Cytotoxic Effect of Hinokitiol and Tropolone on the Growth of Mammalian Cells and on Blastogenesis of Mouse Splenic T Cells

Yoshihiko INAMORI,*,[a] Hiroshi TSUJIBO,[a] Hirofumi OHISHI,[a] Fumio ISHII,[b] Michinao MIZUGAKI,[b] Hisashi ASO[c] and Nakao ISHIDA[d]

Osaka University of Pharmaceutical Sciences,[a] Kawai, Matubara-shi, Osaka 580, Japan, Department of Pharmaceutical Sciences, Tohoku University Hospital,[b] Seiryo-machi 1-1, Sendai 980, Japan, Department of Animal Products, National Institute of Animal Industry,[c] Ikenodai 2, Kukizaki-cho, Inashiki-gun, Ibaraki 305, Japan and The Sendai Institute of Microbiology,[d] Toho Seimei Building 6F, Ichiban-cho 2-5-22, Aoba-ku, Sendai 980, Japan.

Received September 17, 1992

Hinokitiol (I) and tropolone (II) showed characteristic cytotoxic effects *in vitro* on five kinds of human and murine cell lines and blastic lymphocytes from mouse splenocytes. The cytotoxic effect of I on the growth of murine and human tumor cell lines, including RL♂-1, MH134, HL60, K562 and KATO-III was definite when examined by thymidine incorporation into DNA and its 50% inhibitory concentration (IC_{50}) on all cells was 0.3—0.6 μg/ml. Compound II also showed comparable cytotoxic effects on these cell lines, indicating a little lower activity when compared to I. Furthermore, I and II also completely suppressed the [^{3}H]thymidine ([^{3}H]TdR) incorporation of mitogen-induced blastic lymphocytes. The suppressive activity on mouse lymphocyte proliferative response to concanavaline-A was also found with both compounds at a low concentration of 0.32 μg/ml. As compound I is known to be of fairly low toxicity (LD_{50}: 453+24 mg/kg in mice), the antitumor and immuno-suppressive effect of hinokitiol (I) should be further investigated.

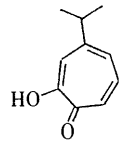
Keywords hinokitiol; tropolone; cytotoxic effect; mitogen response

Hinokitiol (I, Chart 1),[1] the constituent of the wood of *Chamacyparis taiwanensis*, belongs to tropolone (II, Chart 1)[2]-related compounds. For practical use, I was already reported to be effective as an antimicrobial agent[3-7] and a plant growth stimulator.[8] I was also used as a repellent for ticks[9] and II was used to prevent the spread of termites on logs.[10] The germination inhibition was also found with plant seeds treated with I.[11] Recently, the strong inhibitory effect of I and II on the growth of roots of several plants was reported.[12] These results may suggest the common site of action of the two compounds on microorganisms, plants and some insects. It has already been found that I, in spite of its low toxicity in animals,[13] exhibited the inhibitory effect on tumor cells, *e.g.* minimal cytopathogenic concentration is 10 μg/ml on colon 26 cells.[14] There are also papers on the inhibitory effect of II and its derivatives on tumor cells *in vitro*.[15-18] However, no particular work has been conducted to examine the extent of broad inhibitory effects of I in comparison with II.

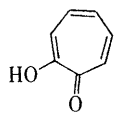
In this work, to develop the range of cytotoxic effects of tropolone-related compounds, the cytotoxic effects of I and II on five kinds of tumor cell lines and blastic lymphocytes were examined *in vitro*. Both compounds were also examined for their suppressive effect on the proliferative response of mouse splenic T cells to concanavalin-A (Con-A).

Materials and Methods

Materials Hinokitiol (I) and tropolone (II) were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Compounds I and II dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 mg/ml were stored at −80 °C.



hinokitiol (I)



tropolone (II)

Chart 1. Chemical Structures of I and II

Mice C3H/He male mice, six-week-old (specific pathogen free), were purchased from the Japan SLC, Hamamatsu, Shizuoka.

Medium Complete RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Flow, Northryde, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, was used throughout the study.

Cells The murine leukemic cells RL♂-1, murine hepatic cells MH134, human stomach cancer cells KATO-III, human leukemic cell K562 and human promyelocytic leukemic cell HL60 were maintained in complete RPMI-1640 medium.

T-Lymphocyte Proliferation Assay Spleen cells (5×10^5 cells/well) from C3H/He mice were incubated in 96-well, flat-bottom microplates for 3 d in a 37 °C, 5% CO_2 humidified chamber, with 2 μg/ml of Con-A (Sigma, St. Louis, MO). Cultures were pulsed with 1 μCi/well of tritiated thymidine ([^{3}H]TdR) for the last 4 h of incubation. Cells were collected with a multiwell harvester and the amount of incorporated radioactivity was counted. Each value was determined by triplicate assay.

