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Laboratory note

Antimalarial activity and structure–activity relationships of protoberberine alkaloids

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Abstract – The thirty-nine protoberberine derivatives including berberine **1** and palmatine **2** were tested for antimalarial activity in vitro against *Plasmodium falciparum* and structure–activity relationships are proposed. The activity of the protoberberine alkaloids was influenced by the type of the quaternary nitrogen atom, the nature and the size of the substituents at the C-13 position, and the type of *O*-alkyl substituents on rings A and D. The activity of the quaternary protoberberinium salts with an aromatic ring C such as berberine was higher than that of the quaternary salts such as the *N*-metho salts or the *N*-oxides of tetrahydro and dihydro derivatives as well as tertiary tetrahydroprotoberberines. Of the 13-alkyl derivatives of **1** and **2**, the activity did not always increase as the length of the aliphatic chain rose in the order methyl, ethyl, propyl, butyl, and hexyl group. 13-Butylberberine (**1Bu**) and 13-propylpalmatine (**2Pr**) were the most active compounds among the 13-alkylberberines and 13-alkylpalmatines, respectively. 13-Hydroxyberberine **3** possessed the same level of activity as **1**. Of **1** and **2** with different substituents types on Ring A, the activity of **1** was significantly higher than that of **2**. Among berberrubines **4** and **5** and their C-9-*O*-alkyl derivatives **6** and **7**, the activity of 9-*O*-ethylberberrubine **6** was the highest. Of the potent protoberberinium salts, the activity decreased in the order: **1**, **3** > **2Pr** > **6** > **1Bu**. A positive effect on the activity might be exerted by the introduction of a more hydrophilic function into the C-13 position of the protoberberinium salts.

in vitro antimalarial activity / structure–activity relationships / protoberberinium salts

1. Introduction

Berberine **1** is a major compound present in Phellodendri Cortex (Obaku in Japanese) and Coptidis Rhizoma (Oren in Japanese), which are used for treating diarrhoea and other gastrointestinal diseases in Japan and other Eastern countries. Berberine and its relatives exhibit several types of biological activity [1]. We have found that some 13-alkyl derivatives of berberine **1** and palmatine **2** possessed antibacterial and antifungal activities comparable to or superior to those of kanamycin sulfate [2, 3]. Various claims have been made concerning the use of berberine in the treatment of malaria [4, 5]. In a very recent in vivo or in vitro study, it has been found that none of ten protoberberines was active in vivo, although some of the tested alkaloids including berberine and palmatine exhibited a potency comparable to that of quinine in vitro [6].

In the present study, the antimalarial activity of 39 tertiary and quaternary protoberberine alkaloids was examined using selectivity indexes as an indication of the activity. The results on the study of the structure–antimalarial activity relationships of the tested alkaloids are described.

2. Chemistry

The protoberberine derivatives used for the antimalarial activity were prepared according to procedures reported in the literature [2, 3, 7–10]. They were as follows: berberine **1** and its 13-alkyl derivatives **1Me**, **1Et**, **1Pr**, **1Bu**, and **1He** [3], palmatine **2** and its 13-alkyl derivatives **2Me**, **2Et**, **2Pr**, **2Bu**, and **2He** [3], 13-hydroxyberberine **3** [7], berberrubine **4** [2], 13-methylberberrubine **5** [2], 9-*O*-ethylberberrubine (**6**) [2], 9-*O*-ethyl-13-ethylberberrubine **7** [2], 13-methyldihydroberberine *N*-metho salt **8** [8], tetrahydroprotoberberines **9** (R_1 = R_3 = H, R_2 = H or OH) [2] and their *N*-metho salts **9** (R_1 = Me, R_2 = H or Me

