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In vitro Antiplasmodial Activity and Cytotoxicity of Vincadifformine and Its Semisynthetic Derivatives

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Abstract

An indole alkaloid with aspidospemane structure possessing a potential antiplasmodial activity, vincadifformine, has been isolated from Aspidosperma *pyrifolium* Mart. Moreover, 10 derivatives were prepared from the vincadifformine. The study was conducted to evaluate the *in vitro* antiplasmodial and cytotoxic activity of the vincadifformine and their semisynthetic derivatives. The *in vitro* antiplasmodial activity was evaluated on *Plasmodium falciparum* chloroquine-resistant (FcM_{29}) and –sensitive (Nigerian) strains after 24-h and 72-h incubation, while cytotoxic activity was estimated on Hela cells and Cytotoxicity Index (CI = IC_{50} on HeLa cells / IC_{50} on FcM_{29} strain) was calculated to evaluate the safety of tested compounds. Experiment results showed that two compounds (4 and 8) exhibited good antiplasmodial activities in comparison with parent compound, vincadifformine and other tested compounds with IC_{50} ranging from 5.3 to 12.8 µM on FcM_{29} strain and 11.4 to 24.0 µM on Nigerian strain. In addition, the CI of two compounds were also lower after 24-h incubation (CI, 2.0 and 4.8) than that of after 72-h incubation (CI, 9.5 and 11.5). Further study will be conducted to evaluate quantitative structure-activity relationship (QSAR) in order to design new antimalarial drugs.

Keywords : vincadifformine - antiplasmodial – *Plasmodium falciparum* – cytotoxic - HeLa

Introduction

The development of new antimalarial drugs is of paramount importance to combat the rapid spread of multi-drug resistant strains of *Plasmodium falciparum*. The growing reality that antimalarial drugs currently available may not be effective any more has led to the investigation of medicinal plant used in traditional medicine for hundreds of years. Medicinal plants for malaria treatment could be a promising source of new antimalarial agents. Significant success was achieved with the new agent extracted from plants like quinine from *Cinchona ledgeriana* or artemisinin from *Artemisia annua* (Li and Rieckmann, 1992).

In South America, medicinal plants play an important role in the treatment of a variety of diseases such as malaria. *Aspidosperma pyrifolium*, Mart. is one of the medicinal plants traditionally used to treat malaria in Bolivia. As *A. pyrifolium* Mart. is used to treat malaria by traditional healers, it is retained as a potential antimalarial candidate. Previous studies reported the presence of numerous indole alkaloids in the *A. pyrifolium*, Mart., among other things indole alkaloid with aspidospermane structure having an potential antiplasmodial activity (Mitaine *et al.*, 1996). Other aspidospremane structure viz. vincadifformine has been isolated from *A. pyrifolium* Mart. and a series of their semisynthetic derivatives have been

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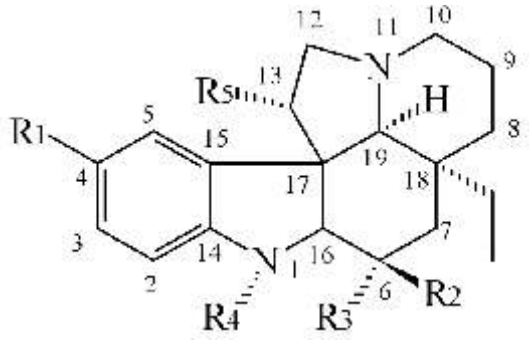
synthesized. The aim of the present study was to investigate the *in vitro* antiplasmodial activity of these compounds on *P. falciparum* culture. These compounds were also tested for cytotoxic activity on HeLa cell lines.

Materials and Methods

Tested compounds

Vincadifformine and 10 semisynthetic derivatives were evaluated in this study (Figure 1). These compounds were supported by Professor Guy Lewin from Department of Pharmacognocy Center for Pharmaceutical Study, Châtenay Malabry, France. The vincadifformine and their semisynthetic derivatives were differentiated from one another by the substitution difference on C_4 (R_1), C_6 (R_2, R_3), C_{13} (R_5) and N_1 (R_4) of the aspidospermane skeleton.