Cytotoxicity Assay Five kinds of tumor cells (1×10^5 cells/well) were incubated in 96-well, flat-bottom microplates for 3 d in a 37 °C, 5% CO_2 humidified chamber, with various concentrations of I or II. Cultures were pulsed with 1 μCi/well of [^{3}H]TdR (ICN Biomedicals, California) for the last 4 h of incubation. Cells were collected with a multiwell harvester and the amount of incorporated radioactivity was counted with a liquid scintillation counter. Each value was determined by triplicate assay. Spleen cells from C3H/He mice were incubated in a 75 cm^2 flask for 3 d in a 37 °C, 5% CO_2 humidified chamber, with 2 μg/ml of Con-A. Cultures were harvested and washed 3 times in complete medium. Con-A stimulated cells (5×10^5 cells/well) were incubated in 96-well microplates for 8 h in a 37 °C, 5% CO_2 hummidified chamber, together with 0—5.0 μg/ml of I or II. Cultures were pulsed with 1 μCi/well of [^{3}H]TdR for the last 4 h of incubation. Cells were collected with a multiwell harvester and the amount of incorporated radioactivity was counted. Each value was determined by triplicate assay.

Results

Cytotoxic Activity of Hinokitiol (I) and Tropolone (II) on Cell Growth of Various Tumor Cells and Blastic Cells *in Vitro* For the first set of experiments, cytotoxic activity of I and II on the cell growth of five kinds of mammalian

