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In vivo Antimalarial and Antitrypanosomal Activity of Strychnogucine B, a Bisindole Alkaloid from *Strychnos icaja* *

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ABSTRACT

Strychnogucine B is a bisindole alkaloid previously isolated from *Strychnos icaja* that possesses promising *in vitro* antiplasmodial properties. This compound was synthesized in four steps from (−)-strychnine. As no acute toxicity was observed at the highest tested cumulative dose of 60 mg/kg, its *in vivo* antimalarial activity was determined intraperitoneally at 30 mg/kg/d in a *Plasmodium berghei* murine model. In the Peters's 4-d suppressive test, this alkaloid suppressed the parasitaemia by almost 36% on day 5 and 60% on day 7 compared to vehicle-treated mice. In addition to this interesting antimalarial activity, it showed moderate *in vitro* antitrypanosomal activity but no *in vivo* activity in an acute *Trypanosoma brucei* model. It was also inactive *in vitro* on *Leishmania mexicana* promastigotes. This highlights its selective antimalarial efficacy and leads to further investigation to assess its potential as new antimalarial lead compound.

Introduction

According to the World Malaria Report [1], 445 000 malaria deaths were reported worldwide in 2016 with about 200 million people affected each year. Malaria is caused by a protozoan parasite, *Plasmodium* sp., and transmitted by female *Anopheles* mosquitoes. Parasite resistance toward available medicines such as chloroquine, mefloquine, but also artemisinin derivatives is gradually increasing, inducing malaria reemergence despite numerous control efforts. Natural products are a validated source of potential new antimalarial drugs to overcome this key threat in the fight against this tropical disease. Indeed, natural biosynthesis produces a large variety of bioactive secondary metabolites with complex structures [2–4], with quinine and artemisinin remaining the best examples to date. Nevertheless, one main limitation of these natural compounds is their supply and efficient isolation in large amount. Semi- or total synthesis based on promising scaffolds represents a prime solution to this shortcoming [5].

* Dedicated to Professor Dr. Robert Verpoorte in recognition of his outstanding contribution to natural product research.

** These authors contributed equally to the work.

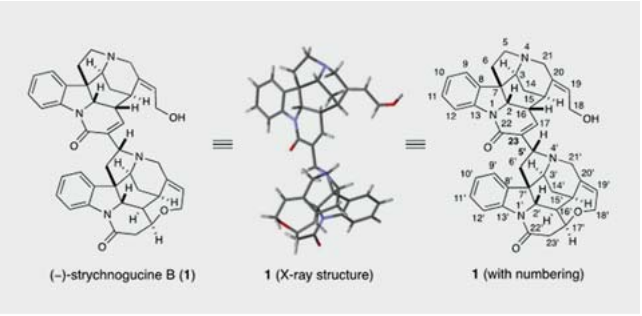
Strychnos icaja Baill. (Loganiaceae) is used as an ordeal poison in the Democratic Republic of Congo and also to treat malaria [6]. The study of their roots and other *Strychnos* species led to the identification of several new antiplasmodial alkaloids, giving information to validate the ethnopharmacological use. Among them, strychnogucine B (▶ **Fig. 1**), a tertiary quasi-symmetric bisindole alkaloid, showed one of the highest selective *in vitro* antiplasmodial activities, especially against a chloroquine-resistant strain (▶ **Table 1**) [7–9]. Nevertheless, because of the low isolated yields of alkaloids from *S. icaja*, only isosungucine was evaluated *in vivo* on the *Plasmodium vinckei petteri* strain and showed good effects [10]. Viewing these results, it seems interesting to evaluate the *in vivo* antimalarial activity of strychnogucine B, the most active (IC_{50} 2–3 times lower than isosungucine) and selective alkaloid identified in *S. icaja*. Furthermore, it showed an insignificant interaction with the glycine receptor, in comparison to strychnine, which obviates the issue of strychnine-like poisoning [11].

