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Metabolism of cryptolepine and 2-fluorocryptolepine by aldehyde oxidase

J. Godfrey P. Stell, Richard T. Wheelhouse and Colin W. Wright

Bradford School of Pharmacy, University of Bradford, Bradford, UK

Keywords
aldehyde oxidase; cryptolepine; *Cryptolepis sanguinolenta*; 2-fluorocryptolepine; *Plasmodium falciparum*

Correspondence
Colin W. Wright, Bradford School of Pharmacy, University of Bradford, Bradford, West Yorkshire, BD7 4ER, UK.
E-mail: c.w.wright@bradford.ac.uk

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Abstract

Objectives To investigate the metabolism of cryptolepine and some cryptolepine analogues by aldehyde oxidase, and to assess the implications of the results on the potential of cryptolepine analogues as antimalarial agents.

Methods The products resulting from the oxidation of cryptolepine and 2-fluorocryptolepine by a rabbit liver preparation of aldehyde oxidase were isolated and identified using chromatographic and spectroscopic techniques. The antiplasmodial activity of cryptolepine-11-one was assessed against *Plasmodium falciparum* using the parasite lactate dehydrogenase assay.

Key findings Cryptolepine was oxidized by aldehyde oxidase give cryptolepine-11-one. Although 2-fluorocryptolepine was found to have less affinity for the enzyme than cryptolepine, it was a better substrate for aldehyde oxidase than the parent compound. In contrast, quindoline, the 11-chloro- , 2,7-dibromo- and 2-methoxy analogues of cryptolepine were not readily oxidized. Cryptolepine-11-one was found to be inactive against *P. falciparum in vitro* raising the possibility that the effectiveness of cryptolepine as an antimalarial, may be compromised by metabolism to an inactive metabolite by liver aldehyde oxidase.

Conclusions Cryptolepine and 2-fluorocryptolepine are substrates for aldehyde oxidase. This may have implications for the design and development of cryptolepine analogues as antimalarial agents.

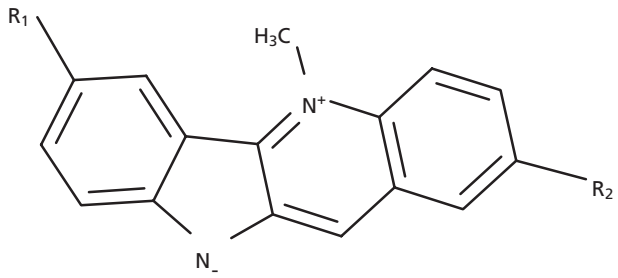
Introduction

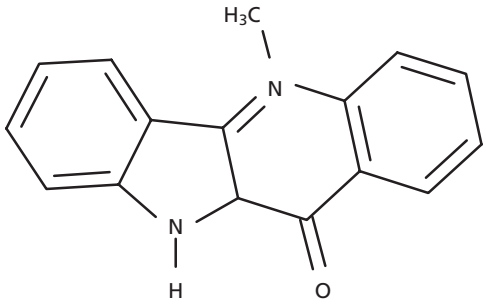
Cryptolepine (Figure 1) is an indoloquinoline alkaloid found in the West African climbing shrub *Cryptolepis sanguinolenta*, the roots of which are used in traditional medicine for the treatment of malaria as well as for various other diseases.[1] Cryptolepine has potent activity against both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* (strain K1) *in vitro*.[2] In mice infected with the murine malaria parasite *Plasmodium berghei*, oral cryptolepine suppressed parasitaemia by 80% at a dose of 50 mg/kg per day, and when given by subcutaneous injection at a dose of 113 mg/kg per day there was no significant reduction in parasitaemia.[3,4] In contrast, a dose of 25 mg/kg per day given by intraperitoneal injection was found to be toxic to the mice.[2] It is possible that the reduced toxicity seen with the oral and subcutaneous routes of administration is due to poor absorption and/or metabolic deactivation of cryptolepine. Since it has been shown that a variety of quinolinium compounds may be oxidized by the molybdenum-containing liver enzyme aldehyde oxidase, it is possible that cryptolepine may also be a substrate for this enzyme.[5,6] In this study we report experiments to determine whether cryptolepine and a number of synthetic analogues were substrates for aldehyde oxidase and have assessed possible implications for the development of cryptolepine analogues as new antimalarial agents. Cryptolepine and cryptolepine analogues are also of interest as lead compounds for the development of anticancer, antitrypanosomal and antileishmanial agents.[7–9] In this paper, we report for the first time in-vitro studies on the metabolism of cryptolepine and some of its analogues by aldehyde oxidase.