© 1993 Pharmaceutical Society of Japan

NII-Electronic Library Service

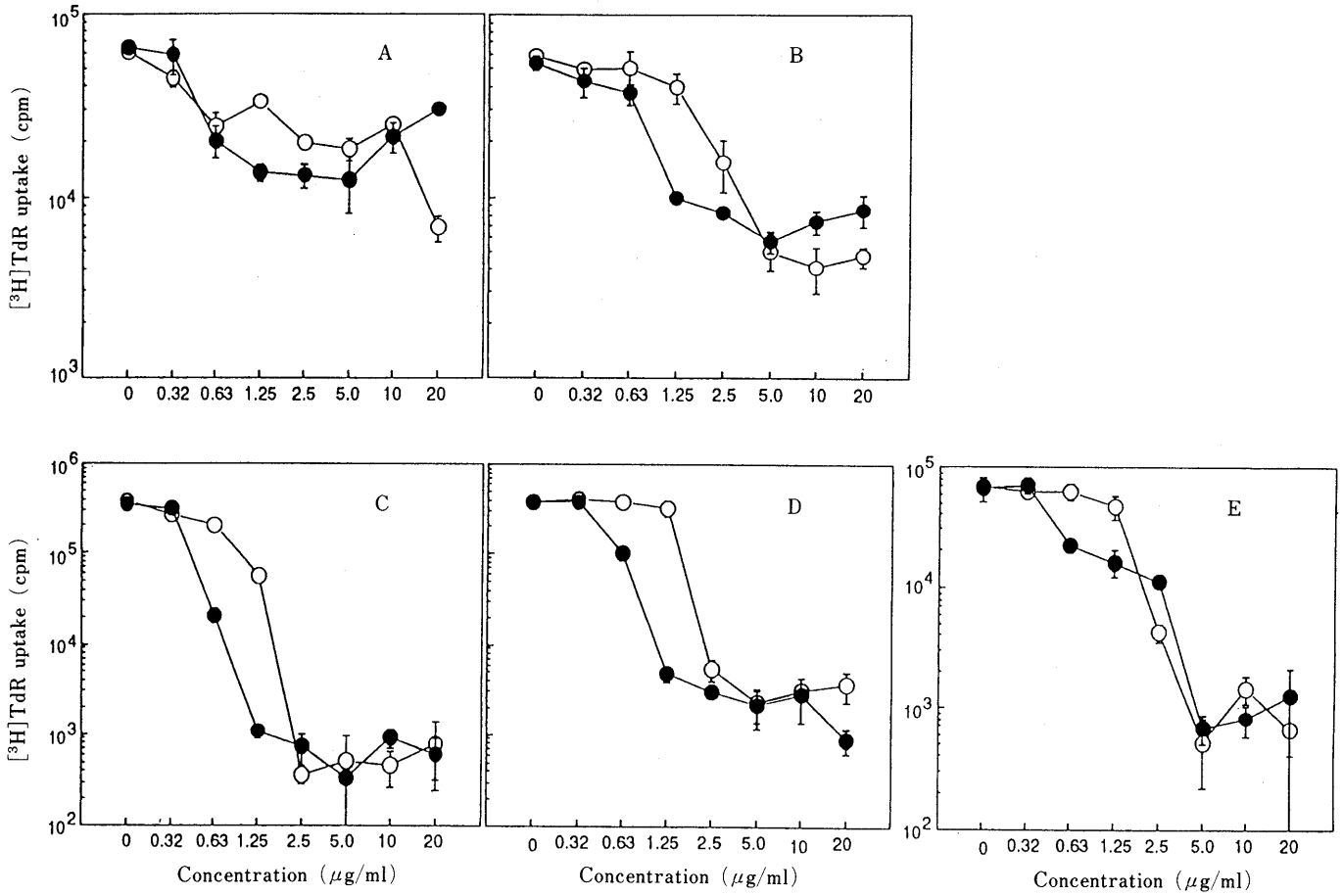


Fig. 1. Cytotoxic Effects of I and II on Cell Growth of Various Tumor Cells *in Vitro*
A: KATO-III. B: K562. C: HL60. D: MH134. E: RLδ-1. ●, I; ○, II.

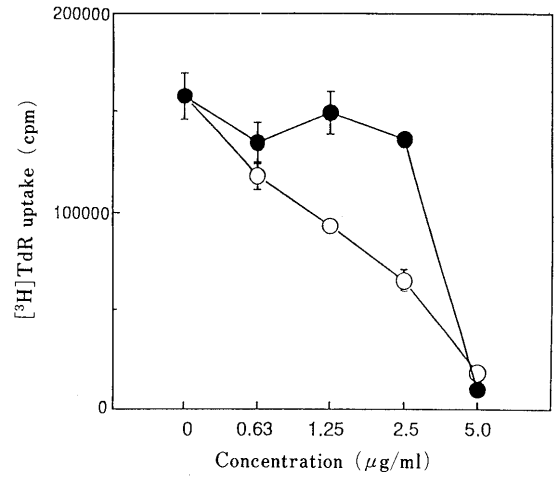


Fig. 2. Cytotoxic Effects of I and II on Blastic Splenocytes
●, I; ○, II.

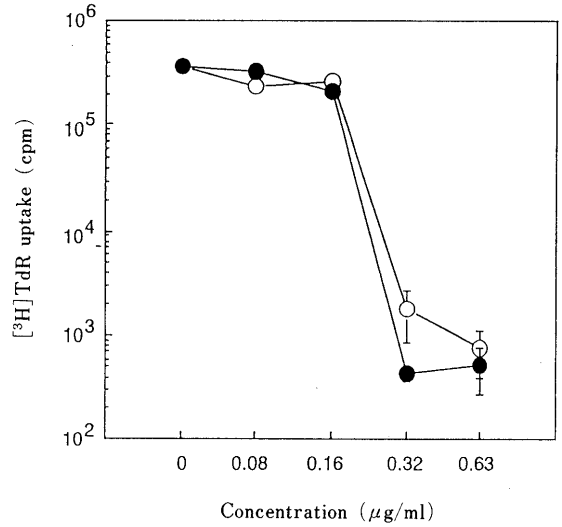


Fig. 3. Suppressive Activity of Con-A Response by I and II
●, I; ○, II.

tumor cells were examined *in vitro*. Compounds I and II were dissolved in DMSO at 25 mg/ml and were diluted in complete medium at 0.31—20 μg/ml. Final concentrations (0.0012—0.08% in complete medium) of DMSO at this time did not influence the cell growth of all tumor cell lines (data not shown). As shown in Fig. 1, the growth of tumor cells by I and II were suppressed in concentration-dependent fashion except for KATO-III cells. The 50% inhibitory concentration (IC_{50}) value of I existed between 0.3—0.6 μg/ml with 5 tumor cell lines. On the other hand, there was a difference of sensitivity against II between these cell lines. The dose required for its IC_{50} value on the cell growth of the same cell lines existed between 0.3—1.23 μg/ml. However, with treatment of 10 μg/ml of I and II, very little remained of the viable cells except for HL60 cells. These results were reproducible and suggested that the cytotoxic activity of I was more potent than II.

Next, we tested the cytotoxic activity of I and II to the blastic cells when Con-A stimulated splenic lymphocytes of normal mice. The blastic cells (5×10^5 cells/well) were

NII-Electronic Library Service

cultured together with I or II at 0.63—5.0 μg/ml for 8 h. As shown in Fig. 2, I and II showed definite cytotoxic activities on blastic cells of normal mice. At this time, influences of DMSO were not observed (data not shown).

