Antiamœbic and Antiplasmodial Activities of Alkaloids Isolated from *Strychnos usambarensis*

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Abstract

Seven alkaloids isolated from *Strychnos usambarensis* have been assessed for *in vitro* activities against *Entamœba histolytica* and *Plasmodium falciparum* and for *in vivo* activity against *Plasmodium berghei* in mice. Strychnopentamine and 3',4'-dihydrousambarensine were highly active against *P. falciparum in vitro*, but were inactive and non-toxic against *P. berghei in vivo*. Usambarensine, usambarine, and 18,19-dihydrousambarine were highly active against *E. histolytica in vitro*, but were less active against *P. falciparum in vitro*. N_b-Methylusambarensine was less active against both protozoa than was usambarensine, and akagerine possessed little antiprotozoal activity. Structure-activity relationships are discussed in the context of the reported cytotoxic and pharmacological properties of these alkaloids.

Key words

Strychnos usambarensis, antiamœbic activity, antiplasmodial activity, *Entamœba histolytica*, *Plasmodium falciparum*, *Plasmodium berghei*, bisindole monoterpene alkaloids.

Introduction

Strychnos usambarensis (Loganiaceae) is a tree used traditionally by people of the Banyambo tribe who live along the Akagera river on the border between Rwanda and Tanzania. The leaves and roots are used as the main ingredient of an arrow poison (1). This species contains a number of bisindole monoterpene alkaloids (2), one of which, 3',4'-dihydrousambarensine has been reported to possess activity against the pathogenic protozoa *Entamœba histolytica* and *Trichomonas vaginalis in vitro* (3). Protozoa are responsible for a number of diseases for which adequate drug therapy is not available, of which amœbiasis and malaria are among the most important (4). In this paper we report the results of studies in which a number of alkaloids isolated from *S. usambarensis* were tested against the malaria parasites *P. falciparum (in vitro)*, *P. berghei (in vivo)*, and against the pathogenic amœba *E. histolytica (in vitro)*.

Materials and Methods

Isolation of alkaloids

Alkaloids were isolated from *S. usambarensis* and identified as previously reported (2, 5, 6).

In vitro testing against Entamœba histolytica

Activity against *E. histolytica in vitro* was assessed using a recently developed microplate method (7). Ethanol (50 µl) was added to samples of alkaloids ($\approx$ 1 mg), followed by enough culture medium to obtain concentrations of 1 mg/ml. Samples were dissolved or suspended by mild sonication in a Sonicleaner bath (Ultrasonics Ltd.) for a few minutes and then further diluted with medium to concentrations of 0.1 mg/ml or less as required. The maximum concentrations of ethanol in the test did not exceed 0.25 %, at which level no inhibition of amœbal growth occurred. Two-fold serial dilutions were made in the wells of 96-well microtitre plates (Linbro; Flow Laboratories, Inc.) in 170 µl of medium. Each plate included emetine as a standard amœbicidal drug, control wells (culture medium plus amœbae), and a blank (culture medium only). A suspension of amœbae was prepared from a confluent culture by pouring off the medium, adding 2 ml of fresh medium, and chilling the culture on ice to detach the organisms from the side of the flask. The number of amœbae per ml was estimated with a haemocytometer, and trypan blue exclusion was used to confirm viability. Fresh culture medium was added to dilute the suspension to 10^5 organisms/ml, and 170 µl of this suspension was added to the test and control wells in the plates so that the wells were completely filled (total volume, 340 µl). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth took place in control wells. Plates were sealed with expanded polystyrene ($\approx$ 0.5 cm thick), secured with tape, placed in a modular incubating chamber (Flow labs), and gassed for 10 minutes with nitrogen before incubation at 37 °C for 72 h.

Assessment of antiamœbic activity

After incubation, the growth of amœbae in the plates was checked with a low power microscope. The culture medium was removed by inverting the plates and shaking them gently. Plates were then immediately washed once in 0.9 % sodium chloride solution at 37 °C. This procedure was completed quickly,

and the plates were not allowed to cool in order to prevent the detachment of amœbae. The plates were allowed to dry at room temperature, and the amœbae were fixed with methanol and, when dry, stained with 0.5 % aqueous eosin for 15 minutes. Stained plates were washed once with tap water and then twice with distilled water and allowed to dry. A 200 µl portion of 0.1 N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader (MR-700; Dynatech Laboratories, Inc.). The % inhibition of amœbal growth was calculated from the optical densities of the control and test wells and was plotted against the logarithm of the dose of the drug or extract being tested. Linear regression analysis was used to determine the best-fitting straight line from which the IC_{50} value was found.