Materials and Methods

Cryptolepine derivatives

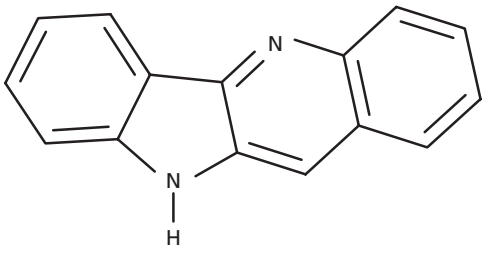
Cryptolepine, its 11-chloro- and 2,7-dibromo- analogues, and quindoline were synthesised as previously reported.[2] The 2-fluorocryptolepine and 2-methoxycryptolepine derivatives were prepared as described by Ablordeppey *et al.*[10]





Cryptolepine-11-one

Cryptolepine,	$R_1 = R_2 = H$
2, 7-Dibromocryptolepine,	$R_1 = R_2 = Br$
2-Fluorocryptolepine,	$R_1 = H$; $R_2 = F$
2-Methoxycryptolepine,	$R_1 = H$; $R_2 = CH_3O$



Quindoline

Figure 1 Structure of cryptolepine and its analogues.

Cryptolepine-11-one was derived from cryptolepine by oxidation with *m*-chloroperbenzoic acid (MCPBA) as described previously.[11]

Spectroscopy

Enzyme reactions were monitored using a Pye Unicam SP8-200 spectrometer using cuvettes with 1 cm light path at 30°C. Mass spectra were run on an AEI MS902 spectrometer equipped with an MSS data acquisition system, version 10 (Mass Spectrometer Services). Accurate mass data for 2-fluorocryptolepine were provided by the EPSRC National Mass Spectrometry Service Centre, Chemistry Department, University of Wales, Swansea, UK. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 600 NMR spectrometer at 600.17 and 150.91 MHz, respectively.

Spectral data for 2-fluorocryptolepine-11-one

^{1}H NMR ($CDCl_3$, δ): 4.40 (3H, s, NCH_3), 7.22 (1H, ddd, $J_{7,8} = 6.7$ Hz, $J_{8,9} = 8.4$ Hz, $J_{6,8} = 1.0$ Hz, 8-*H*), 7.51 (1H, ddd, $J_{6,7} = 8.3$ Hz, $J_{7,8} = 6.7$ Hz, $J_{7,9} = 1.0$ Hz, 7-*H*), 7.57 (1H, br d, $J_{6,7} = 8.3$ Hz, 6-*H*), 7.71 (1H, ddd, $J_{1,3} = 3.3$ Hz, 3-*H*), 8.07–8.10 (2H, m (7 lines), 1-*H*, 4-*H*), 8.42 (1H, br d, $J = 8.4$ Hz, 9-*H*), 12.0 (1H, br s, N*H*). ^{13}C NMR ($CDCl_3$, δ): 36.9 (*C*H_3), 109.7 (C-4), 113.6 (C-6), 116.7 (C9a), 119.3 (C-1), 119.8 (C-8), 120.4 (C-3), 123.9 (C-9), 124.9 (C-11a), 128.1 (C-7), 131.5 (C-5a), 137.8 (C-4a), 139.7 (C-6a), 157.5 (C-2, $^1J_{C,F} = 265.4$ Hz); C-10a and C11 could not be found due to the small sample size. Accurate mass, $[M + H]^+$ found, 267.0927, calculated for $C_{16}H_{12}FN_2O$, 267.0928.

