Growth Inhibition of Stilbenoids in Welwitschiaceae and Gnetaceae through Induction of Apoptosis in Human Leukemia HL60 Cells

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Fifty-six stilbenoids isolated from the families of Welwitschiaceae and Gnetaceae were screened for growth inhibitory activity against HL60 cells, and two compounds (gemonol G and gnetin I) among them exhibited a strong activity with IC_{50} of 10.0 μM and 12.2 μM at 48 h incubation, respectively. The growth suppression by gemonol G and gnetin I was found to be in part due to apoptosis which was assessed by morphological findings such as nuclear condensation and fragmentation, and DNA ladder formation in human leukemia HL60 cells.

Key words gemonol G; gnetin I; stilbenoid; Welwitschiaceae; Gnetaceae; apoptosis

Recent reports on stilbenoids have revealed several interesting biological activities such as anti-bacterial,1–3 anti-fungal,4,5 anti-inflammatory,6 anti-oxidant,7 and cancer prevention,8 and most recently the anti-cancer (apoptosis-inducing) activity of a stilbene tetramer (vaticanol C) from the family of Dipterocarpaceae.9 These findings have led us to screen stilbenoids contained in the families of Welwitschiaceae and Gnetaceae for anti-cancer activity. Welwitschia mirabilis is a monotypic member of the Welwitschiaceae, a weird plant with natural habitat only in the Namib Desert of West Africa. It is classified along with Gnetaceae and Ephedraceae in the Order Gnetale. Several stilbenoids have been isolated from the families of Welwitschiaceae and Gnetaceae, but none from Ephedraceae. The stilbene oligomers contained in these two families are unique in their structural formation. They are composed by heterogenic oligomerization of several stilbene monomers such as oxyresveratrol, isorhapontigenin, piceatannol and resveratrol, and sometimes occurred as glucosides. Many species of Gnetum are used as food as well as medicine.10–21 In the present study, we screened 56 compounds in the two families, and two compounds (gemonol G and gnetin I) were found to exhibit a significant growth inhibitory activity on HL60 cells with IC_{50} of 10.0 and 12.2 μM at 48 h incubation, respectively.

MATERIALS AND METHODS

Plant Materials The stem and root of a cultivated W. mirabilis was used for the experiment. Three species of Gnetum (G. gemonoides, G. gnemon and G. latifolium) were collected at Bogor Botanical Garden in Indonesia, G. parvipilum was obtained from Ishikawa Botanical Garden, Tokyo University, Tokyo, Japan and G. africanaum was collected at Nsukka, Nigeria. The voucher specimens were deposited at Gifu Prefectural Institute of Health and Environmental Sciences, Kakamigahara, Japan. The materials were extracted with acetone and methanol and the structures of the compounds were determined as previously described.9–21

Cell Culture and Treatments Human leukemia HL60 cells obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. 5—40 μM concentrations of the fifty-six compounds were prepared in DMSO and added to the cell culture with the final concentrations of DMSO (<0.4%) that showed no significant effect on the growth (data not shown). Viable cell number was measured by trypan-blue dye exclusion test using a Burker-Turk type cell count chamber.

Morphology Assay For the morphological examination of cell death, the cells were stained with Hoechst 33342 (Calbiochem, Sandiego, CA, U.S.A.). Hoechst 33342 was added to the cultured medium at a concentration of 5 μg/ml. After incubation for 30 min, the cells were collected and washed with phosphate buffered saline (PBS) and then observed under a fluorescence microscope, Olympus BX-50 (Olympus, Tokyo, Japan).

DNA Extraction and Agarose Gel Electrophoresis The cultured cells were treated with 20 μM concentration of either gemonol G or gnetin I and the control cells were treated with DMSO alone. The cells were collected and washed with PBS and then incubated at 37 °C overnight with 100 μM Tris–HCl (pH 7.4), 5 mM EDTA 200 μM NaCl, 0.2% SDS and 200 μg/ml proteinase K (TaKaRa, Ohtsu, Shiga, Japan) and then extracted with phenol/chloroform. DNA was precipitated with ethanol and treated with 0.1 mg/ml RNase (Sigma) and then analyzed by electrophoresis on 2% agarose gel.

RESULTS AND DISCUSSION

Fifty-six stilbenoids including stilbene oligomers and stilbene glucosides in W. mirabilis and five species of Gnetaceae were screened in vitro for anti-cancer activity on HL60 cells with resveratrol as a positive control. HL60 cells in 96-well plates (1×10^3 cells per well) were treated with the compounds at varying concentrations (5—40 μM). After 48 h incubation, we found that most of the stilbenoids induced cytotoxic activity, and that gemonol G and gnetin I (Fig. 1)
among them induced significant growth inhibition at the concentrations between 10 and 20 μM (Figs. 2, 3). The 50% inhibitory activity (IC50) values at 48 h incubation are shown in Table 1. The cytotoxic activity of both was approximately 2-fold higher than that of resveratrol. To examine the participation of gnemonol G and gnetin I in apoptotic inducing activity, we carried out the Hoechst 33342 nuclear staining in the cells treated with each compound. As shown in Fig. 4, the cells treated with each compound at 20 μM for 24 h exhibited chromatin condensation and fragmentation, indicating that these two compounds exhibited considerable cell growth inhibitory activity through apoptotic process. Furthermore, the DNA ladder formation was observed at 12 and 24 h after the same treatment (Fig. 5), which also confirmed that the two

Table 1. IC50 of Gnemonol G, Gnetin I and Some Selected Stilbenoids from *Welwitschia* and *Gnetum*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gnemonol G</th>
<th>Gnetin I</th>
<th>Gnetin C</th>
<th>Gnemonol K</th>
<th>Gnemonol L</th>
<th>Mirabiloside B</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>12.2</td>
<td>10.0</td>
<td>13.0</td>
<td>24.1</td>
<td>24.1</td>
<td>34.0</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Fig. 1. Chemical Structure of Stilbenoids in the Families of Welwitschiaceae and Gnetaceae

Fig. 2. Effect of Gnemonol G or Gnetin I on Cell Growth of Human Leukemia HL60 Cells

Time course of viable cell numbers after treatment with gnemonol G or gnetin I was evaluated by the trypan-blue dye exclusion test. Each value represents the mean of the results obtained in three independent experiments.

Fig. 3. Concentration-Dependent Cell Growth Inhibition at 48 h after Treatment with Gnemonol G or Gnetin I in HL60 Cells

Fig. 4. Gnemonol G or Gnetin I-Induced Apoptosis in HL60 Cells

Morphological features of HL60 cells treated with each compound for 24 h. The cells were stained with Hoechst 33342 (5 μg/ml) for 30 min and then examined by fluorescence microscopy. (a) cells treated with DMSO alone (control); (b) treatment with 20 μM gnemonol G; (c) treatment with 20 μM gnetin I.
compounds induced apoptotic cell death.

In conclusion, we have found that the stilbenoids with smaller molecular sizes have higher cytotoxic activity, at least in part, through apoptosis as compared with those with higher molecular sizes and that stilbene glucosides induced no significant cytotoxic activity.

REFERENCES

7) “Medicinal Herb Index in Indonesia,” PT. EISAI, Indonesia, 1995, p. 5.