Antimalarial Activity and Nucleoside Transport Inhibitory Activity of the Triterpenic Constituents of *Cimicifuga* spp.

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The *in vitro* antimalarial activity against human malaria parasite (*Plasmodium falciparum*, FCR-3 strain) was examined using 59 triterpenoids obtained during studies on the triterpenic constituents of *Cimicifuga* spp. The 50% effective concentration values (EC₅₀) of 25 active triterpenoids were 1.0–3.0 μ M, and 19 of the compounds had a common 16, 23 : 23, 26 : 24, 25-triepoxy group in the side-chain moieties. Among the active triterpenoids, 9 also showed significant inhibition of nucleoside transport in mouse splenocytes. A relationship between the antimalarial activity and the inhibition of nucleoside transport involving these triterpenoids is discussed.

Key words *Cimicifuga*; Ranunculaceae; 9,19-cyclolanostane glycoside; antimalarial activity; nucleoside transport inhibition; thymidine-³H uptake

The rhizomes of *Cimicifuga* (*C.* *simplex*, *C.* *dahurica*, *C.* *heracleifolia*, and *C.* *foetida*) have been used as a traditional Chinese medicine, *Cimicifugae Rhizoma* [“Shoma” in Japanese] prescribed for its anti-inflammatory, analgesic and antipyretic effects.¹⁾ As some of the chemical constituents of *Cimicifuga* spp., highly oxygenated 9,19-cyclolanostane glycosides, chromones, caffeic acid derivatives, and cimicifugic acids have been reported.^{2–6)}

We reported previously that cimicifugoside and its congeners, obtained from *Cimicifuga* species, have potent inhibitory effects on the uptake of thymidine and adenosine into phytohemagglutinin (PHA)-stimulated human peripheral lymphocytes (IC₅₀: 4–5 ng/ml), and some other cell lines. The mechanism has been proved to be potent and selective inhibition of nucleoside transport at the plasma membrane site of mammalian cells.^{7–9)} The inhibitory effect may be promising for reducing the cell growth of the malarial parasite, which relies solely on purine salvage for its survival.¹⁰⁾

Therefore, we examined the antimalarial activity of cimicifugoside and its congeners, comparing it with that of other triterpenoids from *Cimicifuga* spp. We also examined the inhibitory activity of thymidine uptake of the new triterpenoids along with cimicifugoside. In this report we describe the antimalarial activity of these triterpenoids, and the correlation observed between antimalarial activity and the inhibition of thymidine uptake.

MATERIALS AND METHODS

Chemicals Fifty-nine triterpenic compounds (1–59) of 5 structural groups were examined: cimicifugoside group (group I), shengmanol group (II), hydroshengmanol group (III), cimigenol group (IV), and others (V), as shown in Fig. 1.

Group I: Cimicifugoside (1) and cimicifugenin (17) were obtained from *C. simplex*.¹¹⁾ Actein (2) was obtained from *C. racemosa* and *C. simplex*.¹²⁾ Bugbanosides A (3) and B (4) were obtained from *C. simplex*.¹³⁾ 26-Deoxycimicifugoside (15)¹⁴⁾ was isolated from *C. simplex* and *Actaea spicata*.

Their related compounds, 5–12, were derived by treatment of 1–4 with *p*-TsOH in MeOH.^{12,13)} Compounds, 13, 14 and 16, were obtained by oxidation of 1, 3 and 4 with pyridine–CrO₃ solution, and genuine aglycones, 18 and 19, were obtained by hydrolysis of 3 and 4 with cellulase.¹³⁾

Group II: 23-*O*-Acetylshengmanol 3-*O*- β -D-glucosyl-(1 $\rightarrow$ 3)- β -D-xyloside (20), 23-*O*-acetylshengmanol-3-*O*- β -D-xyloside (21),¹⁵⁾ 7 β -hydroxy-23-*O*-acetylshengmanol 3-*O*- β -D-xyloside (22),¹⁶⁾ and 23-*O*-acetyl-7,8-didehydro shengmanol 3-*O*- α -L-arabinoside (23),¹⁷⁾ were isolated from the aerial parts of *C. simplex*. Their aglycones, 24, and 25, were obtained by hydrolysis with cellulase.^{15–17)}

Group III: 7 β -Hydroxy-25-*O*-methyl-24-*O*-acetylhydroshengmanol 3-*O*- β -D-xyloside (28),¹⁸⁾ 24-*epi*-24-*O*-acetyl-7,8-didehydroshengmanol 3-*O*- β -D-galactoside (30),¹⁷⁾ and the related compounds, 26–28, were obtained from the aerial parts of *C. simplex*. The aglycones, 31–35, were obtained by hydrolysis with cellulase or lactase.^{17,18)}