Enzyme preparation

Partially-purified aldehyde oxidase was prepared from the livers of New Zealand White female rabbits (Charles River International, Margate, Kent) to the ammonium sulphate precipitation stage, as described previously.[6] This work was carried out in accordance with University of Bradford Ethics Committee regulations. Small samples of the enzyme preparation were stored in liquid nitrogen until required. Before use, the activity was checked by adding a suitable volume of the enzyme preparation to a solution of dimethylaminocinnamaldehyde (DMAC) (22 μM in phosphate buffer at pH 7)

and monitoring the decrease in absorbance at 398 nm, which occurs on oxidation to the corresponding acid.[12] Enzyme activity was further characterised by examining the effect of menadione, a potent and specific inhibitor of aldehyde oxidase.[13] With the spectrometer set at a fixed wavelength (398 nm), the rate of oxidation of DMAC was recorded for several minutes and then sufficient menadione was introduced into the reaction cuvette to give a final concentration of 2.5×10^{-5} M. The immediate inhibition of absorbance decrease at 398 nm confirmed that aldehyde oxidase in the enzyme preparation was responsible for the oxidation of DMAC.

Enzyme experiments

A solution of cryptolepine or a derivative (20 μM), was placed in the cuvette and the spectrum recorded from 500 to 200 nm. Enzyme solution (25 μl suitably diluted in phosphate buffer at pH 7) was introduced into both test and reference cuvettes, and spectra were repeatedly recorded at regular intervals. Experiments were carried out to determine the ability of cryptolepine and its analogues to compete with DMAC as a substrate for aldehyde oxidase. For this purpose, the spectrometer was set at a fixed wavelength (398 nm) and the oxidation of DMAC (20 μM) by the enzyme was monitored until approximately 30% of the substrate had been consumed. At this point, a solution of the compound under test (25 μl), was added to the test cuvette and the effect on the rate of decrease in absorbance recorded. Further experiments were carried out to determine whether cryptolepine was a substrate for xanthine oxidase. The oxidation of xanthine (15 μM in phosphate buffer, pH 7.0) to uric acid by xanthine oxidase (EC 1.1.3.22 grade IV from bovine milk; Sigma, Poole, UK), was followed by observing the increase in absorbance at 300 nm.[14]

Chromatography

Thin layer chromatography (TLC) was carried out on glass plates coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany) eluted with chloroform/methanol 95 : 5. HPLC was carried out using a Kratos SF770/GM770 HPLC system equipped with a variable wavelength detector and a C_{18} reversed-phase column (250 × 4.6 mm, 5 μM). The eluting solvent was methanol/water 80 : 20 and the flow rate was 1 ml/min.

Isolation of products from enzyme catalysed oxidations

Oxidations of cryptolepine and 2-fluorocryptolepine by aldehyde oxidase were carried out by dissolving 300 μg substrate in 40 ml phosphate buffer (pH 7) and allowing it to stand at room temperature for 24 h. The reaction mixture was then extracted several times with ether and the combined extracts evaporated to dryness. The residue was then subjected to thin layer chromatographic, and spectroscopic analyses.