The objective of this work was to test strychnogucine B *in vivo* to assess its potential as a new antimalarial lead compound for further drug development and also obtain some safety data. This highly active compound was also evaluated on other parasites, *Leishmania mexicana* and *Trypanosoma brucei*, to define its antiparasitic activity/selectivity. As its isolation furnished very low yields, a concise semisynthetic method in four steps from (−)-strychnine was used to yield enough material [12].

Results and Discussion

The acute toxicity of strychnogucine B given i. p. was evaluated according to the highest tolerated dose model [13]. Body weight, hematocrit, general aspect, and behavior remained stable. Moreover, main organ weight was unaltered compared to control (▶ **Fig. 2**), and macroscopic observations did not show any particular signs of toxicity. Overall, this alkaloid did not induce any acute toxic symptoms at a total i. p. cumulative dose of 60 mg/kg, meaning that the efficacy test can be performed at a maximum dose of 30 mg/kg without any risk of acute toxicity.

Given this and its selective *in vitro* antiplasmodial activity (IC_{50} < 1 µM and selectivity index [SI] from 25 to 180, ▶ **Table 1**) [9], strychnogucine B was evaluated *in vivo* on *Plasmodium berghei* and its antimalarial activity is presented in ▶ **Fig. 3**. The classical 4-d suppressive test of Peters [14] was performed, which is the procedure recommended by the World Health Organization as the first-line screen for *in vivo* testing of potential antimalarial leads. In this test, strychnogucine B was administrated i. p. for four successive days to malaria-infected mice. This administration mode was chosen in a first time to allow comparison with other tested bisindole alkaloids and to secure substantial bioavailability and action speed. At 30 mg/kg/d, parasitaemia was reduced on day 5 by 35.8% and on day 7 by 60.3% (with a p-value of 0.002 versus negative control) and survival was increased by at least 24 h for 80% of the mice. The parasitaemia in the chloroquine group on day 7 was reduced by 67% compared to the control, a similar very significant inhibition percentage than the tested compound. Nevertheless, at day 10, only 50% of the mice treated with strychnogucine B were still alive (0% in negative control and 100% in chloroquine group), implying that the dose should be adjusted.



▶ **Fig. 1** Chemical structure of (−)-strychnogucine B.

Therefore, our results show that strychnogucine B possesses interesting antimalarial activity, both *in vitro* against different strains and *in vivo* against a chloroquine-sensitive murine strain. For its parent compound, isosungucine, previously tested on a *P. vinckei* model, parasitaemia reduction was observed earlier but apparently on a shorter period (75% and 47% on days 2 and 4, respectively) with only one mouse of six still alive at day 7. This can be related to activity (IC_{50} two times higher = 1.32 µM *in vitro* on FCA 20), bioavailability, or metabolization differences but also *in vivo* model [10] and has to be explored in the future. Overall, these results enhance the drug development potential of strychnogucine B as it fills the criteria for lead candidate [15] but also strengthens the validation of *S. icaja* use in traditional medicine.

In the literature, many other indole alkaloids were identified in *Strychnos* species and other natural sources with strong antiplasmodial activity (IC_{50} < 2 µM) and some were tested *in vivo* [16–19]. Among bisindole alkaloids, isostrychnopentamine (usambarine-type) was more active on the chloroquine-sensitive FCA 20 strain but less on the resistant W2 one than strychnogucine B (IC_{50} = 0.12 and 0.15 µM compared to 0.62 and 0.09 µM, respectively). It also showed activity on all parasite stages with a preferential activity on the early ring stage at low concentration and did not accumulate in the digestive vacuole. Moreover, at day 5, an i. p. 30 mg/kg/d treatment significantly inhibits parasitaemia of 46% and 61.8% in *P. berghei* or *P. vinckei petteri*-infected mice, respectively [20]. This inhibition difference can be related to the different numbers of daughter parasites in mature schizonts, lower for *P. vinckei*, that will influence virulence. In comparison with strychnogucine B, isostrychnopentamine showed a higher inhibition at day 5, but parasitaemia at day 7 was not reported. Concerning survival, 40% versus 50% of mice were still alive at day 10, respectively. Antimalarial efficacy seems thus comparable on the *P. berghei* species. However, the α-epimer, strychnopentamine, was inactive in the same condition by oral and subcutaneous routes on this strain [21] even if it displayed similar *in vitro* antiplasmodial activities. The same result was observed for dihydrousambarensine (IC_{50} = 0.032 µM on W2 strain, SI = 375, inactive *in vivo* at 30 mg/kg i. p. and s. c.). [7]. This can be due to the administration mode, different targets, structural interaction or mechanisms of action. The last bisindole alkaloid reported with strong antiplasmodial activity and tested *in vivo* is voacamine, less active *in vitro* on W2 strain than strychnogucine B (IC_{50} =

