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In Vitro Inhibitory Effects of Hinokitiol on Proliferation of *Chlamydia trachomatis*

Hiroaki Yamano,[1,2] Tsutomu Yamazaki,[1,3]* Kozue Sato,[1] Sadashi Shiga,[1] Toshikatsu Hagiwara,[1] Kazunobu Ouchi,[4] and Toshio Kishimoto[1]

National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640,[1] Department of Research and Development, Fuji Latex Co., Ltd., Tochigi, Tochigi 328-0006,[2] Department of Pediatrics, Saitama Medical School, Iruma, Saitama 350-0495,[3] and Department of Pediatrics 2, Kawasaki Medical School, Kurashiki,[4] Japan

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The inhibitory effects of hinokitiol (β-thujaplicin) on *Chlamydia trachomatis* D/UW-3/Cx were shown by MIC, minimum lethal concentration (MLC), and preinoculation minimal microbicidal concentration assays using HeLa 229 cells. The MIC and the MLC were both 32 μg/ml. Further evaluation of hinokitiol as a topical agent against *C. trachomatis* is warranted.

Chlamydia trachomatis is the most frequent cause of sexually transmitted diseases (4). Considering possible drug resistance (9, 14, 15), the availability of several approaches such as topical microbicides (2, 3, 10, 11, 16) to control chlamydial infections would be useful. Hinokitiol (β-thujaplicin) is a tropolone-related compound that is present in the heartwood of several cupressaceae, such as *Chamaecyparis obtusa* Sieb. et Zucc and *Thuja plicata* D. Don. Hinokitiol has antimicrobial activity against several microorganisms (6), such as influenza virus (12), *Staphylococcus aureus*, *Staphylococcus epidermidis* (1, 7, 8, 13), and *Schistosoma mansoni* (5). The objective of this study was to clarify the in vitro inhibitory effects of hinokitiol on *C. trachomatis*.

Serovar D of *C. trachomatis* D/UW-3/Cx and HeLa 229 cells were used in this study. Hinokitiol (β-thujaplicin; Fig. 1) was kindly provided by Takasago Int. Co. (Tokyo, Japan). MIC, minimum lethal concentration (MLC), and preinoculation minimal microbicidal concentration (MCC) assays were used to determine the susceptibility of *C. trachomatis* to hinokitiol. The inclusion count (% of control) was determined by the following formula: inclusion-forming units (IFU) in test sample ÷ IFU in control × 100%.

After being inoculated onto a monolayer of HeLa 229 cells, 1×10^5 IFU per ml of *C. trachomatis* strains was incubated for 2 h at 37°C. Then the inocula were aspirated and 1 ml of the culture medium (Eagle's minimum essential medium containing 10% fetal calf serum and 1 μg per ml of cycloheximide) containing serially diluted hinokitiol was replaced. After incubation in 5% CO_2 at 37°C for 72 h, the cultures were fixed with methanol and stained with fluorescein isothiocyanate-conjugated monoclonal antibody specific for *C. trachomatis* (Syva MicroTrak; Syva Company, San Jose, CA). Inclusions were counted by fluorescence microscopy, and the condition of cells was noted. The MIC was defined as the lowest concentration at which no inclusions were found. The MLC was defined as the lowest concentration of hinokitiol that resulted in no inclusions after passage with hinokitiol-free culture medium.

The MCC assay is similar to the preinoculation method previously described by Lampe et al. (11). In brief, 1.0×10^4 IFU of *C. trachomatis* strain was incubated with serial dilutions of hinokitiol at 37°C for 60 min. The mixture was inoculated onto HeLa 229 cells and incubated at 37°C for 2 h. After the inocula were removed, culture medium without hinokitiol was added and incubated for 72 h. The preinoculation MCC was defined as the lowest concentration of hinokitiol at which no inclusions were found.

To determine whether hinokitiol had any effect on HeLa 229 cells before infection with *C. trachomatis*, we added 1 ml of serially diluted hinokitiol to HeLa 229 cells and incubated them at 37°C for 60 min. Hinokitiol was then removed, and *C. trachomatis* was inoculated as described above.

The toxic effect of hinokitiol on HeLa 229 cells was evaluated by a cell counting kit (Dojindo Laboratory Co., Ltd., Kumamoto, Japan), incorporating a colorimetric assay for cell proliferation and viability using 2-(4-Iodophenyl)-3-(4-

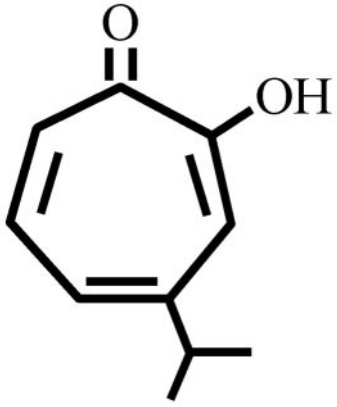

FIG. 1. Structural formula of hinokitiol (β-thujaplicin).

* Corresponding author. Mailing address: Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo, Japan 162-8640. Phone: 81-3-5285-1111. Fax: 81-3-5285-1208. E-mail: tochaben@gray.plala.or.jp.

