Purification and Characterization of Eugeniin as an Anti-herpesvirus Compound from Geum japonicum and Syzygium aromaticum

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ABSTRACT

The hot-water extract of Geum japonicum has been shown to exhibit prophylactic and therapeutic anti-herpes simplex virus (HSV) activity in murine infection models. Eugeniin was purified as an anti-HSV compound from the extract and also was isolated from another herbal extract (Syzygium aromaticum) that had exhibited anti-HSV activity in mice. Thus the anti-HSV action of eugeniin was characterized. The effective concentration (5.0 μg/ml) for 50% plaque reduction of eugeniin for wild HSV type 1 (HSV-1) on Vero cells was 13.9-fold lower than its 50% cytotoxic concentration determined by a yield-reduction assay. Eugeniin also inhibited the growth of acyclovir-phosphonoacetic acid-resistant HSV-1, thymidine kinase-deficient HSV-1 and wild HSV type 2. Eugeniin as well as phosphonoacetic acid inhibited viral DNA and late viral protein syntheses in their infected Vero cells, but not cellular protein synthesis at its inhibitory concentrations. Purified HSV-1 DNA polymerase activity was inhibited by eugeniin noncompetitively with respect to dTTP. Its apparent K_i value for eugeniin was 8.2- and 5.8-fold lower than the K_i values of purified human DNA polymerases α and β, respectively. Thus one of the major target sites of inhibitory action of eugeniin is viral DNA synthesis; the inhibitory action for viral DNA polymerase activity was novel compared with anti-HSV nucleoside analogs.

We previously selected 12 herbal extracts with oral therapeutic antiviral activity against cutaneous HSV-1 infection in mice from 142 herbal medicines (Kurokawa et al., 1993b). Among the 12 herbal extracts, oral administration of four extracts augmented the therapeutic efficacy of ACV in mice, and showed potent anti-HSV-1 activity against ACV-PAAR resistant (AP') HSV-1 and wild HSV-2 strains in vitro and in vivo (Kurokawa et al., 1995a, b). These four herbal extracts also exhibited prophylactic efficacy against recurrent HSV-1 disease in mice (Kurokawa et al., 1997). Their anti-HSV action inhibited HSV DNA synthesis and the mode of anti-HSV action was different from those of ACV and PAA (Kurokawa et al., 1995b). Among these four herbal extracts, the EtOAc-extractable fraction of the herbal extract of Geum japonicum Thunb. exhibited anti-HSV-1 activity in a yield-reduction assay in vitro and a cutaneous HSV-1 infection model in mice (Kurokawa et al., 1993a). Thus, the EtOAc-

Anti-herpesvirus agents, mainly nucleoside analogs such as ACV, have been developed (Elion et al., 1977) and used for the treatment of HSV infection in humans (Dunkle et al., 1991; Fiddian et al., 1982; Whitley et al., 1991). However, the appearance of ACV-resistant HSV strains has become evident in immunosuppressed patients, such as organ transplant recipients and patients with acquired immunodeficiency syndrome (Birch et al., 1990; Coen, 1994; Erlich et al., 1989; Norris et al., 1988; Nugier et al., 1992; Oliver et al., 1989; Pelosi et al., 1992; Reusser et al., 1996; Sibrack et al., 1982). Those ACV-resistant viruses were also resistant to the other nucleoside analogs (Nugier et al., 1992). Thus the development of new therapeutic agents with different mode of anti-HSV action is required.

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ABBREVIATIONS: ACV, acyclovir; AP', acyclovir-phosphonoacetic acid-resistant; Ara-A, 9-β-D-arabinofuranosyladenine; BSA, bovine serum albumin; CC_50, 50% cytotoxic concentration; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EC_50, effective concentrations for 50% plaque reduction; EtOAc, ethylacetate; G, japonicum, Geum japonicum Thunb.; HEL, human embryonic lung; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; MEM, Eagle's minimum essential medium; PAA, phosphonoacetic acid; PFU, plaque-forming units; S. aromaticum, Syzygium aromaticum (L.) Merr. et Perry, SDS, sodium dodecyl sulfate; TK', thymidine kinase-deficient; UV, ultraviolet.
extractable fraction may contain anti-HSV compounds that are absorbed from alimentary tracts after its oral administration.