▶ **Table 1** *In vitro* antiparasitic activities of strychnogucine B compared to reference compounds and its selectivity indices.

Parasite	IC_{50} in µM (95% confidence interval)		SI	
	Strychnogucine B	Positive control	WI38	HCT-116*
P. falciparum:				
▪ FCA 20 Ghana	0.617*	0.020[a*]	12.0	24.3
▪ FCB1-R Colombia	0.529*	0.032 [a*]	14.0	28.3
▪ W2 Indochina	0.085*	0.284[a*]	87.1	176.4
T. brucei brucei	2.576 (1.596–4.159)	0.016 (0.007–0.034)[b]	2.8	5.6
L. mexicana mexicana	> 20	0.089 (0.054–0.141)[c]	< 0.4	< 0.8

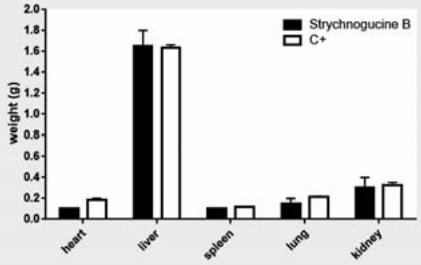
IC_{50} in µM with the 95% confidence interval for newly reported activities and SI of strychnogucine B = IC_{50} WI38 or HCT-116/IC_{50} parasite. *Data reproduced from Frédérich et al. (chloroquine-sensitive, moderately sensitive, and resistant lines respectively) [8, 9]. Reference drugs for positive control: [a]chloroquine, [b]suramine, and [c]pentamidine

0.411 µM) [18], and inducing 43% parasitaemia inhibition in *Plasmodium yoelii*-infected mice at 10 mg/kg/d *per os* on day 4 post-infection with some specificity for trophozoites and schizonts [22].

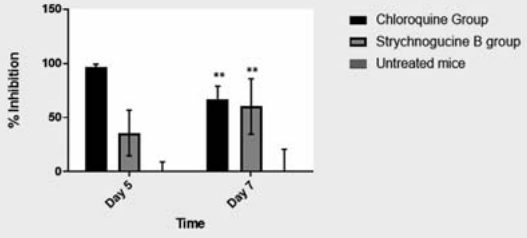
On this basis, it would now be very interesting to investigate the molecular targets affected by strychnogucine B, almost in accordance with the Lipinski's five rule (slightly higher molecular mass), and other related compounds that lead to *Plasmodium* death. For example, fluorescence localization and specific parasite stage assays would identify potential targets. Indeed, it could act with another mechanism than quinoline antimalarials as suggested by its significant higher activity on a resistant strain. The activity evaluation on chloroquine-resistant strain, with the *per os* route and in a cerebral malaria model could also give key information.

As protozoan parasites often share some biological features and face similar challenges [23], strychnogucine B activity was then evaluated *in vitro* on *T. brucei brucei* (African trypanosomiasis) and *L. mexicana mexicana* (cutaneous leishmaniasis). Selectivity index was also calculated compared to newly measured WI38 cytotoxicity. The observed cytotoxicity is slightly higher than the previous reported one (IC_{50} = 7.4 versus 15.5 µM, respectively) [9]. Its determination with a nonlinear regression on a larger concentration range (0.02–40 versus 1–20 µM, respectively), along with biological variability could explain this difference This single value has also to be referred to positive control one (camptothecin IC_{50} = 0.08 µM) to evaluate cells sensitivity. The compound was inactive on *Leishmania* promastigotes (IC_{50} > 20 µM, SI < 0.4) and moderately active on *Trypanosoma* bloodstream forms (IC_{50} = 2.58 µM, SI = 2.8). These activities along with the ones of reference drugs are summarized in the ▶ **Table 1**.

