Supporting Information

Inhibition of Herpes Simplex Virus Type 1 by Thymol-Related Monoterpenoids

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Cell culture

Vero cells were propagated and maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 5% heat-inactivated fetal bovine serum, penicillin, and streptomycin in an atmosphere of 5% CO$_2$ at 37°C.

Mode of antiviral activity

For the analysis of the mode of antiviral action, the test compounds were added individually at different stages during the viral infection cycle. For pretreatment of the virus, HSV-1 was incubated with the test compound for 1 hr at 4°C and then directly inoculated to the Vero cell. The cultures were incubated for 2 hr at 37°C. For analysing the antiviral inhibition during adsorption period, HSV-1 was mixed with the test compound and added to the cells immediately; adsorption of HSV-1 to the cells was carried out at 4°C for 2 hr. At this temperature, the virus can bind, but cannot penetrate the cells. To study the effect on virus penetration, the virus was bound to cells for 1 hr at 4°C. Test compounds were then added, and the cultures were shifted
to 37°C to initiate entry. After 1 hr of incubation, the cells were washed to remove the
drugs, and the wells were treated for 1 min with low-pH citrate buffer (pH 3.0) to
inactivate any remaining extracellular virus. To evaluate the effect against HSV-1
during the replication period, cells were infected with HSV-1 for 2 hr at 37°C, after
which the extracellular virus was washed away. Test compounds were then added, and
the cultures were incubated for an additional 1 hr at 37°C. All test compounds were
dissolved in DMSO and added to the medium at a final concentration of 1% DMSO.
Untreated controls always contained 1% DMSO in order to exclude any effect of
DMSO on cells or viruses. The level of infection was determined using a direct plaque
assay.

**Cytotoxicity assay**

XTT was dissolved in PBS to 1 mg/mL and menadione was dissolved in acetone to 1
mM. The XTT/menadione reagent was prepared fresh prior to each assay and
contained 12.5 parts XTT/1 part menadione. Vero cells were seeded at a density of
10^4 cells/well into 96-well microplates and grown at 37°C for 1 day. The culture
medium was replaced by fresh medium containing test compounds at various
concentrations. After incubation for 4 days, cells were rinsed with PBS, and an aliquot
of XTT/menadione reagent was added. The plate was re-incubated in the dark for an
additional 2 hr to allow formazan production. The colourimetric change at 490 nm
was measured with a microplate reader (Bio-Rad). The addition of the test compound
to XTT/menadione reagent in the absence of cells was used to rule out the false
positive reactions.
**Fig. 1S** Effects of thymol or carvacrol on cell viability. Vero cells were incubated with different concentrations of thymol, carvacrol, or acyclovir at 37°C for 4 days. The cell viability was determined by the XTT method. Data are expressed as the mean ± standard deviation of three independent experiments.

**Fig. 2S** Antiviral activities of varying concentrations of thymol and carvacrol against HSV-1. HSV-1 was pretreated for 1 hour on ice with increasing concentrations of thymol (black bars) or carvacrol (gray bars) and tested in a plaque reduction assay. The percent reduction was calculated relative to the amount of virus in the 1% DMSO-treated virus control. Data are expressed as the mean ± standard deviation of three independent experiments.

**Fig. 3S** Time-dependent activities of thymol and carvacrol against HSV-1. The virus was pre-incubated with 100 μM thymol (black bars) or carvacrol (gray bars) for different time intervals. The residual virus titer was determined by a plaque reduction assay. Data are expressed as the mean ± standard deviation of three independent experiments.
Fig. 1S

![Bar chart showing cell viability (% of control) for different concentrations of acyclovir, thymol, and carvacrol. The x-axis represents concentration (μM) and the y-axis represents cell viability. The data points are accompanied by error bars indicating the standard deviation.](image-url)
Fig. 2S

[Graph showing the percentage of plaque number in relation to serial concentration of indicated compounds, μM. The graph compares thymol and carvacrol concentrations.]
Fig. 3S

![Graph showing the percentage of plaque number over time for Thymol and Carvacrol.](image)