed resistance to artemether (Jambou et al 2005). Moreover, six out of seven isolates that exhibited resistance to artemether also showed a single polymorphism (S769N) in the gene for the sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *PfATPase6* gene, which is significant as studies suggest that SERCA may be a specific target for the action of artemisinins (Eckstein-Ludwig et al 2003). In response to this worrying development, the World Health Organization have called for an immediate halt to the marketing and sale of malaria medicines that contain only artemisinin or one of its derivatives (World Health Organization 2006). It is strongly recommended that artemisinins should always be used in combination with another antimalarial agent (e.g. mefloquine, lumefantrine, amodiaquine), known as artemisinin combination therapies, in order to minimize the risk of resistance development. Even so, there is evidence that resistance to lumefantrine–artemether may be developing in Zanzibar after a short period of use (Sisowath et al 2005), and this underlines the urgent need for new antimalarial drugs to be developed.

In addition to artemisinin, it should not be forgotten that quinine from the bark of *Cinchona* species is a plant-derived antimalarial of considerable importance (Wright 2005). Artemisinin and quinine originate from China and South America, respectively, and although malaria is mainly a problem in Africa and many African plant species are used traditionally for the treatment of malaria, none have yet yielded a clinically useful antimalarial drug. One African plant species that does show potential in this respect is the climbing shrub *Cryptolepis sanguinolenta*, and the purpose of this paper is to review the current status of this species as a possible source of new antimalarial agents.

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C. sanguinolenta

C. sanguinolenta Lindl. Schltr. is a climbing shrub native to West Africa belonging to the family Periplocaceae, but it may also be assigned to the Asclepiadaceae as some authors include members of the Periplocaceae in the Ascepiadaceae (Addae-Kyereme 2004). The leaves are elliptic oblong to ovate or lanceolate in shape, 6–8 cm in length, the margin entire, apices curved and acuminate with symmetrical, obtuse or rounded bases. Flowers are small and yellow but give rise to striking "boomerang" shaped seed pods, about 25 cm in length, that split to release small, flat seeds each with a tuft of fine hairs attached at one end. The roots are tortuous with a yellow brown outer surface and yellow inner part that contains abundant starch-filled parenchyma cells; lacticifers are also present.

Constituents of *C. sanguinolenta*

The roots contain indoloquinoline alkaloids, the main constituent being cryptolepine, which constitutes about 1% of the dried roots. Two alternative structures for cryptolepine (as base), are found in the literature (Figure 1). Figure 1b is preferred by this author as the ^{1}H-NMR signal for the *N*-methyl group (using $CDCl_3$), occurs at δ 4.7 ppm, which is consistent with a methyl group attached to a quaternary aromatic nitrogen atom. Furthermore, cryptolepine has only one protonable nitrogen (N-10), which again is in accord with Figure 1b and, as will be seen later, may have important implications with respect to understanding the antimalarial mode of action of cryptolepine. The preferred structure of the hydrochloride salt of cryptolepine is also shown (Figure 1c).

In addition to cryptolepine, quindoline (the demethylated analogue of cryptolepine), quindolinone and 11-hydroxycryptolepine (Paulo et al 1995) have been isolated from the roots. The latter is tautomeric with cryptolepinone, which is thought to be an artefact arising from the oxidation of cryptolepine (Fort et al 1998). Cryptoheptine (Paulo et al 1995) and homocryptolepinone (Sharaf et al 1995a) have also been isolated from the roots. The cryptolepine isomers neocryptolepine (Cimanga et al 1996a) and isocryptolepine (Grellier et al 1996) are identical to cryptotackeine and cryptosanguinolentine, respectively (Sharaf et al 1996b), the two compounds being isolated in the same year by different groups. In addition, the spirononacyclic alkaloid cryptospirolepine (Tackie et al 1993) and the dimeric alkaloids cryptoepicarboline (Sharaf et al 1995b), biscryptolepine (Cimanga et al 1996b), cryptoquindoline (Paulo et al 1995) and cryptomisrine (Sharaf et al 1996a) have been reported.