Strychnogucine B was also tested *in vivo* on an acute *T. brucei* model in mice. However, an i.p. treatment at 30 mg/kg for five consecutive days from the reappearance of detected parasitaemia did not induce any growth inhibition or life expectancy increase compared to the vehicle-treated mice. Conversely, all mice treated with suramine or diminazen aceturate, two reference drugs (human and veterinary ones), were cured. However, these old drugs possess some limits as administration routes and alternatives with easier administration and wider activity are still needed



▶ **Fig. 2** Main organ weight in the highest tolerated dose assay (performed on two mice). Mean ± standard deviation; C^+ = vehicle-treated control mice.



▶ **Fig. 3** *In vivo* antimalarial activity of strychnogucine B on mice infected by *P. berghei*. Data are shown as mean ± standard deviation of at least four mice per condition. The percentages of parasite growth inhibition were compared at days 5 and 7 (**p < 0.01).

[24, 25]. Another test with a lower infection and a less aggressive strain, for example the AnTaT 1.1E pleiomorphic one, could be tried to evaluate strychnogucine B antitrypanosomal activity, with an earlier treatment. Nevertheless, to our knowledge, it is the first reported bisindole alkaloid with *in vitro* antitrypanosomal activity.

Extracts from *S. icaja* could also be evaluated for antitrypanosomal activity, as this genus contains important antiparasitic potential. Thereby, another species of *Strychnos, Strychnos spinosa*,

traditionally used to treat African trypanosomiasis, was evaluated for its *in vitro* activities: leaves extract displayed strong to moderate activities (IC_{50} = 1.5 and 16.4 µg/mL on *T. brucei brucei* and *rhodesiense*, respectively) related to some identified active triterpenes [26, 27].

Concerning the antileishmanial activity, strychnogucine B was inactive on *L. mexicana* (IC_{50} > 20 µM), as were four macroline-pleiocarpamine bisindole alkaloids (IC_{50} > 50 µM) [28]. In accordance with these results, no notable docking scores with the 24 tested *Leishmania* proteins were reported for strychnogucine B in an *in silico* molecular docking study compared to the other evaluated indole alkaloids derived from plants [29]. Finally, a moderate activity on *L. amazonensis* promastigotes (IC_{50} = 24.9 µg/mL) was described for *Strychnos pseudoquina* A. St.-Hil. but activity seems to be related to another phytochemical class with an identified flavonoid, strychnobiflavone (IC_{50} = 3.2 µM, SI = 39.6 compared on murine macrophages and active on infected macrophages) [30].

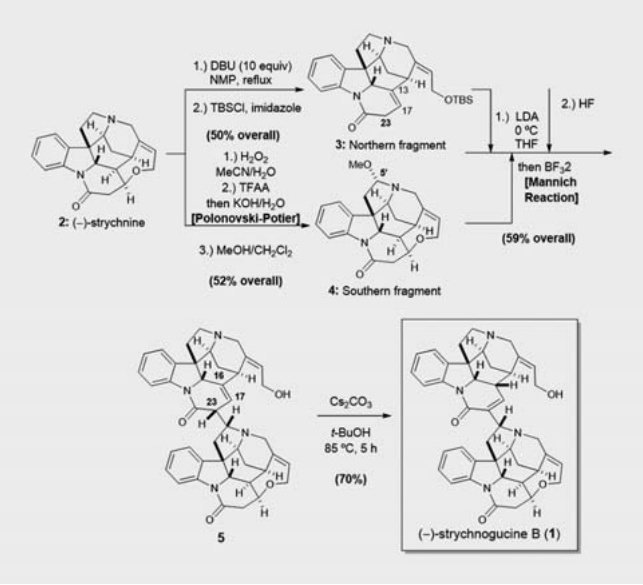
Materials and Methods

Strychnogucine B (**1**) is a natural product found in *S. icaja* as described previously by Frédérich et al. [9]. The one used for this *in vivo* study was prepared from commercially available strychnine [12]. The identity of the synthetic compound was assessed by comparison of HPLC chromatograms and ^{1}H NMR spectra with the isolated natural compound. Its purity was assessed by HPLC (> 95%). Spectrum and chromatogram are available as Supporting Information.