Antiplasmodial assay of cryptolepine-11-one

Malaria parasites were maintained in human A^+ erythrocytes suspended in RPMI 1640 medium supplemented with A^+ serum and D-glucose according to published methods.[15,16] Cultures containing predominantly early ring stages were used for testing. Cryptolepine-11-one was dissolved in dimethyl sulfoxide (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 microtitre plates in duplicate and infected erythrocytes were added to give a final volume of 100 μl with haematocrit 2.5% and 1% parasitaemia. 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 nitrogen 93%, oxygen 3%, carbon dioxide 4% and incubated at 37°C for 48 h. Parasite growth was assessed by measuring lactate dehydrogenase activity.[17] The reagent used contained the following in each ml: 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, phenazine ethosulphate 0.5 mg. Fifty microlitres of this reagent was added to each well and mixed, and plates were incubated for 15 min at 37°C. Absorbances were read at 550 nm using a Dynatech Laboratories (Billingshurst, UK) MRX microplate reader and% inhibition of growth was calculated by comparison with control values. The values of the dose at which the cell growth rate was inhibited by 50% (IC50) were determined using linear regression analysis (Microsoft Excel).

Statistical analysis

For the determination of K_m (Michaelis-Menten constant) values, a minimum of three separate determinations were carried out for each of the six substrate concentrations used. The values for 1/v (reciprocal of the reaction rate) and $-1/K_m$ were determined by linear regression analysis and standard deviations were applied to each data point (Microsoft Excel). Three separate experiments were carried out to determine the effect of cryptolepine on the oxidation of DMAC by aldehyde oxidase and the standard deviation was calculated.

Results and Discussion

Oxidation of cryptolepine by aldehyde oxidase

When the rabbit liver aldehyde oxidase preparation was added to a solution of cryptolepine, spectrophotometric

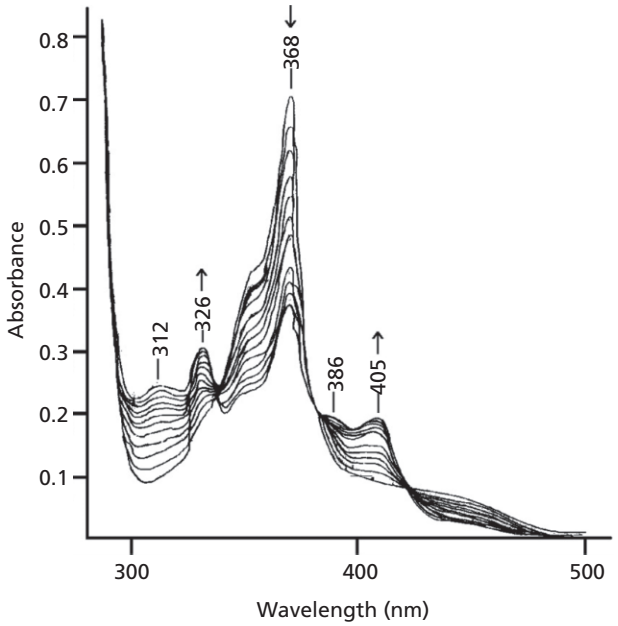


Figure 2 Oxidation of cryptolepine by partially–purified rabbit liver aldehyde oxidase. The test cuvette contained 1.5 ml cryptolepine hydrochloride solution (20 μM) and 75 μl enzyme preparation in 1.4 ml phosphate buffer at pH 7.0 and the reference cuvette contained 75 μl enzyme in 2.9 ml phosphate buffer at pH 7.0. The reaction at 30 °C was monitored by scanning from 200 to 500 nm every 20 min. The reduction in amplitude of the substrate peak at 368 nm and the appearance of peaks at 326 and 405 nm indicates the formation of cryptolepine-11-one.

monitoring showed a steady reduction of the characteristic peak for the substrate at 368 nm and the concomitant appearance of new peaks at 326 and 405 nm (Figure 2) indicating the oxidation of cryptolepine to cryptolepine-11-one. After 1.5 h most of the cryptolepine (20 μM) had been transformed. However, the oxidation of cryptolepine was found to be ~40-fold slower than that of DMAC. From a plot of 1/v against 1/[S] (Figure 3), the K_m value for the oxidation of cryptolepine was found to be 7.04 μM, which was comparable with values previously published for a number of quinolinium compounds and the v_{max} was found to be 0.25 μM/min.[5] The addition of cryptolepine (36 μM) to a reaction mixture of DMAC plus rabbit enzyme inhibited the oxidation of DMAC by 47 ± 0.17% ($n = 3$), suggesting that, under the conditions of this experiment, cryptolepine may have acted as a competitive substrate for the enzyme (to confirm this, kinetic experiments need to be carried out but unfortunately it was not possible to do this as part of this study). Chromatographic analysis of the product obtained following the oxidation of cryptolepine with aldehyde oxidase showed that it was less polar than cryptolepine (which did not migrate) and identical to synthetic cryptolepine-11-one (Figure 1); (TLC, $R_f = 0.5$, under UV light, quenching at 254 nM, purple fluorescence at 365 nM; HPLC analysis, retention time = 5 min).