Group IV: 25-*O*-Acetylcimigenol 3-*O*- β -D-glucosyl-(1 $\rightarrow$ 3)- β -D-xyloside (36),¹⁵⁾ 1 α -hydroxy-25-*O*-acetylcimigenol 3-*O*- β -D-xyloside (38),¹⁸⁾ 12 β -hydroxycimigenol 3-*O*- α -L-arabinoside (39),¹⁹⁾ 7 β -hydroxycimigenol (40),¹⁶⁾ 7,8-didehydrocimigenol 3-*O*- β -D-galactoside (43)¹⁷⁾ and the related compounds, 41 and 42, were isolated respectively from the aerial parts of *C. simplex*. Cimigenol (49) and 25-*O*-acetylcimigenol-3-*O*- β -D-xyloside (37) were obtained from *C. simplex*, *C. acerina* and *C. japonica*.²⁰⁾ 15-Deoxycimigenol (51) was derived from 39. The aglycones, 45–50, were obtained by hydrolysis of the glycosides with cellulase or lactase.^{15–17)}

Group V: Cimiaceroside (52) was isolated from *C. simplex* and *C. acerina*.²¹⁾ Cimiacerol (53)²¹⁾ and *O*-methylcimiacerol (54)²²⁾ were obtained from *C. acerina*. Acerinol (55) and acerinol acetate (56)²³⁾ were obtained from *C. acerina*, *C. japonica*, and *C. simplex*, *O*-methylcimicimol (57) and simplexol (59)²⁰⁾ were derived from 41, and isodahurinol (58)²⁴⁾ was isolated from *C. acerina*.

Malaria Parasites *P. falciparum* strain FCR-3 (ATCC-30932) was used in this study. The strain was maintained *in vitro* at 37°C in RPMI 1640 medium (Gibco, NY) supple-

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mented with 10% human serum containing human red blood cells (type A) at 5% hematocrit using 24-well microplates.²⁵⁾ The microplates were placed in a CO₂ incubator (5% CO₂, 5% O₂, 90% N₂) at 37 °C and the medium was changed daily.

Mammalian Cells A wild-type mouse mammary tumor FM3A cell line (subclone F-28-7), was supplied by the Japanese Cancer Research Resources Bank (JCRB). FM3A cells were maintained in a suspension culture at 37 °C in a 5% CO₂ atmosphere in plastic bottles containing ES medium supplemented with 2% heat-inactivated fetal bovine serum (Gibco, NY).²⁶⁾

In Vitro Antimalarial Activity The following procedures were used for routine assay of antimalarial activity. Various concentrations of triterpenoids in distilled water or dimethyl sulfoxide (DMSO) were prepared. 10 µl of each solution was added to the individual wells of a 24-well plate. Erythrocytes with 0.3% parasitemia were added to each well containing 990 µl of culture medium to give a final hematocrit level of 3%. The plates were incubated at 37 °C in a 5% CO₂ incubator (5% CO₂, 5% O₂, 90% N₂). To evaluate the antimalarial activity of the triterpenoids, thin smears were prepared from each culture, stained with Giemsa (E. Merck, Germany), and more than 10000 erythrocytes were examined by microscopy. All the test compounds were assayed in duplicate at each concentration. Drug-free control cultures were run simultaneously. All data points represent the mean of at least two experiments. The fifty percent effective concentration EC₅₀ was determined by comparison with drug-free controls incubated under the same conditions.

Toxicity against a Mammalian Cell Line The population doubling time for FM3A Cells was 12 h. Prior to exposure to drugs, cell density was adjusted to 5×10⁴ cells/ml. A cell suspension of 990 µl was dispensed to the test plate, and compounds at various concentrations suspended in either 10 µl of distilled water or DMSO, were added to the individual wells of a 24-well plate. The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. Cell numbers were measured using a blood cell counter CC-108 (Toa Medical Electric Co., Japan). All data points represent the mean of at least two experiments. The EC₅₀ was determined by comparing with drug-free controls incubated under the same conditions.

Uptake Assay of Radioactive Compounds, Reagents and Procedures The mitogens were concanavalin A (Con A): Sigma, cat. no. C2010, U.S.), lipopolysaccharide (LPS): Difco Laboratories, cat. no. 3920-25-2, U.S., PHA: Gibco, cat. no. 670-0576AD, U.S. Thymidine-³H (³H-TdR): spec. act., 5 Bq/mmol, and uridine-³H (³H-UdR): spec. act., 27 Bq/mmol were obtained from Amersham, U.S.