In this study, we isolated and identified an anti-HSV compound, eugeniin, from the herbal extracts of not only *G. japonicum* but also *Syzygium aromaticum* (L.) MERR. et PERRY, which are two of the four herbal extracts with anti-HSV activity in *vitro* and in *vivo* reported previously (Kurokawa *et al.*, 1993a, 1993b, 1995a, 1995b, 1997). Thus the action of the anti-HSV compound isolated coincidentally from the two different medicinal herbs was characterized. One of the major target sites of inhibitory action of eugeniin was found to be viral DNA synthesis. It showed higher specificity for the inhibition of HSV-1 DNA polymerase activity than that of cellular DNA polymerases. Its specificity for HSV-1 DNA polymerase as well as its therapeutic index was higher than those of Vidarabine (Ara-A) that has been used clinically for the treatment of herpetic disease (Coen *et al.*, 1982; Seidlin and Straus, 1984; Whitley *et al.*, 1986). The inhibitory action of eugeniin for HSV-1 DNA polymerase was noncompetitive, such as a non-nucleoside inhibitor of reverse transcriptase of HIV-1, Nevirapine (Carr and Cooper, 1996; Kilby and Saag, 1996; Kopp *et al.*, 1991; Romero *et al.*, 1991). Thus eugeniin may be a promising novel anti-HSV agent which is different from anti-HSV nucleoside analogs.

**Materials and Methods**

**Viruses and cell lines.** The HSV strains used were the wild-type 7401H HSV-1 (Kurokawa *et al.*, 1993b), TK− B2006 HSV-1 (Dubb and Kit, 1964), AP+ HSV-1 (Kurokawa *et al.*, 1995a) and the wild-type HSV-2 (Ito-1262) (Kurokawa *et al.*, 1995a, 1995b). These virus stocks were prepared from infected Vero cells as reported previously (Kurokawa *et al.*, 1995a, 1995b). Vero cells were grown and maintained in Eagle’s MEM supplemented with 5% and 2% calf serum, respectively. For the preparation of HSV-1 DNA polymerase and cellular DNA polymerases α and β, HEL cells were grown and maintained in MEM supplemented with 10% and 2% fetal bovine serum, respectively.

**Fractionation of herbal extracts and identification of purified compounds.** Anti-HSV compounds were purified from herbal extracts with use of different chromatographic fractionations guided by anti-HSV activity. Hot-water extracts were prepared from dried *G. japonicum* and then extracted with EtOAc and methanol extractions and successive chromatographic separations as reported by Takechi and Tanaka (1981).

**Plaque-reduction assay and cytotoxicity assay.** Fractions separated in each step for fractionations of herbal extracts were examined for anti-HSV activity in the plaque-reduction assay. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU of wild HSV-1 for 1 h. The cells were overlaid with 1 ml of nutrient methylcellulose (0.8%) medium containing various concentrations of samples and then cultured at 37°C for 2 days. The cells were fixed and stained, and the numbers of plaques were counted as described previously (Kurokawa *et al.*, 1995a). The effective concentrations for 50% plaque reduction (EC₅₀) were determined from a curve relating the plaque number to the concentration of samples.

Cytotoxicity of each fraction was examined by measuring its effect on the incorporation of [methyl-³H]thymidine into the DNA of Vero cells as described previously (Kurokawa *et al.*, 1995a). Vero cells (2.5 × 10⁴ cells/well) were grown in 24-well plates for 2 days. The culture medium was replaced with fresh medium containing 37 kBq/ml of [methyl-³H]thymidine (3.11 TBq/mmol, Amersham, Buckinghamshire, UK) and eugeniin at various concentrations. After an 18-h exponential growth period of the cells, the cells were lysed. The lysates were spotted onto paper filters, and then radioactivity on the washed and dried filters was determined in a liquid scintillation counter. For a growth-inhibition assay, HEL cells and Vero cells were seeded at a concentration of 5 × 10⁴ cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced by fresh medium containing eugeniin at various concentrations, and the cells were grown further for 2 days. The cells in triplicate wells for each concentration of eugeniin were treated with trypsin, and the number of viable cells was determined by a trypan blue exclusion test. The 50% cytotoxic concentration (CC₅₀) was determined graphically (Kurokawa *et al.*, 1995a).

**Yield-reduction assay.** Eugeniin was compared for its antiviral activity against wild HSV-1, AP+ HSV-1, TK− HSV-1 and wild HSV-2 strains in the yield-reduction assay. Monolayers of Vero cells in 25-cm² plastic flasks were infected with each HSV strain at 5 to 10 PFU/cell for 1 h. The cells were washed three times with MEM and incubated in maintenance medium containing various concentrations of eugeniin at 37°C for 24 h. The cultures were frozen and thawed and then centrifuged at 3,000 rpm for 15 min. Virus titers in culture medium were determined by the plaque assay on Vero cells (Kurokawa *et al.*, 1995a).