In vitro testing against Plasmodium falciparum

This was carried out as described previously, (8) using a method which is based on that of Desjardins et al. (9). Cultures of *P. falciparum*, (chloroquine resistant strain K-1), were maintained in human erythrocytes using a method developed by Trager and Jensen, (10) and modified by Fairlamb et al. (11). Ethanol, (50 µl), was added to samples of pure alkaloids which were then dissolved or suspended in RPMI 1640 medium with the aid of mild sonication in a Sonicleaner bath (Ultrasonics Ltd.), and further diluted as required in medium. The ethanol concentration for tested dilutions was not greater than 0.1 %. 50 µl aliquots of diluted extracts were placed into the wells of 96-well microtitre plates or serial dilutions were made in the plates to give 10, 4 or 2-fold dilutions. All tests were performed in duplicate. To each well was added 50 µl of a suspension of human red blood cells in medium (O+ve, 5 % haematocrit) with 1 % parasitaemia. Dilutions to prepare 1 % parasitaemia were made using uninfected washed red blood cells. After incubation in a 3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen atmosphere for 18–24 h at 37 °C, 5 µl [³H]-hypoxanthine (40 µCi/ml, Amersham) were added to each well and incubation continued at 37 °C for a further 18–24 h. Each test included control, uninfected and infected blood cells, and chloroquine as an internal standard.

Harvesting

Red blood cells were washed out from the wells with normal saline using a Titertek cell harvester, (Flow Laboratories), through a glass fibre filter moistened with saline. Blood cells were lysed by flushing with distilled water for 20 sec and then saline was passed through for 20 sec to remove remaining traces of haemoglobin. After further washing with distilled water and saline (20 sec each), the filter was dried and the glass fibre disc for each well was pushed out into scintillation vials (4 ml, polypropylene). 4 ml of scintillation fluid (Packard toluene scintillator) were added and the vials were counted for 2 min at about 30 % efficiency. The % inhibition of incorporation of [³H]-hypoxanthine at each dose level was calculated and linear regression analysis used to determine the best fitting straight line from which IC_{50} values were determined.

In vivo testing for antimalarial activity

The 4-day suppressive test against *P. berghei* infection in mice described by Peters et al. (12) was used. This work was carried out as previously described (13). Mice were inoculated with *P. berghei* strain N on the first day of the experiment and dosed daily for four consecutive days with the extract or drug under test. Five dose levels were used. On the fifth day of the test a blood smear was taken and the animals were sacrificed. ED_{50} and/or ED_{90} values were computed by comparing the parasitaemias present in infected controls with those of test animals. Deaths that occur during the test are considered to be due to the toxicity of the extract or drug.

Results

The alkaloids tested can be conveniently divided into four groups according to their chemical structures as shown in Fig. 1 (respective activities are given in Table 1).

a. Usambarensine type

The structures and *in vitro* activities of usambarensine and two derivatives are shown in Fig. 1 and Table 1. Usambarensine was found to possess marked activity against *E. histolytica*, (IC_{50} = 0.49 µg/ml), which is three times that of emetine (IC_{50} = 1.70 µg/ml). However, the 3',4'-dihydro and the N_b-methyl derivatives were 4 and 8 times less active, respectively, than usambarensine against *E. histolytica*. In contrast, 3',4'-dihydrousambarensine was 30 times more active than usambarensine and some 200 times more active than the N_b-methyl derivative against *P. falciparum in vitro*.

b. Usambarine type

As shown, both usambarine and 18,19-dihydrousambarine oxalate were highly active against *E. histolytica in vitro*, (three times more potent than emetine), but, in contrast, were relatively inactive against *P. falciparum in vitro*, their respective IC_{50} values being 5 and 9 times more than that of chloroquine (IC_{50} = 0.20 µg/ml).
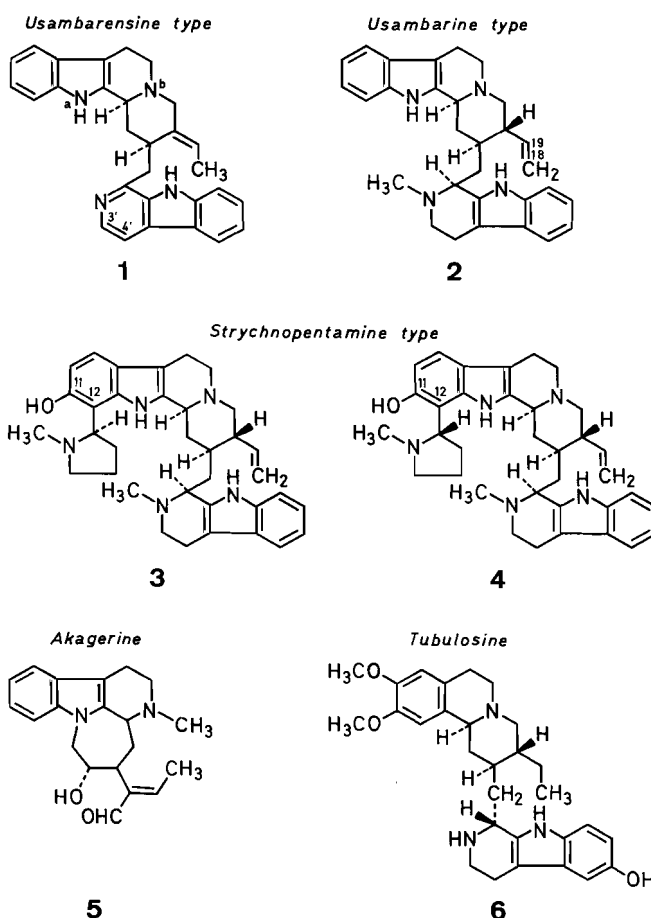