Compound	MW	Substitution				
		R_1	R_2	R_3	R_4	R_5
1. (Vincadiff-ormine)	338	H	CO_2CH_3	-	H	H
2.	383	NO_2	CO_2CH_3	-	H	H
3.	325	H	NO_2	-	H	H
4.	370	NO_2	NO_2	-	H	H
5.	404	Br	NO_2	-	H	H
6.	383	H	NO_2	CH_2CH_3	-	H
7.	462	Br	NO_2	CH_2CH_3	-	H
8.	372	H	Cl	CH_2CH_3	-	H
9.	417	NO_2	Cl	CH_2CH_3	-	H
10.	451	H	Cl	CH_2CH_3	-	Br
11.	352	H	CH_3	CH_2CH_3	-	H

Figure 1. Structure of vincadifformine and its semisynthetic compounds.

Plasmodium falciparum strains and *in vitro* culture

Two strains of *P. falciparum* were used to evaluate the *in vitro* antiplasmodial activities of the compounds. A FcM_{29} strain (chloroquine-resistant strain with IC_{50} for chloroquine about 230 ng/mL) and a Nigerian (chloroquine-sensitive strain with chloroquine IC_{50}, about 41 ng/mL) were cultured continuously according to Trager and Jensen (1976). The IC_{50} values of chloroquine were checked every two months and we observed no significant variations.

The parasites were maintained *in vitro* in human red blood cells (O^+), diluted to 1% hematocrit in RPMI 1640 medium (GIBCO BRL) supplemented with 25-mM Hepes and 30-mM $NaHCO_3$ (GIBCO BRL) and complemented with 5% human AB^+. Parasite cultures were synchronized by flotation on gelatin (Plasmagel) according to Jensen (1978) followed by 5% of D-sorbitol lysis (Merck) as reported by Lambros and Venderberg (1979).

Assay for antiplasmodial activity

The antiplasmodial activity of the compounds was evaluated by a radioactive method described by Desjardins *et al.*, (1979). Testing of each compound was performed tree times in triplicate in 96-well culture plates (TPP). Compounds testing were dissolved initially in dimethyl sulphoxide (DMSO) and then diluted in serum-free culture medium to provide stock solutions. Further dilutions were made before testing each compound at various concentrations. One hundred µL of vigorously growing culture with a predominance of young ring stages (synchronization interval, 16 h) at 0.5-1% parasitemia (hematocrit, 1%) were distributed in 96-well culture plates and 100 µL of the serum-free culture medium containing compounds at various concentrations were added. Parasite culture was then incubated with each compound for

two time interval, 24-h and 72-h before adding [^{3}H]-hypoxanthine (0.25 µCi per well). Following incubation, parasites were harvested onto glass fiber filters using semiautomatic cell harvester (Skatron). Parasite growth was estimated by [^{3}H]-hypoxanthine incorporation and compared to controls. The control parasite cultures freed from any compounds were referred to as 100% growth. Incorporation of [^{3}H]-hypoxanthine was determined by liquid scintillation using a Beckman LS 6000 LL scintillation counter (Beckman Instruments Ltd.). Computer programs were used to convert dpm (disintegrations per minute, proportional to the amount of [^{3}H]-hypoxanthine incorporated by the parasites) for each well to percentage inhibition and then plot this as function of the logarithm of drug concentration. Linear regression analysis was applied to those parts of sigmoidal curves obtained where response (percent inhibition) was depending upon concentration. IC_{50} values (concentration of the drugs at which inhibition of parasite growth represents 50%) were derived for each drug.

Assay for cytotoxicity

Cytotoxicity of the compounds was estimated on HeLa cells as conducted by Valentin *et al.* (1997). Cells were cultured in the same conditions as for *P. falciparum*, except for the 5% human serum, which was replaced by 5% fetal bovine serum (Boehringer). For the determination of compounds *in vitro* toxicity, 100 µL cell culture were distributed in 96-well plates at 2×10^{-4} cells per well and 100 µL of culture medium containing compounds at various concentration were added. Cell growth was estimated by [^{3}H]-hypoxanthine incorporation after 24-h and 72-h incubation exactly as for the *P. falciparum* contact period. The [^{3}H]-hypoxanthine incorporation in the presence of compounds was compared with that of control cultures without compounds. IC_{50} values were also derived for each drug.

Results and Discussion

Among strategies in the antimalarial development is structure modification of existing agents by substitution of various functional groups to parent compound. In previous study, modification of vincadifformine aspidospermane skeleton has been conducted by the substitution difference on C_4 (R_1), C_6 (R_2, R_3), C_{13} (R_5) and N_1 (R_4) and 10 vincadifformine derivatives have been obtained. In this study, *in vitro* antiplasmodial activities of the vincadifformine derivatives and their cytotoxicity were evaluated.