Semisynthesis of strychnogucine B from strychnine

The synthesis of bisindole alkaloid **1** is shown in ▶ **Fig. 4**. The successful realization of this goal required two subgoals. The first entailed access to two monoterpene indole fragments–northern fragment **3** and southern fragment **4**–each derived from commercially available (−)-strychnine (**2**) (Sigma-Aldrich, > 98%). The second included the strategic coupling of both fragments, in addition to functional group transformations to access (−)-strychnogucine B (**1**). Thus, when **2** was treated sequentially with 1,8-diazabicyclo[5.4.0]undec-7-ene at 200 °C in deaerated *N*-methyl-2-pyrrolidone, then *t*-butyldimethylsilyl chloride and imidazole, northern fragment **3** was isolated in 50% overall yield. Alternatively, site-selective oxidation of (−)-strychnine (**2**) to access southern fragment **4** was realized with the venerable Polonovski-Potier reaction. Thus, treatment of **2** with H_2O_2 afforded strychnine *N*-oxide. Sequential treatment with trifluoroacetic anhydride, aqueous KOH, and methanolic dichloromethane afforded southern fragment **4** in 52% overall yield.

The coupling of fragments **3** and **4** was accomplished via a stereoselective Mannich reaction. Specifically, northern fragment **3** was first treated with lithium diisopropylamide to generate the lithium enolate. In tandem, southern fragment was activated with BF_3-Et_2O. Addition of the former to the latter smoothly afforded the dimeric bisindole framework of target molecule **1** as a mixture of two diastereomers at C23. This fact was inconsequential as treatment of the intermediate with HF-Pyridine both removed the *t*-butyldimethylsilyl group and equilibrated the mixture to



▶ **Fig. 4** Semisynthesis of (−)-strychnogucine B (**1**) from (−)-strychnine (**2**).

the thermodynamically more stable diastereomer **5**. The final task in the synthesis of **1** was the isomerization of the alkene from the C16–C17 position to the C23–C17 position. To this end, we reacted **5** with Cs_2CO_3 in deaerated *t*-BuOH at 85 °C to give (−)-strychnogucine B (**1**) in 70% yield. Spectral data for synthetic **1** (e.g., ^{1}H and ^{13}C NMR, IR), in addition to R_f value, were in complete agreement with natural (−)-strychnogucine B (**1**) as reported by Frédérich et al. (**Fig. 1S** and **2S**, Supporting Information) [9].

In vivo approvals

All animal husbandry and handling conditions were in accordance with the Principles of Laboratory Animal Care and the Belgian Regulation. The present *in vivo* studies were designed according to the internationally recognized guidelines and approved by the ethics committee for the animals use at the University of Liège (n°16/1873, February 24, 2017) and at the Health Sciences Sector of the Catholic University of Louvain (2017/UCL/MD/017, July 24, 2017).

In vivo acute toxicity

The assessment of the highest tolerated dose was based on a DNDi protocol and described in Beaufay et al. [31]. Four increasing doses of strychnogucine B were given i. p. to two female NMRI mice (6 wk, 29.8 ± 1.9 g, Envigo) every 2 h: 5–10–15–30 mg/kg. Samples were prepared from a stock solution of 9 mg/mL in sterile distilled water with 10% of tween 80-EtOH (7 : 3). Control group received the vehicle. If nontoxic symptoms are observed (any health problem or behavioral changes, hematocrit, body, and main organs weights), 50% of the total injected dose can be used without toxicity risk in the acute *in vivo* antiparasitic efficacy test.