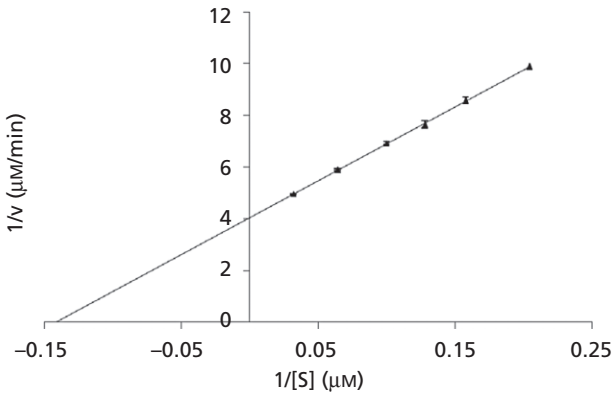


Figure 3 Double reciprocal plot showing the oxidation of cryptolepine by rabbit liver aldehyde oxidase preparation. Each point is the mean of three separate experiments and standard deviations are shown. $K_m = 7.04$ μM.

The UV and mass spectra of the product were identical to those of synthetic cryptolepine-11-one and to published values (UV, CH_3OH, λ_{max} 217, 233, 272, 311, 326, 368(sh), 386, 405 nm; EIMS *m/z* 248 [M^+]).[11] Cryptolepine-11-one has also been produced as an oxidation artefact that may arise during the extraction of cryptolepine from *C. sanguinolenta* and it is tautomeric with 11-hydroxycryptolepine, previously reported to be a constituent of *C. sanguinolenta*.[11,18] Human liver also contains the enzyme xanthine oxidase which, like aldehyde oxidase, contains molybdenum and the two enzymes show some overlap with respect to substrate specificity, although charged heterocycles have been shown to be substrates for xanthine oxidase at high pH values.[19] To confirm that the activities reported above were not due to xanthine oxidase, experiments were carried out in which cryptolepine was incubated with xanthine oxidase under the same conditions as those used with the aldehyde oxidase preparations. No change in the spectrum of cryptolepine was observed after 1 h and it was shown that the conversion of xanthine to uric acid by xanthine oxidase was not affected when cryptolepine was added to the reaction mixture. Taken together, the above results clearly demonstrated that cryptolepine was a substrate for aldehyde oxidase, but not for xanthine oxidase.

Cryptolepine analogues as substrates for aldehyde oxidase

Under the same conditions, 2-fluorocryptolepine was found to be readily oxidised by aldehyde oxidase (Figure 4), but at a 4-fold faster rate than cryptolepine ($v_{max} = 1.05$ and 0.25 μM/min, respectively) and its K_m value was 20.2 μM (Figure 5), indicating that it had less affinity for the enzyme than cryptolepine ($K_m = 0.74$ μM). The product was isolated as for cryptolepine-11-one above and the mass spectrum showed