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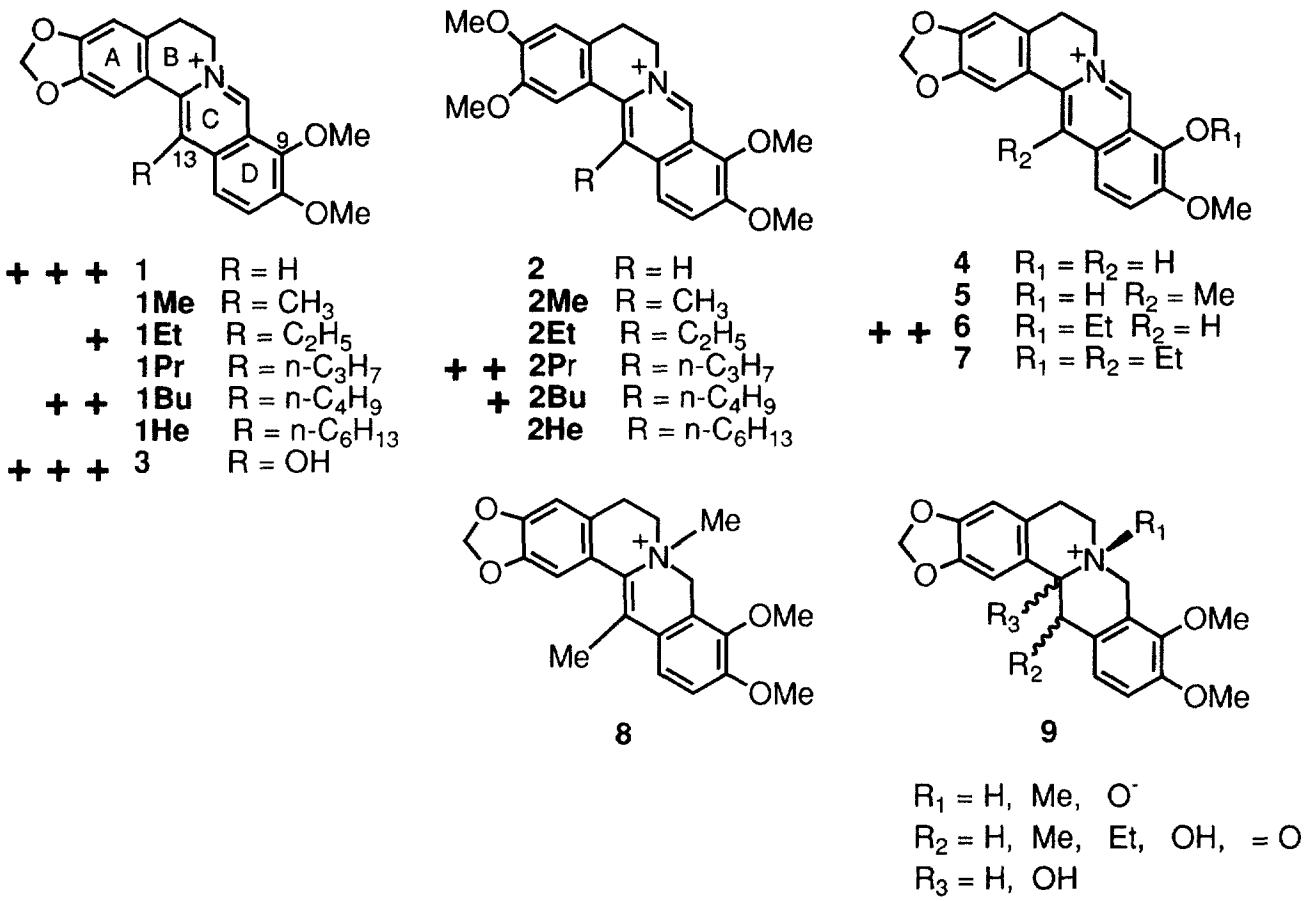

Figure 1.

or Et or OH, R_3 = H) [2], tetrahydroberberine *N*-oxides **9** ($R_1 = O^-$, $R_2 = R_3$ = H) [9], protopines **9** (R_1 = H, R_2 = H or = O, R_3 = OH) [2], and allocryptopine *N*-oxide **9** ($R_1 = O^-$, R_2 = H, R_3 = OH) [10].

3. Results and discussion

39 protoberberines were tested in vitro against human malaria *Plasmodium falciparum* FCR-3. The antimalarial activity of each compound was determined as percentage reduction. The compound concentration required to inhibit cell growth by 50% was expressed as IC_{50} (*table I*). From the evaluation of the toxicity of the compounds for mammalian cells, the concentration causing a 50% reduction of cell growth (GI_{50}) of mouse mammary FM3A cells, a model of the host, was determined (*table I*). The GI_{50}/IC_{50} ratios for the compounds were calculated as selectivity indexes (*table I*). These ratios were used as an evaluation of antimalarial activity. The results are presented in *table I*.