Responder cells: mouse BDF1 (Japan SLC, Inc., Shizuoka, Japan)-splenocyte, male, 8 weeks. The lymphocytes were cultured in microculture plates (Sumitomo Bakelite, cat. no. MS3096-F, Japan). Each well of the microplate received 5×10⁵ lymphocytes in 200 µl culture medium containing Con A (5 µg/ml), or LSP (10 µg/ml), or PHA (1%), together with various concentrations of the test triterpenoids. After 54 h incubation at 37 °C in humidified 5% CO₂ in air, 0.5 µBq ³H-TdR, or 0.5 µBq ³H-UdR was added to each well. Cells were harvested 18 h later using a multiple semi-automated cell harvester (Labo Mash, Japan), and the dried glass filter disk in 10 ml Econoflor-2 (Packard Institute, U.S.)

was counted in a liquid scintillation counter (Aloka, Japan). The labeled TdR, or UdR incorporation was determined in triplicate cultures and expressed as a relative percentage inhibition (IC₅₀ value) with dried free controls incubated under the same conditions.

³H-TdR Uptake Assay Responder cells were human PBL cells obtained from a healthy donor. Each well of the microplate received 2×10⁵ lymphocyte cells in 200 µl culture medium containing 1% PHA and the test compound. After 102 h incubation at 37 °C humidified in the usual way, 0.5 µBq ³H-TdR was added to each well. Cells were harvested 18 h later and the ³H-TdR uptake was measured as the same as described above.

RESULTS AND DISCUSSION

Relationship between Chemical Structure and Antimalarial Activity The *in vitro* antimalarial activity of 59 triterpenoids (1—59, Fig. 1) obtained during the studies on the triterpenic constituents of *Cimicifuga* spp. were examined against FCR-3 strain (*P. falciparum*) malarial cultures. Almost all compounds were found to have antimalarial activity in a final concentration range 1—56 µM (data not shown). The EC₅₀ values of cimicifugoside-related compounds, 1—19, and some potently active compounds, 20, 22, 36, 44, 46 and 49, among these triterpenoids were found to be in the concentration range 1.0—13.0 µM, while those of quinine, chloroquine and pyrimethamine as positive controls were 110, 18.0 and 1.0 nM (Table 1). These data suggest that cimicifugoside (1) and its related compounds have potent antimalarial activity among the triterpenoids of *Cimicifuga* species, although they were less potent than positive controls such as quinine, chloroquine and pyrimethamine by 2—3 orders of magnitude. Among the 25 triterpenoids which showed potent antimalarial activity, 19 (1—19) were classified in group I, 4 (36, 44, 46, 49) in group IV, and 2 (20, 22) in group II, while those classified in group V did not show such potent activity.

Their selective toxicity in terms of antimalarial activity (EC₅₀) and cytotoxicity against FR3A were not so high, while the positive control compounds had indexes of 450—12000. These results showed that 12 compounds, 1, 2, 4, 9, 10, 13, 14, 15, 16, 20, 22 and 46, have relatively potent cytotoxicity against FM3A, which seemed inconsistent with the conclusion of a previous report that 5 µg/ml (7.5 µM) cimicifugoside (1) had no effect on the growth of L-5178Y cells, cl 1D cells, Raji cells and HeLa S3 cells.⁹⁾ The EC₅₀ of 1 was 5.0 µM against FCR-3 and 30.0 µM against FM3A suggesting that 1 should be cytotoxic against both in these concentration range, although 1 inhibited thymidine uptake at almost one-thousandth the above concentrations, as mentioned later.

Nucleoside Transport Inhibitory Activity in Vitro The inhibitory effect on ³H-TdR uptake in Con A-stimulated mouse splenocytes was examined using 19 triterpenoids (Table 2). Nine compounds (1, 3—8, 10, and 12) of group I were found to have a strong ³H-TdR transport inhibitory effect on Con A-stimulated lymphocyte proliferation and their IC₅₀ values were in the concentration range 12—1050 ng/ml (17.8 nM—1.7 nM). Compounds 9 and 11, and 8 compounds, 21, 22, 35, 37—40, and 52, of other groups showed far weaker inhibitory effects.