Influences of I and II on the Blastogenesis Splenic Lymphocytes We examined the influences of I and II on the blastogenesis of mouse splenocytes in mitogen responses. Compounds I and II showed the definite suppressive activities on mitogen responses. The blastogenesis of splenocytes in mitogen responses was completely suppressed by 0.32 μg/ml of both I and II (Fig. 3).

Discussion

In our experiment, hinokitiol (I) and tropolone (II) showed definite cytotoxic effects *in vitro* on all tumor cell lines and blastic cells so far examined (Fig. 1). I was already reported to exhibit strong cytopathogenic effects on the established tumor cell line such as colon 26, RK and MDCK cells, and minimal cytopathogenic concentration was reported to be around 10 μg/ml.[14] However, a broader and much potent cytotoxic activity of I on various tumor cell lines and blastic cells of normal mouse splenocytes *in vitro* were first noticed in these experiments. When the low toxicity of I given intraperitoneally in mice[13] is taken into consideration, such strong cytotoxic effects of two compounds on mammalian tumor cell lines, blastic cells and blastogenesis of mouse splenic lymphocytes *in vitro* are of considerable interest. The cytotoxic activity of I against tumor cell growth and blastic splenocytes was higher than that of II (Figs. 1 and 2). However, the mechanisms of the cytotoxic effect of I and II are not fully understood.

A strong suppressive effect on mouse lymphocyte proliferation response to Con-A was also confirmed with both compounds at a low concentration of 0.32 μg/ml (Fig. 3). The findings suggest that both compounds may suppress the mouse lymphocyte immunity which is mediated by T cells. Judging from the suppressive effect of I and II on the immune response, both compounds are thought to be effective as immunosuppressive agents. Further basic studies on the immunological activities of I and II *in vitro* seem to be desirable.

References and Notes

1) T. Nozoe, *Bull. Chem. Soc. Jpn.*, **11**, 295 (1936).
2) M. J. S. Dewar, *Nature* (London), **155**, 50 (1945).
3) K. Okazaki and A. Homma, *Yakugaku Zasshi*, **74**, 174 (1954).
4) Y. Taga and M. Ozaki, *Sogo Igaku*, **12**, 1106 (1955).
5) S. Katsura, K. Tamura, S. Hatori and S. Maeda, *Tohoku J. Exp. Med.*, **49**, 357 (1948).
6) I. Shibasaki and G. Terui, *J. Ferment. Technol.*, **33**, 216 (1955).
7) H. Erdtmann and J. Gripenberg, *Nature* (London), **161**, 719 (1953).
8) A. Kafuku, Japan Kokai, 7622819 (1976) [*Chem. Abstr.*, **85**, 15349*z* (1976)].
9) H. Hara, Jpn. Kokai Tokkyo Koho Japan. Patent 01242508 (1988) [*Chem. Abstr.*, **89**, 153749*f* (1988)].
10) M. Fukada, Japan Kokai, 100696 (1974) [*Chem. Abstr.*, **82**, 81717*g* (1975)].
11) T. Okabe, Y. Otomo and K. Kasahara, Jpn. Kokai Tokkyo Koho Japan. Patent 153023 (1989) [*Chem. Abstr.*, **111**, 227241*p* (1989)].
12) Y. Inamori, K. Nishiguchi, N. Matsuo, H. Tsujibo, K. Baba and N. Ishida, *Chem. Pharm. Bull.*, **39**, 2378 (1991).
13) S. Ri, *Niigata Igakkai Zasshi*, **65**, 566 (1951).
14) T. Okabe, K. Saito and Y. Otomo, *A Technical Journal on Food Chemistry & Chemicals*, **2**, 45 (1988).
15) M. Yamato, Jpn. Kokai Tokkyo Koho Japan. Patent 59134744 (1984) [*Chem. Abstr.*, **101**, 210746*c* (1984)].
16) M. Yamato, Jpn. Kokai Tokkyo Koho Japan. Patent 59134745 (1984) [*Chem. Abstr.*, **101**, 210747*d* (1984)].
17) M. Yamato, K. Hashigaki, N. Kokubu, T. Tsuruo and T. Tashiro, *J. Med. Chem.*, **27**, 1749 (1984).
18) M. Yamato, K. Hashigaki, N. Kokubu, T. Tashiro and T. Tsuruo, *J. Med. Chem.*, **29**, 1202 (1986).

NII-Electronic Library Service