Among the tested protoberberinium salts with the aromatic ring C, the potent active compounds were **1** and **3**. **1Bu**, **2Pr** and **6** were less active derivatives, and the other protoberberinium salts showed much less activity (*table I*). 13-Methyldihydroberberine *N*-metho salt, the tetrahydroprotoberberines and their *N*-metho salts and *N*-oxides, the protopines and their *N*-oxides were inactive (*table I*).

Aromatization of ring C is necessary to increase the activity, because the quaternary salts with the aromatic ring C showed higher activity than the quaternary salts such as the *N*-metho salts or the *N*-oxides of dihydro- and tetrahydroprotoberberine and tertiary tetrahydroprotoberberines.

The activity of 13-alkylprotoberberinium salts did not always increase as the length of the aliphatic chain

Table I. In vitro antimalarial activity of 13-substituted protoberberinium salts.

Compound	50% growth inhibition (mol)		Selectivity indexes
	Plasmodium falciparum FCR-3 IC_{50}	Mouse mammary cells FM3A GI_{50}	GI_{50}/IC_{50}
1	2.7E–07	> 1.2E–05	> 44
1 Me	2.1E–06	> 1.1E–05	> 5.2
1 Et	1.7E–06	1.4E–05	8.2
1 Pr	1.5E–06	6.5E–06	4.3
1 Bu	6.0E–07	> 1.1E–05	> 18
1 He	1.0E–06	3.3E–06	3.3
2	6.4E–06	> 6.4E–05	> 4
2Me	7.0E–06	> 2.5E–05	3.5
2 Et	8.0E–06	> 2.8E–05	> 3.5
2 Pr	1.0E–06	> 2.7E–05	> 27
2 Bu	2.0E–06	> 2.3E–05	> 11.5
2 He	1.3E–06	7.0E–06	5.4
3	2.2E–07	> 9.4E–06	> 43
4	2.2E–06	5.0E–06	2.3
5	4.9E–06	2.3E–06	0.5
6	5.1E–07	> 1.3E–05	> 25.5
7	3.6E–06	1.1E–05	3.1
8	4.0E–06	> 1.0E–05	> 2.5

rose in the order methyl, ethyl, propyl, butyl, and hexyl group. This finding is different from the result that antibacterial activity increased as the length of the alkyl chain increased [2, 3]. At the 13-position of berberine **1**, introduction of the alkyl group reduced the activity. The activity greatly diminished by substitution of methyl or ethyl or propyl groups, recovered by that of the butyl group, and decreased again by that of the hexyl group (*table I*). Of the palmatine-type derivatives, parent base **2**, **2Me**, and **2Et** displayed low activity, **2Pr** the highest activity, **2Bu** less activity, and **2He** much less activity (*table I*). 13-Butylberberine **1Bu** was the most active compound among the 13-alkyl derivatives.

Introduction of the hydroxyl group at the C-13 position of berberine caused no decrease of the activity (*table I*). It is suggested that introduction of more hydrophilic groups into the C-13 position of the protoberberinium salts increases the activity. On berberrubine **4** derivatives, the antimalarial activity did not always augment with increasing of *O*-alkyl chains at the C-9 position. Replacement of hydrogen of the hydroxyl group at the C-9 position by a methyl group increased the activity (compare **1** and **1Me** with **4** and **5**, respectively) (*table I*). The activity decreased by substituting the C-9-OMe group with the C-9-OEt group (compare **1** and **1Et** with **6** and **7**, respectively) (*table I*).

The inhibitory effects of some protoberberines against *P. falciparum* are different from those previously reported [6]. The inhibitory effect of berberine **1** was similar to that described in the previous report [6]. The inhibitory effects of palmatine **2**, tetrahydroberberine **9** ($R_1 = R_2 = R_3 = H$), and its *N*-oxide **9** ($R_1 = O^-$, $R_2 = R_3 = H$) and *N*-metho salt **9** ($R_1 = Me$, $R_2 = R_3 = H$) were less than those previously described [6]. The inhibitory effect of 13-hydroxyberberine **3** was comparable to that of berberine **1**. The inhibitory effects of phenolbetaine form **3** ($R = O^-$) has been found to be much less than that of **1** [6].