Table 1. Antimalarial Activity and Cytotoxicity of Triterpenoids from *Cimicifuga* spp. and Their Derivatives

Compound	Antimalarial activity (EC ₅₀) ^{a)} (μ M)	Cytotoxicity (EC ₅₀) ^{b)} (μ M) (Cell growth) ^{c)}	Selective toxicity index ^{d)}
Cimicifugoside (1)	5.0	30.0 (50)	6
Actein (2)	10.0	>10.0 (90)	>1
Bugbanoside A (3)	2.6	>3.0 (97)	>1
Bugbanoside B (4)	10.0	>12.0 (100)	>2
(26 <i>S</i>)- <i>O</i> -Methylcimicifugoside (5)	1.3	2.2 (50)	2
(26 <i>R</i>)- <i>O</i> -Methylcimicifugoside (6)	3.0	4.5 (86)	1.5
(26 <i>S</i>)- <i>O</i> -Methylactein (7)	1.0	>1.4 (87)	>1
(26 <i>R</i>)- <i>O</i> -Methylactein (8)	1.8	3.3 (50)	2
(26 <i>S</i>)- <i>O</i> -Methylbugbanoside (9)	5.0	>5.3 (96)	>1
(26 <i>R</i>)- <i>O</i> -Methylbugbanoside (10)	6.0	>11.0 (81)	>2
(26 <i>S</i>)- <i>O</i> -Methylbugbanoside (11)	3.0	>2.9 (86)	>1
(26 <i>R</i>)- <i>O</i> -Methylbugbanoside (12)	9.0	>2.7 (100)	—
1-Ketobugbanoside A (13)	6.4	>6.4 (89)	>1
1-Ketobugbanoside B (14)	9.2	>9.2 (68)	>1
26-Deoxycimicifugoside (15)	3.1	>3.0 (100)	>1
Cimicifugoside lactone (16)	2.1	>6.7 (68)	>3
Cimicifugenin (17)	13.0	15.0 (50)	1
Bugbanogenin A (18)	8.0	—	—
Bugbanogenin B (19)	8.2	—	—
23- <i>O</i> -Acetylshengmanol-glc-xyl (20)	4.7	>11.0 (100)	>2
23- <i>O</i> -Acetyl-7 β -OH-shengmanol-xyl (22)	12.0	>27.0 (96)	>2
25- <i>O</i> -Acetylcimigenol-glc-xyl (36)	13.0	11.0 (50)	1
25- <i>O</i> -Acetylcimigenol (44)	1.7	1.5 (50)	1
25- <i>O</i> -Acetyl-1 α -OH-cimigen (46)	13.0	22.0 (50)	2
Cimigenol (49)	8.0	5.0 (50)	0.6
Quinine	0.11	50.0 (50)	450
Chloroquine	0.018	32.0 (50)	1780
Pyrimethamine	0.001	12.0 (50)	12000

a) *Plasmodium falciparum* [FCR-3 (ATCC30932)]; b) mouse mammalian cell (FM3A); c) FM3A cell growth % at final compound concentration; d) EC₅₀ for FM3A Cell/EC₅₀ for *Plasmodium falciparum* (FCR-3).

Table 2. IC₅₀ of the Triterpenes and Their Derivatives from *Cimicifuga* spp. on Con A-Stimulated Lymphocyte Proliferation (³H-TdR Uptake Assay)

Compound	IC ₅₀ (ng/ml)
Cimicifugoside (1)	12
Bugbanoside A (3)	1050
Bugbanoside B (4)	750
26(<i>S</i>)- <i>O</i> -Methylcimicifugoside (5)	210
26(<i>R</i>)- <i>O</i> -Methylcimicifugoside (6)	25
26(<i>S</i>)- <i>O</i> -Methylactein (7)	55
26(<i>R</i>)- <i>O</i> -Methylactein (8)	22
26(<i>S</i>)- <i>O</i> -Methylbugbanoside A (9)	7800
26(<i>R</i>)- <i>O</i> -Methylbugbanoside A (10)	660
26(<i>S</i>)- <i>O</i> -Methylbugbanoside B (11)	7700
26(<i>R</i>)- <i>O</i> -Methylbugbanoside B (12)	440
23- <i>O</i> -Acetylshengmanol-xyl (21)	10000
23- <i>O</i> -Acetyl-7 β -OH-shengmanol-xyl (22)	>10000
24- <i>O</i> -Acetylhydroshengmanol-7-xyl (35)	10000
25- <i>O</i> -Acetylcimigenol-xyl (37)	9900
25- <i>O</i> -Acetyl-1 α -OH-cimigenol-xyl (38)	10000
12 β -Hydroxycimigenol-xyl (39)	9500
7 β -Hydroxycimigenol-xyl (40)	8500
Cimiaceroside (52)	>10000

Mouse splenocytes (5 $\times$ 10⁵ cells/well) stimulated with Con A (5 μ g/ml). After incubation for 54 h, ³H-TdR was added. Cells were harvested after further incubation for 18 h.