Two strains of *P. falciparum* (FcM_{29} and Nigerian strains) were used to evaluate the *in vitro* antiplasmodial activities of the vincadifformine and its derivatives (1 – 11). The results are summarized in Table 1. This screening showed that all the compounds were less active against *P. falciparum* chloroquine-sensitive strain (Nigerian) than chloroquine-resistant strain (FcM_{29}). The IC_{50} values were higher (ranging from 7.8 - 104.4 µM) for FcM_{29} strain than for Nigerian strain (IC_{50}, 11.4 - 226.7 µM). It was suggested that these compounds inhibit *P. falciparum* growth by mean of a fundamentally different mechanism from that chloroquine. With regard to the incubation time, almost all of the tested compounds have the IC_{50} (5.3 – 38.6 µM) lower after a 72-h contact between parasites and compounds than after a 24-h contact (IC_{50}, 7.8 – 104.4 µM) indicating cumulative effect observed.

Among the 10 vincadifformine derivatives tested, three compounds (4, 8 and 9) showed good antiplasmodial activities in comparison with other tested compounds. These compounds had IC_{50} ranging from 5.3 to 12.8 µM on FcM_{29} strain and 11.4 to 24.0 µM on Nigerian strain (Table 1). Five compounds (2, 3, 5, 10 and 11) had

antiplasmodial activities (IC_{50}, 12.4 – 28.5 µM) similar with vincadifformine (IC_{50}, 18.4 – 24.3 µM) on FcM_{29} strain. However, these compounds were more active (IC_{50}, 14.4 – 84.6 µM) than vincadifformine (IC_{50}, 26.4 – 139.8 µM) against Nigerian strain. Two compounds (6 and 7) were less active (IC_{50}, 18.2 – 226.7 µM) than vincadifformine (IC_{50}, 18.4 – 139.8 µM) against both FcM_{29} and Nigerian strains.

Table 1. *In vitro* antiplasmodial activity (IC_{50} in µM) of vincadifformine and its semisynthetic derivatives on *P. falciparum* chloroquine-resistant (FcM_{29}) and –sensitive (Nigerian) strains after 24-h and 72-h incubation.

Compounds	F_cM_{29}		Nigerian	
	24-h	72-h	24-h	72-h
1. (Vincadif-formine)	24.3±6.0	18.4±6.4	139.8±31.4	26.4±7.5
2.	28.5±3.7	29.0±11.3	34.8±5.0	31.4±8.2
3.	27.3±0.4	14.9±10.3	84.6±25.1	26.4±6.7
4.	12.8±5.2	5.3±5.8	14.5±2.8	18.5±5.8
5.	16.4±6.0	15.6±7.3	23.9±7.9	22.9±4.6
6.	104.4±61.0	38.6±18.5	226.7±180.1	180.1±53.0
7.	89.5±89.7	18.2±3.7	172.3±44.0	187.0±36.3
8.	7.8±2.0	10.0±6.8	24.0±3.8	21.3±3.7
9.	12.1±2.2	11.0±4.5	13.8±7.3	11.4±3.5
10.	19.0±9.9	12.4±1.7	20.8±4.9	14.4±5.9
11.	20.4±9.2	18.9±9.6	62.7±10.1	26.6±5.6

The vincadifformine is indole alkaloids with aspidospermane skeleton. The indole alkaloids have been isolated from some plants and their antiplasmodial activity and cytotoxicity have also been evaluated by some authors. Wright *et al.* (1992) evaluated *in vitro* antiplasmodial activity nine indole alkaloids isolated from *Alstonia angustifolia* roots. The more active alkaloids were macrocarpamine, macralstonine and villastonine with IC_{50} values ranged from 2.92 to 9.36 µM. The antipalsmodial activity of the macrocarpamine and the villastonine has been confirmed by Keawpradub *et al.* (1999) after he evaluated this indole alkaloids obtained from other *Alstonia* spp viz. *A. scholaris, A. macrophylla* and *A. glaucescens*. In addition, Wright *et al.* (1994) also evaluated other indole alkaloids from *Strychnos* species (Loganiaceae) and showed that among twenty alkaloids evaluated, 3',4'-dihydrousambarensine was the most active against *P. falciparum* (IC_{50} = 0.023 µM). The *in vitro* antiplasmodial activity of indole alkaloids with aspidospermane skeleton was reported firstly by Mitaine *et al.* (1998) after investigate 12 alkaloids on *P. falciparum*. The results showed that the tetracyclic alkaloids possessing a free ethyl chain such aspidospermine were more active (IC_{50} ranged 3.2 – 15.4 µM) than the pentacyclic alkaloids with ethyl chain included in a tetrahydrofuran such haplocine (IC_{50} ranged 22.6 – 52.6 µM). The cytotoxicity of aspidospermane indole alkaloids including vincadifformine have also reported by Lewin (1995) and Lewin *et al*. (2002). The cytotoxicity of these compounds varied depending on their structures.