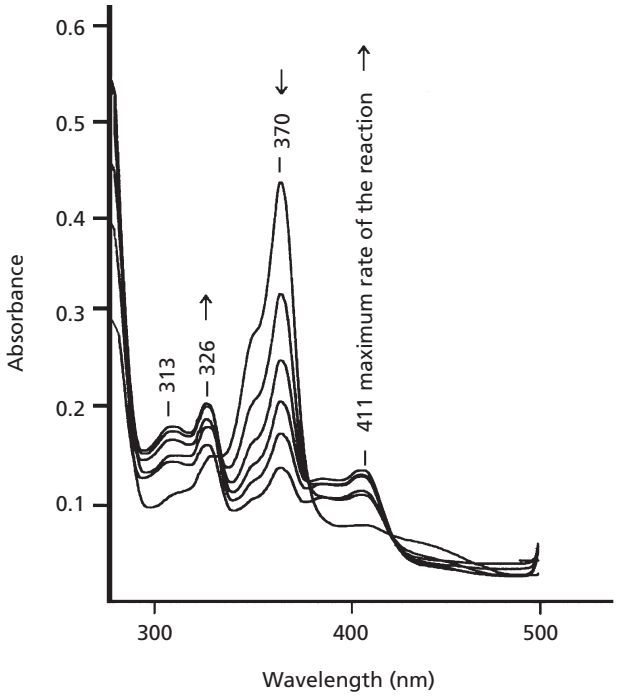


Figure 4 Oxidation of 2-fluorocryptolepine by partially–purified rabbit liver aldehyde oxidase. The test cuvette contained 1.5 ml 2-fluorocryptolepine hydrochloride solution (20 μM) and 75 μl enzyme preparation in 1.4 ml phosphate buffer at pH 7.0 and the reference cuvette contained 75 μl enzyme 2.9 ml. The reaction at 30°C was monitored by scanning from 200 to 500 nm every 7 min for five scans and then after 10 min for the final scan. The reduction in amplitude of the substrate peak at 370 nm and the appearance of peaks at 326 and 411 nm indicates the formation of 2- fluorocryptolepine-11-one.

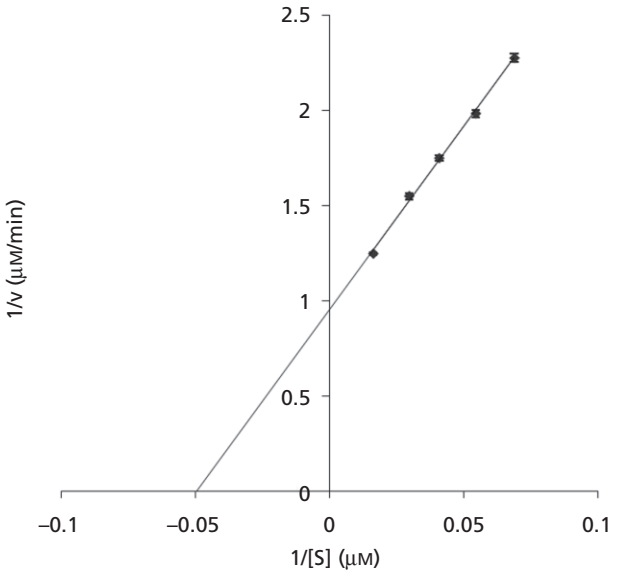


Figure 5 Double reciprocal plot showing the oxidation of 2-fluorocryptolepine by rabbit liver aldehyde oxidase preparation. Each point is the mean of three separate experiments and standard deviations are shown. $K_m = 20.2$ μM.