In vivo antiplasmodial assay

P. berghei NK173 strain was provided by Prof. Ph. Grellier (Museum National d'Histoire Naturelle, Paris, France). It was maintained in mice by syringe passage. Female Swiss mice (10 wk age, 20 ± 2 g, Charles River Laboratories), were maintained under controlled conditions of temperature and illumination. Based on the protocol described by Frédérich et al. [20], mice, randomly divided in group of five, were infected i. p. on day 1 with 150 µL of infected mice blood (parasitaemia of about 50 %), diluted two-fold in physiological saline. After 2 h, the treatment was started i. p. once daily for a total of four successive days, as described by Jansen et al. [32]. The treatment dose was prepared by dissolving strychnogucine B in tween 80 and EtOH (7 : 3) diluted at 10 % in saline at 30 mg/kg/100 µL, based on international guidelines for *in vivo* antimalarial activity in rodent malaria models [33]. On day 5 and 7, thin blood smears were made from mouse-tail blood. Slides were stained with Giemsa and the parasitaemia was determined under the microscope by counting at least 500 erythrocytes. Chloroquine diphosphate salt (Sigma-Aldrich, > 98 %) at 4 mg/kg (i. p.) was used as positive control and the vehicle as negative one. The inhibition of *Plasmodium* growth was calculated by parasitaemia normalization with the negative control values obtained for 80 % responder mice (parasitaemia between 35 % and 50 %). All the parasitaemia and inhibition percentage values are given as supporting information (**Table 1S**). Statistical significance between treatments was set at $p < 0.05$ and analyzed with Student's t-test on GraphPad Prism.

Other antiparasitic assays

All *in vitro* assays were done as previously reported [34, 35]. Strychnogucine B was evaluated in eight serial three-fold dilution from a DMSO stock solution of 4 mM (final concentration ranges: 20–0.009 and 40–0.018 µM for antiparasitic and cytotoxicity assays, respectively). Suramine sodium and pentamidine isethionate salts (Sigma-Aldrich, ≥ 99 % and 98 % of purity, respectively) were used as reference drugs with a stock solution at 2 mg/mL (final concentration range: 10–0.0046 µg/mL). For cells assay on WI38, eight serial five-fold dilution of camptothecin (Sigma-Aldrich, approximatively 95 % of purity) was tested from a stock solution of 10 mg/mL (final concentration range: 25–0.0003 µg/mL). The maximum used DMSO concentration (0.5 %) was verified to be nontoxic in all biological assays. Antiparasitic tests were performed at least in triplicate with two wells/concentration and cytotoxicity, already reported, was evaluated once with three wells/concentration to calculate SI. The IC_{50} were calculated by nonlinear regression with GraphPad Prism 7.0 based on the sigmoidal dose-response curves (**Fig. 3S**, Supporting Information).

The *in vivo* antitrypanosomal activity was evaluated based on a DNDi protocol. Female NMRI mice (7–8 wk, 30.4 ± 4.6 g, Envigo) were inoculated i. p. on day 0 with 10^4 bloodstream forms of *T. brucei brucei* (strain 427) obtained from a parasitized mouse (100 µL diluted in a PBS solution). The treatment was performed during five consecutive days from the reappearance of detected parasitaemia (day 3–7 post-infection). Strychnogucine B was administered i. p. at 30 mg/kg/d to five mice. The seven untreated mice received the previously cited vehicle and the positive control group were treated either with Suramin (Sigma, four mice) at 0.5 mg/kg/d or once with Veriben (Ceva, three mice) at a dose of 1.6 mg/kg diminazene diaceturate. Parasitaemia was monitored almost every day along with precise daily individual observation. The Herbert and Lumsden matching method was used to estimate parasitaemia by counting at least three fields for each wet blood films from tail blood [36]. Animals were assessed for their survival time and level of infection compared to negative control.

Supporting Information

The ^{1}H NMR spectrum and HPLC chromatogram of strychnogucine B (**1**) along with the *in vivo* percentage values and the dose-response curves for newly calculated IC_{50} values are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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