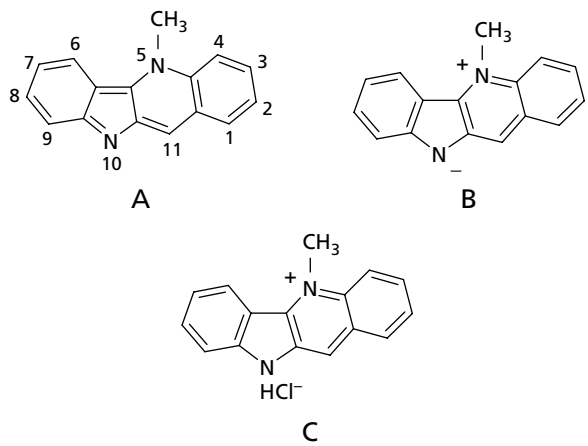

Figure 1 Structures of cryptolepine; A and B, alternative depictions of the cryptolepine base; C, cryptolepine hydrochloride.

Rationale for cryptolepine as a lead antimalarial agent

In West Africa, decoctions of the roots of *C. sanguinolenta* are used in traditional medicine to treat malaria as well as a variety of other infectious diseases, including respiratory and sexually transmitted infections (Boye & Ampofo 1983). A commercial herbal tea preparation containing the powdered roots known as Phyto-Laria is also available (Addae-Kyereme 2004). In 1989, the effectiveness of the aqueous extract of the roots in the treatment of uncomplicated falciparum malaria was compared with chloroquine in a small, open, randomized clinical trial in adult patients (cited in Addae-Kyereme 2004). It was concluded that the decoction was of comparable efficacy to chloroquine but it should be noted that the patients, being adults, were likely to be semi-immune to malaria and it cannot be assumed that the decoction would be similarly effective in children with little or no immunity to malaria.

In support of the effectiveness of *C. sanguinolenta* in malaria treatment, the major alkaloid present, cryptolepine, has potent in-vitro antiplasmodial activity against both chloroquine-sensitive (strain HB3) and chloroquine-resistant (strain K1) strains of *P. falciparum*, the species of malaria parasite responsible for most deaths due to malaria (IC50 = 0.27 and 0.44 μM, respectively) (Wright et al 2001). However, cryptolepine has been shown to be moderately cytotoxic to a number of human cancer cell lines and the mechanisms involved may include intercalation into DNA, inhibition of topoisomerase II and DNA synthesis as well as the induction of apoptosis (Dassonneville et al 2000). Interestingly, cryptolepine was found to have a previously unknown mode of intercalation as the crystal structure of a cryptolepine–DNA complex showed that it intercalates preferentially between non-alternating G-C sequences (Lisgarten et al 2002). These data suggest that cryptolepine would be an unlikely candidate as a lead towards new antimalarial agents as it is likely to lack selectivity.

In addition to the need for selectivity, another key requirement for antimalarial agents is to have demonstrable in-vivo activity, but the results of studies in which cryptolepine was assessed for antimalarial activity in mice infected with the rodent malaria parasite *Plasmodium berghei* have been mixed. Using Peters' 4-day test (Peters 1975), parasitaemia was suppressed by 80.5% by oral treatment with 50 mg kg^{-1}/day for 4 days (Wright et al 1996) but subcutaneous administration with higher doses (113 mg kg^{-1}/day) was ineffective (Kirby et al 1995). With intraperitoneal administration, parasitaemia was suppressed by 80% with doses of 2.5 mg kg^{-1}/day in mice infected with *Plasmodium vinckei-petteri* (Grellier et al 1996), although it should be noted that this species of parasite is more sensitive to antimalarials such as chloroquine than is *P. berghei*. In mice infected with *P. berghei*, intraperitoneal