TABLE 1. Inhibitory effect of hinokitiol on serovar D/UW-3/Cx of *C. trachomatis*

Assay	Mean ± SD inclusion count (% of control)[a] after exposure to hinokitiol concn (μg/ml) of:				
	2	4	8	16	32
MIC	81 ± 6	36 ± 1	28 ± 3	12 ± 3	0 ± 0
MLC	ND[c]	96 ± 12	56 ± 9	5 ± 2	0 ± 0
MCC	ND	ND	82 ± 1	74 ± 5	70 ± 4
Cell[b]	ND	ND	99 ± 5	100 ± 3	98 ± 3

[a] Inclusion count (% of control) = IFU in test sample ÷ IFU in control × 100%.
[b] Cell, preincubation with HeLa 229 cells prior to infection.
[c] ND, not done.

nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt (WST-1). Cell activity was determined in accordance with the manufacturer's instructions. In brief, serial dilutions of hinokitiol were dispensed onto microtiter plates containing HeLa 229 cells and incubated at 37°C for 72 h. After the hinokitiol solution was removed, cell activity was counted and the condition of the monolayer cells stained with Giemsa stain was assessed at ×400 magnification.

The results of MIC, MLC, and MCC assays are shown in Table 1. The MIC and the MLC of hinokitiol for *C. trachomatis* were both 32 μg/ml. The number of *C. trachomatis* inclusions in the MCC assay decreased with increasing concentrations of hinokitiol. At a concentration of 128 μg/ml of hinokitiol, the number of inclusions was 75% of that in control HeLa 229 cells. However, the number of inclusions decreased only 2% when HeLa 229 cells were preincubated with 32 μg/ml of hinokitiol prior to infection with *C. trachomatis* (Table 1). No morphological changes were observed in the HeLa 229 cells.

The cytotoxic effects of hinokitiol on HeLa 229 cells as assessed by the WST-1 assay are shown in Fig. 2. Cell activity decreased with increasing concentrations of hinokitiol. Although no decrease in activity was observed at hinokitiol concentrations of less than 1 μg/ml, activity decreased to 50% of the control value at 32 μg/ml of hinokitiol; however, no morphological changes were apparent by Giemsa staining at 32 μg/ml.

Results of the MIC assay demonstrate that hinokitiol has an in vitro inhibitory effect on *C. trachomatis* at a concentration of 32 μg/ml. However, the concentration of hinokitiol required for complete inhibition of chlamydial proliferation is relatively high in comparison to the MICs of antibiotics such as tetracyclines, macrolides, and fluoroquinolones. The MICs of hinokitiol for *S. aureus* were reported to vary as follows: 1.56 to 3.13 μg/ml (1), 8.2 μg/ml (7), and 6.25 to 12.5 μg/ml (8). For clinical applications, therefore, hinokitiol could be used topically.

Our MIC and MCC assays both showed that hinokitiol has antimicrobial activity against *C. trachomatis*, indicating inhibitory effects both outside and inside HeLa 229 cells. This implies the potential clinical utility of hinokitiol for the prevention of *C. trachomatis* infection. We suggest that continuous or repeated topical application of hinokitiol as an appropriate gel or jelly would be helpful in inactivating *C. trachomatis*.

Although changes in the morphology of HeLa 229 cells were not observed at 32 μg/ml of hinokitiol, cell activity decreased at hinokitiol concentrations of more than 4 μg/ml after an incubation of 72 h. In addition, preincubation of HeLa 229 monolayers with hinokitiol for 60 min had little effect on the number of chlamydial inclusions and no morphological changes were observed. The most appropriate concentration of hinokitiol for application to a mucous membrane remains to be determined.

In conclusion, hinokitiol has inhibitory effects on *C. trachomatis* and may be clinically useful as a topical drug; however, the mechanisms of its inhibitory activity should be investigated further.

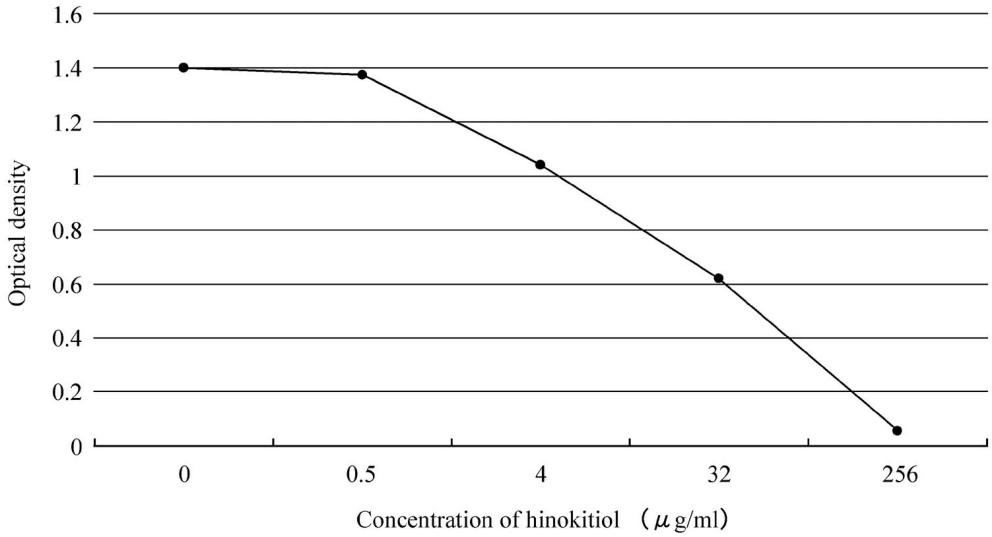

FIG. 2. Cytotoxic effects of hinokitiol on HeLa 229 cells, as assessed by the WST-1 assay. HeLa 229 cells were incubated with serial concentrations of hinokitiol in 5% CO_2 at 37°C for 72 h. Cell activity decreased to 50% at a concentration of 32 μg/ml of hinokitiol. However, morphological effects were not observed at this concentration by Giemsa staining.

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