These results were similar to the reported data of **1** and its related compounds in PHA-stimulated human peripheral lymphocytes and some other cell lines,^{7,8)} and suggested that the 16, 23:23, 26:24 25-triepoxy group in the side-chain was responsible for the activity. It is interesting that compounds, **6**, **8**, **10**, and **12**, with the 26*R* configuration were

Fig. 2. Effect of Cimicifugoside (1) on Mitogen-Induced Proliferation of Mouse Splenocytes

TdR (Con A), ³H-TdR uptake inhibition into Con A-stimulated splenocytes; UdR (Con A), ³H-UdR uptake inhibition into Con A-stimulated splenocytes; TdR (LPS), ³H-TdR uptake inhibition into LPS-stimulated splenocytes; TdR (PHA), ³H-TdR uptake inhibition into PHA-stimulated splenocytes, the procedures and conditions of the ³H-uptake inhibition assay are described in "Materials and Methods." Each point represents the mean $\pm$ S.E. of three experiments.

3—18 times more active than compounds, **5**, **7**, **9**, and **11**, with the 26*S* configuration.

Effect of Cimicifugoside (1) on Uptake of ³H-TdR, and ³H-UdR into Mitogen-Stimulated Human and Mouse Lymphocytes Cimicifugoside (**1**) inhibited ³H-UdR and ³H-TdR uptake into Con A-stimulated mouse splenocytes

Fig. 3. Relationship between ^3H -TdR Transport Inhibition (IC_{50}) and Antimalarial Activity (EC_{50}) of Compounds **1**, **3**–**12**, **21**, **22**, **37**–**40** and **52**

The procedures and conditions of the ^3H -TdR transport inhibition assay are described in "Materials and Methods," and the data on the transport inhibition and antimalarial activity are as described in Tables 2 and 3. An asterisk indicates significance by the bivariate normal distribution t -test, $p < 0.05$.

Table 3. Effects of Cimicifugoside (**1**) on Mitogen-Induced Proliferation of Different Cell Lines

Conc. (ng/ml)	% Inhibition	
	Human PBL ^{a)}	Mouse-splenocyte ^{b)}
2000	97.7	92.5
200	92.2	87.9
20	69.4	63.1
2	54.2	39.6
0.2	35.8	25.0

% Inhibition is expressed as a relative percentage of inhibition with dried free controls incubated under the same conditions; a) Human PBL (2×10^5 cells/well) stimulated with PHA (1%). After incubation for 54 h, ^3H -TdR was added. Cells were harvested after further incubation for 18 h; b) Mouse splenocytes (5×10^5 cells/well) stimulated with Con A ($5 \mu\text{g}/\text{ml}$). After incubation for 54 h, ^3H -TdR was added. Cells were harvested after further incubation for 18 h.

and PHA-stimulated human peripheral lymphocytes. Additionally, **1** inhibited the ^3H -TdR uptake in a dose-dependent manner into splenocytes stimulated with LPS and PHA (Fig. 2, Table 3). These results were similar to those reported with PHA-stimulated human peripheral lymphocytes.⁷⁾

Almost all tested compounds had antimalarial activity and 25 compounds (**1**–**20**, **22**, **36**, **44**, **46** and **49**) had EC_{50} values of 1.0 – $13.0 \mu\text{M}$. Among them, 9 (**1**, **3**–**8**, **10** and **12**) had a strong inhibitory effect on nucleoside transport into mitogen-stimulated splenocytes. Because the 9 compounds belong to group I, the partial structure, a 16, 23 : 23, 26 : 24, 25-triepoxy group in the side-chain may be involved in both biological activities. As shown in Fig. 3, there was a relationship between ^3H -TdR transport inhibition (IC_{50}) and antimalarial activity (EC_{50}) ($r = 0.679$). Statistical evaluation of this correlation coefficient was made using the bivariate normal distribution t -test.

Previously we reported that the cimicifugoside (**1**) and its derivatives were significant inhibitors of ^3H -TdR uptake into mitogen-stimulated lymphocytes,^{7,8)} and the mode of the inhibitory effect on ^3H -TdR uptake proved to be selective inhibition of nucleoside transport of mammalian cells, but not inhibition of DNA synthesis. Therefore, these compounds had

little effect on mammalian cell growth, such as human L-5178Y cells, cl 1D cells, Raji cells and HeLa S3 cells.⁹⁾ These results did not support the hypothesis that the inhibitors of nucleoside transport such as **1** and its congeners might selectively inhibit the cell growth of a malarial parasite, which relies solely on purine salvage for its survival.¹⁰⁾ *In vivo* experiments will be needed before a conclusion can be made whether the above inhibitors have selective antimalarial effects or not.

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