**Analysis of viral DNA synthesis.** The effects of eugeniin and PAA on viral DNA synthesis were compared in Vero cells infected with wild HSV-1 or AP+ HSV-1 strains. Monolayers of Vero cells in
Purification of eugeniin from herbal extracts. Eugeniin was isolated from the herbal extract of *G. japonicum*, and the chemical structure was identified by physicochemical analyses. The herbal extract was first extracted with EtOAc and the EtOAc-extractable fraction was further extracted with methanol to remove methanol-insoluble materials mainly elagic acid with cytotoxicity. The methanol-soluble fraction was separated into 63 fractions by Sephadex LH-20 column chromatography. The fractions 50 and 51 showed the strongest anti-HSV-1 activity among the 63 fractions, and their EC<sub>50</sub> values were 16.5 and 17.5 μg/ml, respectively (table 1). These fractions were separated further into six subfractions (P1 to P6, table 1) by HPLC. Among the six fractions, fraction P4 exhibited the strongest anti-HSV-1 activity (EC<sub>50</sub> value, 5.0 ± 0.61 μg/ml). Its CC<sub>50</sub> values for Vero and HEL cells were 69.3 ± 9.4, 66 and 77 μg/ml. These CC<sub>50</sub> values were 13.9-, 13.2- and 15.4-fold, respectively, higher than the EC<sub>50</sub> value. Chemical structure for a compound obtained from fraction P4 was identified as eugeniin (fig. 1), a 1,2,3-trigalloyl 4,6-hexahydroxydiphenoyl β-D-glucopyranose, molecular weight, 938 daltons), which is a light yellow amorphous solid showing a quasi molecular ion peak at m/z 939 [M+H]<sup>+</sup>; [α]<sub>D</sub>+64.5° (c = 0.067, acetone) and soluble in water. This compound was also isolated as an anti-HSV-1 compound from the herbal extract of *S. aromaticum* with anti-HSV activity in vitro and in vivo (Kurokawa et al., 1993a, 1995a, 1995b, 1997) (data not shown). We coincidentally purified eugeniin as a major anti-HSV compound from

### TABLE 1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; µg/ml</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; µg/ml</th>
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<tbody>
<tr>
<td>Fraction 50</td>
<td>16.5</td>
<td>–</td>
</tr>
<tr>
<td>Fraction 51</td>
<td>17.5</td>
<td>–</td>
</tr>
<tr>
<td>Fraction P1</td>
<td>&gt;20</td>
<td>–</td>
</tr>
<tr>
<td>Fraction P2</td>
<td>&gt;20</td>
<td>–</td>
</tr>
<tr>
<td>Fraction P3</td>
<td>&gt;20</td>
<td>–</td>
</tr>
<tr>
<td>Fraction P4 (eugeniin)</td>
<td>5.0 ± 0.61</td>
<td>69.3 ± 9.4** (66***,77****)</td>
</tr>
<tr>
<td>Fraction P5</td>
<td>16.5</td>
<td>–</td>
</tr>
<tr>
<td>Fraction P6</td>
<td>&gt;20</td>
<td>–</td>
</tr>
</tbody>
</table>
both extracts of *G. japonicum* and *S. aromaticum*, which belong to different families of plants.

**Anti-HSV activity of eugeniin.** Anti-HSV activity of eugeniin was compared with wild HSV-1, AP*HSV-1*, TK⁻HSV-1 and wild HSV-2 strains in the yield-reduction assay with Vero cells (fig. 2). All four strains used were similarly susceptible to eugeniin and their virus yields were reduced more than 1,100-fold in the presence of 20 μg/ml eugeniin compared with its absence. Thus this compound exhibited potent antiviral activity against AP*HSV-1*, TK⁻HSV-1 and wild HSV-2 strains as well as the wild HSV-1 strain.

**Effect of eugeniin on viral DNA synthesis.** Eugeniin was examined for the inhibition of viral DNA synthesis of wild HSV-1 and AP*HSV-1* strains in Vero cells by slot-blot hybridization to assess the mode of its anti-HSV action. As shown in figure 3, eugeniin dose-dependently inhibited DNA synthesis of wild HSV-1 and AP*HSV-1* strains, although AP*HSV-1 DNA was synthesized in the presence of PAA. These viral DNA syntheses were strongly suppressed in the presence of 20 μg/ml eugeniin, and the levels of DNA amounts detected were similar to those in the HSV-1-infected cells immediately after virus adsorption and in the presence of 150 μg/ml PAA, which indicated the complete inhibition of viral DNA synthesis. DNA syntheses of TK⁻HSV-1 and wild HSV-2 strains were not detectable in the presence of 20 μg/ml eugeniin either (data not shown). Eugeniin inhibited DNA synthesis of all HSV strains examined.