peaks at *m/z* 250 (base peak) and at 235 ($M^+ - 15$), consistent with 2-fluorocryptolepine-11-one and this was further confirmed by accurate mass measurement (measured, 267.0927, calculated for $C_{16}H_{11}FN_2O + H$, 267.0928). The identity of the product was conclusively shown to be 2-fluorocryptolepine-11-one using 2-D NMR experiments; ^{1}H-^{1}H COSY, HMQC with ^{13}C-decoupling and gradient-enhanced HMBC were employed to determine ^{1}H-^{1}H, 1-bond C-H and 3-bond C-H correlations, respectively. On TLC the product showed a quenching at 254 nm and purple fluorescence at 364 nm ($R_f = 0.55$) and its retention time on HPLC was 5.5 min. The UV spectrum resembled that of cryptolepine-11-one and showed peaks at 234, 276, 313, 326, 390 and 411 nm. In contrast to cryptolepine, quindoline (5-desmethylcryptolepine) did not appear to be a substrate for cryptolepine. Similarly, and not surprisingly since the position of oxidation was blocked, 11-chlorocryptolepine was not a substrate for aldehyde oxidase. Two other analogues, 2, 7-dibromocryptolepine and 2-methoxycryptolepine did not appear to be oxidised by aldehyde oxidase but slight changes to their spectra were seen on prolonged incubation with the enzyme, and the TLC of an ether extract of the reaction mixture containing 2, 7-dibromocryptolepine showed two very feint fluorescent spots under UV light at 365 nm that were less polar than the substrate (which did not migrate), and of similar R_f values to that of cryptolepine-11-one. It was possible that the latter compounds were very slowly metabolised by aldehyde oxidase or possibly by other enzymes present, but further work will be needed to investigate this.

Implications for the development of cryptolepine analogues as antimalarial agents

As aldehyde oxidase is present in human liver, it is possible that metabolism of cryptolepine to cryptolepine-11-one could occur in humans. Since the latter has been shown to be inactive against *P. berghei* in mice, oxidation of cryptolepine or of some cryptolepine analogues could result in diminished *in vivo* activity of compounds that have potent *in vitro* antiplasmodial activity. In our laboratory, cryptolepine-11-one was found to be devoid of activity against *P. falciparum* (strain K1) *in vitro* (IC50 > 100 μM in two experiments), although its tautomer 11-hydroxycryptolepine was reported to have in-vitro activity similar to that of cryptolepine.[20] With respect to the development of new antimalarial agents, 2,7-dibromocryptolepine is one of the most promising cryptolepine analogues as it has potent in-vitro antiplasmodial activity as well as activity against malaria parasites *in vivo*.[2,21] It is interesting in this context to note that the latter analogue did not appear to be oxidised by aldehyde oxidase as the above

results suggested that 11-oxidation may lead to a marked reduction in antiplasmodial activity *in vitro*. In a preliminary study in rats, orally administered cryptolepine (10 mg/kg bodyweight) was found to be slowly absorbed into the blood with maximum serum concentrations (0.42 ± 0.08 μM) attained after 5 h and it was still detectable 10 h after administration.[22] However, no metabolites of cryptolepine were detected. These results suggested that cryptolepine metabolism in the rat, if it occurs, was not rapid. The metabolic fate of cryptolepine in man is unknown, but if it is metabolised this may have implications for the use of decoctions of *C. sanguinolenta* roots for the treatment of malaria in West Africa and studies to investigate this are needed.

Conclusions

Cryptolepine was oxidised by rabbit liver aldehyde oxidase to its 11-one analogue and 2-fluorocryptolepine was similarly metabolised but at a faster rate ($v_{max} = 0.25$ and 1.05 μM/min, respectively), although the affinity of cryptolepine for the enzyme was greater than that for 2-fluorocryptolepine ($K_m = 0.74$ and 20.2 μM, respectively). Cryptolepine was not a substrate for xanthine oxidase. In contrast, quindoline, the 11-chloro-, 2,7-dibromo- and 2-methoxy- analogues of cryptolepine did not appear to be substrates for aldehyde oxidase, but the latter cryptolepine analogues may undergo very slow metabolism. Unlike cryptolepine, cryptolepine-11-one was inactive against malaria parasites *in vitro* and it is possible that cryptolepine and some analogues could be inactivated by oxidation *in vivo*. This finding could have implications for the efficacy of *Cryptolepis sanguinolenta* root decoctions which are used in West Africa for the treatment of malaria. The results of this study have shown that 2- or 11- substitution may block the action of aldehyde oxidase and this may be helpful in the design of antimalarial cryptolepine analogues.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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