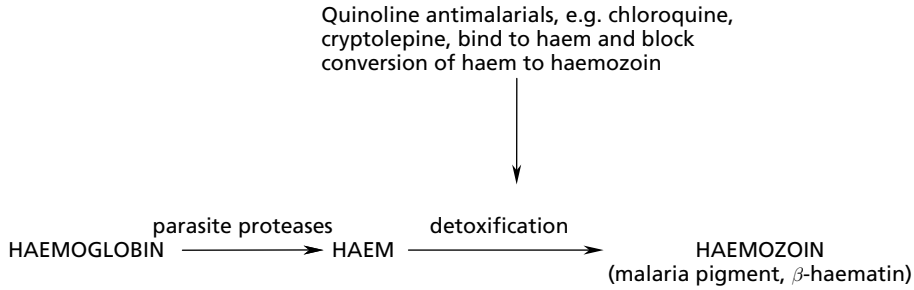

Figure 2 Mode of action of quinoline antimalarials.

administration of cryptolepine (20 mg kg^{-1}/day) was found to be toxic to the mice (Wright et al 2001). Unfortunately, none of the above studies showed that cryptolepine could cure malaria infection in mice (i.e. suppress parasitaemia by 100%) and intraperitoneal administration in mice was associated with toxicity at the higher dose used.

Further studies investigating the mode of action of cryptolepine revealed the possibility that its antiplasmodial mode of action may be different from its cytotoxic mode of action. In red blood cells infected with malaria parasites, haemoglobin is digested to provide a source of amino acids for the parasites, but the haem remaining is toxic and so it is detoxified by conversion into haemozoin, also known as malaria pigment (Figure 2). Haemozoin is identical to β-haematin which may be prepared in the laboratory from haemin and this process is used as the basis for assays to determine the ability of compounds to inhibit β-haematin (haemozoin) formation (Basilico et al 1998). In common with chloroquine and related quinoline antimalarial drugs, cryptolepine was found to bind to haem and prevent its conversion to haemozoin (Wright et al 2001). The drug–haem complex is believed to be toxic to the parasite. This finding opened up the possibility that it may be possible to prepare analogues of cryptolepine that have potent antiplasmodial properties but without DNA-interacting/cytotoxic properties.

Synthesis of cryptolepine analogues

Unusually, the synthesis of cryptolepine was reported (Fichter & Boehringer 1907) before the alkaloid was isolated as a natural product from *Cryptolepis triangularis* (Clinquart 1929) and later from *C. sanguinolenta* (Gellert et al 1951). Several methods for the preparation of cryptolepine analogues have been described (Bierer et al 1998; Fan & Ablordeppey 2001), but the one that has proved to be most useful to the author is based on the synthesis of quindoline by Holt & Petrow (1947). In this method, *O, N*-acetylindoxyl is refluxed with isatin in strongly alkaline conditions in the absence of oxygen (to avoid the formation of indigo from indoxyl) to give quindoline-11-carboxylic acid (Figure 3). Decarboxylation of the latter by heating in diphenylether gives quindoline, which is then methylated using iodomethane in sulfolane to give cryptolepine (5-methylquindoline) as its hydroiodide salt. If substituted indoxyl and/or isatin derivatives are used as starting materials, a wide range of cryptolepine analogues may be made, although it should be noted that the first step of the synthesis does not work if strongly electron-withdrawing substituents such as $-NO_2$ are present. It is interesting to note that indoxyl and isatin may be derived from the woad plant, *Isatis tinctoria*, which from ancient times has been used as a source of the blue dye, indigo (Clark et al 1993). All of the cryptolepine analogues discussed in this paper were synthesized in the author's laboratory, fully characterized using spectroscopic methods and shown to be pure by means of C, H and N analysis (Wright et al 2001; Onyeibor et al 2005).

Biological evaluation

Compounds were evaluated for in-vitro antiplasmodial activity against chloroquine-sensitive (strain HB3) as well as chloroquine-resistant (strain K1) malaria parasites using the colorimetric parasite lactate dehydrogenase assay (Makler et al 1993). Interactions with DNA were determined using thermodenaturation techniques in which ΔT_m values are measured (Plumbridge & Brown 1979). When DNA is heated, the two strands of the double helix separate at a temperature known as the melting point, but molecules that intercalate into DNA increase the melting point as a result of stabilization. The ΔT_m value is the increase in melting point seen when a compound is added to the DNA solution; values of above 5°C are suggestive of DNA intercalation, while values of 5°C or less suggest non-specific binding to the outside of the DNA helix. Cytotoxicity tests using the MTT assay (Mosmann 1983) were also carried out. The in-vitro antiplasmodial and cytotoxic activities together with in-vivo antimalarial activities of selected compounds are shown in Table 1.