**Effect of eugeniin on viral protein synthesis.** Because PAA inhibits the synthesis of HSV DNA and late HSV proteins but permits the synthesis of early HSV proteins (Honess and Roizman, 1973; Honess and Watson, 1977), the synthesis of HSV proteins was examined in the presence of eugeniin and PAA to compare the effects of PAA and eugeniin on protein synthesis. As shown in figure 4 and table 2, the synthesis of late viral proteins was noticeably reduced in cells infected with wild HSV-1 and HSV-2 strains in the presence of 150 μg/ml PAA compared with its absence.

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**Fig. 2.** Inhibition of the growth of various HSV strains by eugeniin. Eugeniin was compared for its antiviral activity with wild HSV-1, AP*HSV-1*, TK⁻HSV-1 and wild HSV-2 strains in the yield-reduction assay. Vero cells were infected with the HSV strains at 5 to 10 PFU/cell and incubated in the presence of 0, 10, 20 and 30 μg/ml of eugeniin at 37°C for 24 h. The cultures were frozen, thawed and then centrifuged at 3,000 rpm for 15 min. Virus titers in the supernatants were determined by the plaque assay. The titers represent the average values with standard error for triplicate samples.

**Fig. 3.** Inhibition of DNA synthesis of various HSV strains by eugeniin. Eugeniin was examined for its effect on viral protein synthesis in Vero cells infected with wild HSV-1 or AP*HSV-1* strains. The cells were infected with the HSV-1 strains (5–10 PFU/cell) and then incubated in the presence of 0 (lane 2), 5 (lane 3), 10 (lane 4) and 20 (lane 5) μg/ml eugeniin and 150 μg/ml of PAA (lane PAA) at 37°C for 8 h. The cells were lysed and then DNA was prepared from the lysates. DNA was blotted to nylon filters at 6.25, 25, 100 and 400 ng/slot and fixed by UV irradiation. In lane 1, DNA was prepared from infected cells immediately after virus adsorption for 1 h. In lane 6, DNA was prepared from uninfected cells as a negative control. In lane pTK, plasmid pTK involving the DNA fragment of the HSV-1 TK gene was blotted at 10^4, 10^5 and 10^6 copies/slot as a positive control for HSV-1 DNA. In lane PAA, DNA prepared from infected cells in the presence of 150 μg/ml of PAA was blotted at 400 ng/slot. The filters were hybridized with denatured radioactive probes as described in the text. The hybridized filters were washed and the dried filters were exposed to X-ray films at –80°C.

**Fig. 4.** Inhibition of protein synthesis of various HSV strains by eugeniin. Eugeniin was examined for its effect on viral protein synthesis in Vero cells infected with HSV strains. The cells were mock-infected (M) or infected with wild HSV-1, AP*HSV-1* or wild HSV-2 strains and incubated in the presence of 0 (lane 1), 5 (lane 3), 10 (lane 4) and 20 (lane 5) μg/ml of eugeniin or 150 μg/ml of PAA (lane 2) at 37°C. The infected and mock-infected cells were labeled with [³⁵S]methionine and [³⁵S]cysteine for 5.5 to 7.5 h postinfection in the presence of eugeniin or PAA as described in the text. Immunoprecipitates from wild HSV-1, AP*HSV-1* and wild HSV-2 infected cells (Wild HSV-1, AP*HSV-1* and Wild HSV-2) and total cell lysates of mock-infected cells (Cell lysate) were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Marker proteins (30, 46, 69, 97.4 and 200 kdaltons) were co-electrophoresed in each gel. Arrow heads indicate late viral proteins whose production was reduced in the presence of eugeniin as well as PAA.

Eugeniin dose-dependently inhibited the synthesis of viral proteins such as 53-, 60-, 67-, 89- and 155-kdalton proteins for wild HSV-1 and 48-, 57-, 64-, 71-, 80-, 115- and 145-kdalton proteins for wild HSV-2. The quantitative analysis of...
autoradiograms showed that the intensities of those bands of wild HSV-1 and HSV-2 proteins synthesized in the presence of 20 μg/ml eugeniin were reduced similarly to those in the presence of 150 μg/ml PAA (table 2). In AP r HSV-1-infected cells, the synthesis of AP r HSV-1 proteins was not markedly modified in the presence or absence of 150 μg/ml PAA, but eugeniin dose-dependently reduced the synthesis of late viral proteins of AP r HSV-1 strain. The concentrations (5–20 μg/ml) of eugeniin used did not inhibit the synthesis of host cellular proteins (fig. 4 and table 2). Thus eugeniin as well as PAA selectively inhibited the synthesis of late HSV proteins without apparent cytotoxicity.