Fig. 1 Structures of the *Strychnos* alkaloids **1–5** and tubulosine **(6)**.

Alkaloid	E. histolytica IC_{50}(95% C.I.) µg/ml	P. falciparum IC_{50}(95% C.I.) µg/ml
a. Usambarensine type		
Usambarensine (1)	0.49 (0.296–0.825)	0.38 (0.261–0.549)
3',4'-Dihydrousambarensine	2.18 (1.74–2.72)	0.01 (0.008–0.019)
N_b-methylusambarensine chloride	4.12 (2.53–6.71)	2.39 (1.43–4.99)
b. Usambarine type		
Usambarine (2)	0.46 (0.189–1.13)	≈ 1.85
18,19-Dihydrousambarine oxalate	0.65 (0.560–0.763)	1.07 (0.86–1.33)
c. Strychnopentamine type		
Strychnopentamine (3)	7.70 (4.12–14.4)	0.09 (0.051–0.180)
Strychnopentamine methanesulphonate	10.10 (6.99–17.6)	0.09 (0.057–0.143)
Isostrychnopentamine base (4)	10.70 (7.69–14.9)	Not tested
d. Akagerine and Tubulosine		
Akagerine (5)	17.1 (7.59–38.4)	6.98 (3.80–12.77)
Tubulosine (6)	Not tested	0.020 (0.019–0.021)

Table 1   *In vitro* activities of the alkaloids against *E. histolytica* and against *P. falciparum*.

c. Strychnopentamine type

Strychnopentamine was found to have only weak activity against *E. histolytica in vitro*, but was about twice as active as chloroquine against *P. falciparum in vitro*. The methanesulphonate of this alkaloid was similarly active. Isostrychnopentamine was also weakly active against *E. histolytica in vitro* but has not been tested against *P. falciparum*.

d. Akagerine

This alkaloid was found to possess little activity against either protozoon.

In vivo activity against Plasmodium berghei

Strychnopentamine and dihydrousambarensine were tested in mice as these were the most active alkaloids against *P. falciparum in vitro*. Both were tested, orally and by subcutaneous injection, but neither showed activity at the highest dose tested, (30 mg/kg), and no deaths occurred.

Discussion

With the exception of akagerine, the alkaloids tested do not differ markedly in their structures. However, it is clear from the results that in some cases a minor structural change results in significantly different biological activities. This is well illustrated with reference to the usambarensine derivatives in which the 3',4'-dihydro compound is more active than the parent against *P. falciparum in vitro*, but the N_b-methyl derivative is markedly less active against the same parasite. On the other hand, usambarensine and usambarine have very similar activities against *E. histolytica in vitro* although the former is about 4 times more active against *P. falciparum in vitro*. Interestingly, the activities of these alkaloids against *E. histolytica* do not parallel their activities against *P. falciparum*, and minor changes in structure may increase activity against one of the organisms and reduce it against the other. For example, usambarine is highly active against *E.*

histolytica in vitro and weakly active against *P. falciparum in vitro*, but strychnopentamine which has a C-11 hydroxy and an *N*-methylpyrrolidine group at C-12, has low antiamœbic but high antiplasmodial activity. These findings could possibly reflect differences between the two protozoa with respect to the uptake or the modes of action of these alkaloids. If these compounds have a similar mode of action in both parasites there may be subtle differences in the sites at which they act.

The lack of activity of strychnopentamine and dihydrousambarensine *in vivo* against *P. berghei* is disappointing. Presumably this is due to the pharmacokinetics and/or metabolism of these compounds *in vivo*, and may be worthy of investigation since such data could lead to the development of compounds which are effective but relatively non-toxic. This is illustrated with reference to the amœbicidal alkaloid emetine and its dehydro-derivative. Dehydroemetine has similar activity to emetine *in vitro*, but is less toxic *in vivo* because it is cleared more rapidly from the body (14).