In the first series of test compounds, cryptolepine was found to be active against malaria parasites (IC50 = 0.44 μM against strain K1), was toxic to human colon carcinoma (DLD-1) cells (IC50 = 1.44 μM), and increased the melting point of DNA by 9°C (ΔT_m value) (Wright 2001). Quindoline, quindoline-11-carboxylic acid and quindoline-11-amide were inactive against malaria parasites, suggesting that the *N*-5 methyl group in cryptolepine is essential for antiplasmodial activity. The antiplasmodial activity of three halogenated analogues, 2-bromo-, 7-bromo- and 11-chlorocryptolepine were found to be slightly higher (~2-fold) than that of cryptolepine. Since 2- and 7- bromo-substitution appeared to enhance antiplasmodial activity, 2,7-dibromocryptolepine was synthesized and this was found to be nearly 10-fold more potent than cryptolepine against both chloroquine-sensitive and chloroquine-resistant malaria parasites (IC50 = 0.026 and 0.049 μM, respectively). However, this strategy was not

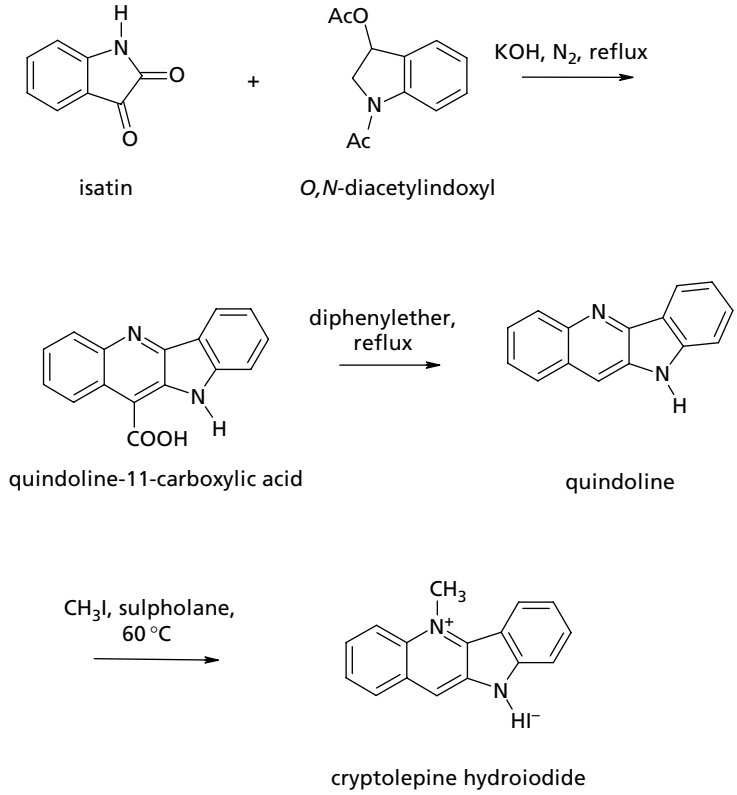

Figure 3 Synthetic scheme for cryptolepine based on the method of Holt & Petrow (1947). Derivatives may be conveniently prepared by using substituted isatin and/or *O, N*-diacetylindoxyl derivatives.