This difference might result from the structural change. It is noteworthy that berberine exhibited much higher activity than palmatine. This result is different from that previously described [6]. A simple change of the functional groups on ring A thus converted a much less active compound to one with considerable activity. In *P. falciparum*, antimalarial effects have been shown to depend on the time of exposure to the tested compounds during the malaria life cycle [11]. Generally speaking, results obtained using either microscopic examination or ^3H incorporation assays are similar. Microscopy allows scoring of parasite numbers, whereas hypoxanthine incorporation measures the parasite's ability to take up and utilize a metabolic precursor during 18 h of treatment. In the present case, the exposure time to the protoberberines was longer than that in the procedure previously reported [6] and microscopy allows not only scoring of parasite numbers but also observation of reinvasion of parasites in the life cycle. The difference of the results between the present and previous works might be derived from a difference of the experimental method and/or a change of drug susceptibility caused by the different *P. falciparum* strains.

4. Conclusion

From the structure–activity point of view, some features can be pointed out. On the basis of the results derived from examinations with protoberberines, it appears that several structural features such as the type of quaternary structures (positively charged nitrogen atom in the aromatized ring), the size and the nature of substituents at the C-13 position, and the type of substituents on rings A and D had an influence on the antimalarial activity. It has been reported that berberine is a potent inhibitor in vitro of both nucleic acid and protein synthesis in *P. falciparum* [5]. These structural features might influence the interaction and/or intercalation between protoberberines and the receptor of the biopolymers or the synthetic enzymes.

Among the potent protoberberinium salts, the activity decreased in the order: **1**, **3** > **2Pr** > **6** > **1Bu**. Even though selectivity indexes (GI_{50}/IC_{50}) obtained for the two active antimalarial compounds **1** and **3** are not so high, some new derivatives with a higher and selective inhibitory activity might be obtained by introduction of more hydrophilic functions into the C-13 position of the protoberberinium salts.

5. Experimental protocols

5.1. In vitro antimalaria screening

5.1.1. Parasites

In all of the studies described in this report, *P. falciparum* strain FCR-3 (ATCC 30932) was used [12, 13]. Human serum and erythrocytes were obtained from healthy donors, stored at 4 °C and used within 10–14 days from donation. Parasites were cultured in 10% heat-inactivated A^+ human erythrocytes and suspended at a 5% hematocrit in RPMI 1640 medium (Gibco, NY) which contained 50 mg of gentamycin per liter, and 10% group A^+ human serum and was buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES, pH 7.4) and 25 mM $NaHCO_3$ [12, 13]. Cultures were maintained at 37 °C in a gas mixture of 5% oxygen–5% carbon dioxide–90% nitrogen [14].

5.1.2. Drug testing

The following procedure was used for routine assay of antimalarial activity. Various concentrations of compounds, suspended in 10 μL of distilled water were added to individual wells of a 24-well plate. Erythrocytes with 0.3% parasitemia were added to each well in 990 μL of culture medium to give a final hematocrit of 3%. The plates were incubated at 37 °C for 72 h under 5% oxygen–5% carbon dioxide–90% nitrogen. Parasite morphology in drug-treated culture after 72 h was measured by staining with Giemsa, and the number of parasitized red bloodcells per 10 000 erythrocytes was counted and growth rates were calculated. All compounds were run in duplicate at each concentration. Drug-free control cultures were run simultaneously. All data points represent means of at least two experimental tests. The 50% inhibitory concentration (IC_{50}) is defined, by comparison with drug-free controls incubated under the same conditions [15, 16].

5.1.3. Mammalian cells

A wild-type mouse FM3A cell line (subclone F-28-7) was supplied by the Health Sciences Research Resources Bank (Osaka, Japan). FM3A cells were maintained in suspension culture at 37 °C in a 5% CO_2 atmosphere in plastic bottles containing ES medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2% heat-inactivated fetal bovine serum (Gibco, NY) [17, 18].

5.1.4. Toxicity to mammalian cells

The cell line grew with a doubling time of about 12 h. Before being exposed to drugs, cells were seeded at 990 μL of 5×10^4 cells/mL density and various concentrations of compounds dispensed in 10 μL of distilled water were added to individual wells of a 24-well plate. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. Cell numbers were measured using a blood cell counter CC-108 (Toa Medical Electric Co., Japan). All data points represent means of at least two experimental tests. The 50% inhibitory concentration (GI_{50}) is defined and by comparison with that of drug-free controls incubated under the same conditions. Cell growth inhibition is the index of cytotoxicity including cytostatic activity of the test compounds.

5.1.5. Selective toxicity

The selective toxicity was estimated from the EC_{50} ratio (the drug concentration necessary to inhibit the growth rate of cells to 50% of the growth value) between the malaria parasites and mouse mammary FM3A cells which served as a model host [19].

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