In order to obtain an estimate for the therapeutic index, the cytotoxicity of the derivatives showing similar and higher antiplasmodial activities than vincadifformine on HeLa cells were also investigated. The cytotoxicity to HeLa cells has been compared to antiplasmodial activity on FcM29 strain to obtain Cytotoxicity Index (CI = IC_{50} on HeLa / IC_{50} on FcM29 strain). The CI obtained enable the selection of compounds which show some specificity of action in the *in vitro* situation, prior to more detailed *in vivo* investigations. The results of these experiments are summarized in Table 2. The IC_{50} values of the various vincadifformine derivatives on HeLa cells ranged from 16.3 to 213.4 µM. In general, the vincadifformine and their derivatives were more toxic after 24-h

incubation (IC_{50}, 16.3 – 96.8 µM) than that after of 72-h (IC_{50}, 22.1 – 213.4 µM). In addition, the CI of these compounds were also lower after 24-h incubation (CI, 0.9 - 4.8) than that of after 72-h incubation (CI, 3.1 – 11.5). Compounds 4 and 8 showing good antiplasmodial activities were less toxic (CI, 2.0-11.5 and 4.8-9.5 respectively) than the parent compound vincadifformine In contrast, compound 9 showing good antiplasmodial activities was more toxic than vincadifformine with CI ranged 1.4-3.7. It could be seen that the activities of the compounds against HeLa cells were not parallel with the activities against *P. falciparum* suggesting that the mode of action of these compounds as antimalarial was not simple cytotoxic effect and lent support to further investigation of these compounds and their analogues as potential antimalarials drugs.

Table 2. Cytotoxicity (IC_{50} in µM) and Cytotoxicity Index (CI) of vincadifformine and its semisynthetic derivatives on HeLa cell after 24-h and 72-h incubation.

Compounds	HeLa		CI*	
	24-h	72-h	24-h	72-h
1. (Vincadif-formine)	68.7 ± 4.8	115.3 ± 65.5	2.8	6.3
2.	ND	ND	ND	ND
3.	96.8 ± 38.3	213.4 ± 28.5	3.6	8.2
4.	26.0 ± 0.8	60.7 ± 3.7	2.0	11.5
5.	33.6 ± 0.3	28.6 ± 7.5	2.0	1.8
6.	ND	ND	ND	ND
7.	ND	ND	ND	ND
8.	38.0 ± 6.1	95.6 ± 57.1	4.8	9.5
9.	16.9 ± 6.1	40.2 ± 25.3	1.4	3.7
10.	16.3 ± 3.9	22.1 ± 0.3	0.9	6.3
11.	51.8 ± 5.3	59.3 ± 13.3	2.5	3.1

* CI = IC_{50} on HeLa cells / IC_{50} on FcM29 strain; ND : non determined

This study also clearly demonstrated that the antiplasmodial activity and cytotoxicity of the vincadifformine derivatives was influenced by the substitution difference on atoms of the aspidospermane skeleton (Table 1 and 2). Introduction a nitro group to C_4 or replacement of a methyl ester group to C_6 with a nitro group of vincadifformine (compound 2 dan 3) in general did not influence its antiplasmodial activity, but reduced its cytotoxic activity (compound 3). However, the presence nitro groups together to C_4 and C_6 (compound 4) increased its antiplasmodial and cytotoxic activity, although its cytotoxicity decreased. In addition, the presence a nitro groups to C_6 together with a bromide to C_4 (compound 5) increased its antiplasmodial activity and cytotoxicity. It was likely that the alkylation to C_6 (compound 6-11) did not influence its antiplasmodial activity. However, the antiplasmodial and the cytotoxic activity of these compounds was depending on the other substitution to C_4 and C_6. For instance, introduction a halogen atom to C_6 of aspidospermane skeleton such as chlorine in compounds 8, 9 and 10 increased their antiplasmodial and also their cytotoxic activity. Inversely, replacement of this chlorine by a nitro group or a methyl group decreased their activities as shown in compounds 6, 7 and 11. Further study will be conducted to evaluate quantitative structure-activity relationship (QSAR) by computational chemistry in order to design new antimalarial drugs.

The results reported here show those two compounds (4 and 8) exhibite good antiplasmodial activities in comparison with parent compound, vincadifformine and its derivatives indicating that the compounds are prospective for further study.

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