As mentioned above, *S. usambarensis* is used in the preparation of an arrow poison suggesting that highly toxic constituents are present. Previous investigations have shown that the root bark possessed neuromuscular blocking properties (15). This is mainly due to three alkaloids, C-curarine, dihydrotoxiferine, C-calebassine and, to a lesser extent, to afrocurarine (16). These diquaternary ammonium compounds work as true competitive curare-like agents since their inhibitory action is antagonized by eserine (physostigmine). In contrast, usambarensine, its N_b-methyl derivative, and 3',4'-dihydrousambarensine are not active in the tests studied. Nevertheless, usambarensine at 3×10^{-6} M antagonises carbachol-induced contractions in rat intestinal muscle (muscarinic receptors), but is less potent than atropine (16). In contrast, dihydrousambarensine and N_b-methylusambarensine are devoid of significant effects on the rat intestine. Strychnopentamine and usambarine have not been tested on intestinal smooth muscle nor on the skeletal muscle neuromuscular junction. Akagerine and its derivatives were found to have convulsant activity, but were 100 times less potent than strychnine (3).

Obviously, it would not be possible to use any of the *Strychnos* alkaloids clinically unless they were devoid of important effects on the nervous system. The seven alkaloids tested in this study are much less toxic than strychnine which has an LD_{50} value of 0.5 mg/kg (*i.v.* in mice), and the curarizing diquaternary alkaloids which have LD_{50} values in the region of 50 µg/kg (*i.v.* in mice), (3). Indeed, experiments carried out on mice with subcutaneous or intraperitoneal administration at a single dose of 50 mg/kg show that usambarensine, strychnopentamine, and derivatives do not exert lethal effects at this dose, but akagerine causes convulsions (Angenot and Tits, unpublished results). More studies will be needed in order to determine whether the antiprotozoal activities of these alkaloids are related to their pharmacological actions in mammals.

Strychnos alkaloids have also been shown to be toxic to a number of tumour cell lines (17). All of the alkaloids tested above have marked cytotoxic activities with the exception of N_b-methylusambarensine, which, (with the exception of akagerine), was the only alkaloid tested which did not show potent activity against one of the protozoa tested. This suggests that there may be some correlation between cytotoxic and antiprotozoal effects, but it is interesting to note that there are significant differences between the alkaloids tested. For example, strychnopentamine and 3',4'-dihydrousambarensine are highly active against *P. falciparum in vitro* and were found to be the most active to tumour cells (17), whereas usambarine and usambarensine are highly active against *E. histolytica in vitro* but have less cytotoxicity. This indicates that the antiprotozoal and cytotoxic effects of these alkaloids do not always parallel each other.

At present, nothing is known of the mode of action of these alkaloids, but since the emetine alkaloid, tubulosine (6) is similar in structure to usambarine, it is possible that their modes of action may be similar Tubulosine also has potent activity against *P. falciparum in vitro* (IC_{50} = 0.02 µg/ml) (18), and has been shown to have cytotoxic activity against tumour cell lines (19). This alkaloid is a potent inhibitor of protein synthesis in mammalian cells and acts by blocking peptidyl translocation (19). Moreover, some studies have concluded that a requirement for the amoebicidal activity of emetine-like alkaloids is that the molecule must be able to adopt a planar conformation i.e. the two aromatic ring systems must be capable of lying in the same plane (20). Crystallographic studies on usambarensine and strychnopentamine have revealed that these molecules do not have this planar conformation in the crystal state (21, 22); measurements have shown that although the two aromatic ring systems are parallel with each other, they do not lie in the same plane. Unfortunately, crystallographic data for the other alkaloids are not available at present. In addition, usambarensine, dihydrousambarensine, and usambarine do not have electron-withdrawing substituents on the aromatic rings which are considered to be important for biological activity (20). These observations suggest that the *Strychnos* alkaloids may have a different mode of action from that of emetine. If so, the question arises as to whether tubulosine is emetine-like or usambarensine-like in its action.

Conclusions

This study has shown that some of the *Strychnos* alkaloids tested have significant *in vitro* antiprotozoal activities against *E. histolytica* and against *P. falciparum*. Relatively minor structural differences markedly effect these, and the activities against the two protozoa do not parallel each other. The absence of activity against *P. berghei in vivo* is disappointing, but further work to determine the reasons for this may be worthwhile. In addition, more studies are needed on the relationships between the cytotoxic and pharmacological effects of these alkaloids and their antiprotozoal activities.

Acknowledgements

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