Table 1 In-vitro antiplasmodial and cytotoxic activity together with in-vivo antimalarial activity of cryptolepine and some disubstituted analogues

Compound[a]	**Activity vs *Plasmodium falciparum* (K1) IC50 μM ± s.d. (n)**	**Cytotoxicity vs MAC15a cells; 96 h exposure IC50 μM ± s.d., n = 3**	**Activity vs *Plasmodium berghei* in mice; % suppression of parasitaemia (dose mg kg^{-1}/day i.p.)**
Cryptolepine sulfate	0.44 ± 0.22 (3)	3.95 ± 1.1	Toxic (20)
2-Bromocryptolepine	0.26 ± 0.05 (3)	5.79 ± 0.91	5.9 (25)
7-Bromocryptolepine	0.26 ± 0.21 (3)	Not tested	41.5 (20)
2,7-Dibromocryptolepine	0.049 ± 0.017 (3)	6.04 ± 0.49	91.4 (25)
7-Bromo-2-chlorocryptolepine	0.030 ± 0.002 (3)	1.73 ± 0.12	91.9 (25)
2-Bromo-7-nitrocryptolepine	0.07 ± 0.01 (3)	1.03 ± 0.35	90.8 (25)[b]
2,8-Dichlorcryptolepine	0.045 (2)	1.12 ± 0.27	Toxic (25)

Data compiled from Wright et al (2001) and Onyeibor et al (2005). [a]Tested as hydrochloride salts unless otherwise stated; [b]mice appeared to suffer pain and/or irritation for a few minutes after injection; n = number of separate determinations.

successful for 2- and 11-disubstitution as 2-bromo-11-chlorocryptolepine was more than 10-fold less active than cryptolepine. The 2-bromo-, 7-bromo, 11-chloro- and 2,7-dibromocryptolepine analogues were all found to have ΔT_m values of 4°C or less, suggesting that, unlike cryptolepine, they were not able to intercalate into DNA, although cytotoxicity tests showed that they were not markedly less cytotoxic than cryptolepine. As with cryptolepine, 2-bromo-, 7-bromo-, and 2,7-dibromocryptolepine were found to inhibit β-haematin (haemozoin) formation. When the latter three analogues were tested in-vivo against *P. berghei* in mice using Peters' 4-day suppressive test (Peters et al 1975) in doses of 20 mg kg^{-1}/day, no toxicity was seen, in marked contrast to cryptolepine, although 11-chlorocryptolepine was toxic to the mice. Parasitaemia was reduced only slightly by 2-bromocryptolepine (5.9% suppression compared with untreated infected controls), moderately reduced by 7-bromocryptolepine (42% suppression) and markedly reduced by 2,7-dibromocryptolepine (89% suppression). Further experiments with 2,7-dibromocryptolepine showed that suppression of parasitaemia was dose-dependent with an ED90 value of 21.6 mg per day (Onyeibor et al 2005).

The encouraging in-vivo antimalarial activity of the latter analogue coupled with its apparent lack of toxicity in mice

prompted the synthesis of another series of cryptolepine analogues, including a number of disubstituted derivatives. Several of these were found to have potent in-vitro activity against *P. falciparum*, with IC50 < 0.1 μM (Table 1), and three compounds were assessed for in-vivo antimalarial activity. Two compounds, 7-bromo-2-chlorocryptolepine and 2-bromo-7-nitrocryptolepine, suppressed parasitaemia by 90% at intraperitoneal doses of 25 mg kg^{-1}/day with no observed toxicity, although with the latter the mice appeared to suffer pain and/or irritation for a few minutes following injection. In contrast, 2,8-dichlorocryptolepine was toxic to the mice. Interestingly, the active compounds were more cytotoxic in-vitro than cryptolepine, but unlike cryptolepine they were not toxic in-vivo (Table 1), illustrating that there does not appear to be a correlation between in-vitro and in-vivo toxicity with cryptolepine analogues.

Mechanism of action

The synthesis of analogues with promising in-vivo antimalarial activity has shown that it is possible to enhance the antimalarial activity of cryptolepine, but further work is needed in order to find analogues that cure malaria in mice, that is to suppress parasitaemia by 100% with no recurrence after treatment has ceased. One approach would be to continue to synthesize new analogues with the expectation that more potent compounds would be found, but a more rational approach would clearly be desirable. Such an approach might be possible given a better understanding of the antimalarial mechanism of action of the most active compounds; if we could determine why it is that 2,7-dibromocryptolepine is 10-fold more potent than cryptolepine then this would facilitate the design and synthesis of more potent analogues. The enhanced antiplasmodial activity of 2,7-dibromocryptolepine could possibly be due to better binding to haem, resulting in more effective inhibition of haemozoin formation compared with cryptolepine, but it is also possible that it is better able to accumulate into the parasite food vacuole (the site of haemoglobin digestion), or it could have an additional or different mode of action.

