In Vitro Inhibitory Effects of Hinokitiol on Proliferation of Chlamydia trachomatis

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Received 4 October 2004/Returned for modification 14 December 2004/Accepted 18 February 2005

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 Ch. 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% CO2 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).
morphological effects were not observed at this concentration by Giemsa staining.

concentrations of hinokitiol in 5% CO\textsubscript{2} at 37°C for 72 h. Cell activity decreased to 50% 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 \textit{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 \textit{C. trachomatis}, indicating inhibitory effects both outside and inside HeLa 229 cells. This implies the potential clinical utility of hinokitiol for the prevention of \textit{C. trachomatis} infection. We suggest that continuous or repeated topical application of hinokitiol as an appropriate gel or jelly would be helpful in inactivating \textit{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 \textit{C. trachomatis} and may be clinically useful as a topical drug; however, the mechanisms of its inhibitory activity should be investigated further.

![Image](http://aac.asm.org/)

**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\textsubscript{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.
REFERENCES