In an attempt to resolve this question, a series of cryptolepine analogues were assessed for their potency as inhibitors of β-haematin formation with a view to exploring any correlation between the ability of compounds to inhibit β-haematin formation and their in-vitro antiplasmodial activity (Onyeibor et al 2005). However, as these compounds are basic, it is likely that, as with chloroquine, they will accumulate in the acidic parasite food vacuole and therefore this must be taken into account. The antimalarial action of chloroquine depends in part on its accumulation into the parasite food vacuole, which occurs because of the acidic pH of the vacuole and the weakly basic nature of chloroquine. At blood pH, chloroquine is only partially ionized so that non-ionized molecules are able to cross the parasite and parasite vacuole membranes, but they then become protonated in the acidic food vacuole, thus preventing their exit from the vacuole (ion trapping). The extent of accumulation into the food vacuole of compounds may be calculated provided that the pK_a values are known. For cryptolepine and 2,7-dibromocryptolepine, the pK_a values are 11.8 and 9.1, respectively, giving accumulation ratios of 79.4 and 77.9, respectively (i.e. the concentration of drug inside the food vacuole will be 79.4/77.9-fold higher than that outside). This suggests that the increased activity of the 2,7-dibromo- analogue is not likely to be due to enhanced accumulation into the parasite as there is little difference in the predicted accumulation ratios of these two compounds. Interestingly, the predicted accumulation ratio for chloroquine is much higher (5896) as this molecule has two protonable nitrogen atoms ($pK_a = 8.55$ and 9.81), whereas in cryptolepine only one (*N*-10) of the two nitrogen atoms is basic (Figure 1b). If the antimalarial mode of action depends on the accumulation of the molecule into the acidic parasite food vacuole, it may be expected that the addition of a second protonable nitrogen to cryptolepine and/or its analogues would enhance their potency against plasmodia and work is currently in progress to test this hypothesis. The activity of 2,7-dibromocryptolepine as an inhibitor of β-haematin formation was found to be very similar to that of cryptolepine and also to that of chloroquine (50% inhibitory activities in terms of equivalents of drug relative to haemin were 1.64, 1.53 and 1.44, respectively). Taken together, the above data suggest that the enhanced antiplasmodial activity of this analogue is not due to increased accumulation into the parasite food vacuole nor to a better ability to inhibit β-haematin formation. When the β-haematin inhibitory activities of a series of cryptolepine analogues were compared with their in-vitro antiplasmodial activities after the latter had been normalized to take into account their predicted accumulation into the parasite food vacuole by pH trapping, it was found that there was no correlation at all between normalized antiplasmodial activity and the ability of compounds to inhibit β-haematin formation (Onyeibor et al 2005). It is possible that potent antiplasmodial disubstituted cryptolepine analogues such as 2,7-dibromocryptolepine may be acting by an additional or different mechanism of action to that of chloroquine, and, for the 2,7-dibromo- analogue at least, this does not appear to involve interactions with DNA. Further studies are currently in progress to elucidate the mode of action of 2,7-dibromocryptolepine with the expectation that this will facilitate the development of new antimalarial cryptolepine analogues.

Conclusion

Although cryptolepine itself is not suitable for clinical development as an antimalarial agent, the synthesis of several analogues with potent in-vitro and promising in-vivo antimalarial activity suggests that further work on cryptolepine analogues would be worthwhile, particularly as there does not appear to be cross-resistance between these compounds and chloroquine. It is intriguing that the antimalarial mode of action of the disubstituted cryptolepine analogues differs from that of the parent, and further elucidation may facilitate the design and development of potent analogues suitable for preclinical evaluation as new antimalarial drugs.

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