**Effect of eugeniin on DNA polymerase activity.** To examine the effect of eugeniin on the activity of HSV DNA polymerase, HSV-1 DNA polymerase was partially purified from wild HSV-1-infected HEL cells. As shown in fig. 5A, eugeniin inhibited the activity of HSV-1 DNA polymerase (apparent K_m value, 1.67 μM) noncompetitively with respect to dTTP, which indicates that the viral DNA polymerase is a target of eugeniin. Further cellular DNA polymerases α and β were partially purified from HEL cells to clarify the difference in the enzymological properties against eugeniin between HSV-1 DNA polymerase and cellular DNA polymerases. This compound inhibited the activity of cellular DNA polymerases α (apparent K_m value, 7.02 μM) and β (apparent K_m value, 13.1 μM) with respect to dTTP (fig. 5, B and C). However apparent K_i values (4.82 and 3.44 μM) of the α and β DNA polymerases for eugeniin were 8.2- and 5.8-fold higher, respectively, than that (0.59 μM) of the HSV-1 DNA polymerase. Thus eugeniin noncompetitively inhibited the activity of HSV-1 DNA polymerase more strongly than the activities of host cellular DNA polymerases.

**Discussion**

We have isolated an anti-HSV compound, eugeniin, from the extracts of *G. japonicum* and *S. aromaticum* with differential and chromatographic fractionations guided by antiviral assay in this study. In each step of the fractionations, we selected fractions showing the lowest EC_{50} value for the step of next fractionation. Thus eugeniin (EC_{50} 5 μg/ml in table 1), a phenolic compound with diphenyl group, was isolated as a major anti-HSV-1 compound which was quantitatively separable from the herbal extracts and of which the chemical structure was identified, although it is possible that the two herbs contain other minor anti-HSV compounds with much lower EC_{50} values than the EC_{50} of eugeniin. The CC_{50} values of eugeniin determined by [3H]thymidine uptake and/or growth inhibition assays with Vero and HEL cells were 66 to 77 μg/ml (table 1), which indicates the coincidence of cytotoxic concentrations by the different assays. The analysis of protein synthesis of host cells (fig.4 and table 2) showed that eugeniin inhibited late HSV protein synthesis but not cellular protein synthesis at 20 μg/ml and supported our EC_{50} and CC_{50} values. However Kashiwada et al. (1992) have reported a much lower cytotoxic concentration (2.74 μg/ml) for tumor cells by use of an assay condition with a longer exposure time (3 days) of eugeniin. Similarly, Fukuchi et al. (1989) reported that the cytotoxic concentration of ge-raniin, an anti-HSV compound with the EC_{50} value of 0.093

### TABLE 2

Quantitative analysis of representative viral proteins on autoradiograms

<table>
<thead>
<tr>
<th>Autoradiogram and Protein</th>
<th>Eugeniin</th>
<th>PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild HSV-1 155</td>
<td>6.31 (1)*</td>
<td>9.61 (1)</td>
</tr>
<tr>
<td>Wild HSV-1 89</td>
<td>15.2 (2.4)</td>
<td>8.78 (0.9)</td>
</tr>
<tr>
<td>Wild HSV-1 67</td>
<td>15.4 (2.4)</td>
<td>14.8 (1.3)</td>
</tr>
<tr>
<td>Wild HSV-1 60</td>
<td>9.01 (1.4)</td>
<td>2.73 (0.3)</td>
</tr>
<tr>
<td>Wild HSV-1 53</td>
<td>9.98 (1.6)</td>
<td>11.7 (1.2)</td>
</tr>
<tr>
<td>AP r HSV-1 155</td>
<td>7.49 (1)</td>
<td>9.00 (1)</td>
</tr>
<tr>
<td>AP r HSV-1 89</td>
<td>15.3 (2.0)</td>
<td>7.38 (0.8)</td>
</tr>
<tr>
<td>AP r HSV-1 67</td>
<td>13.7 (1.8)</td>
<td>12.3 (1.4)</td>
</tr>
<tr>
<td>AP r HSV-1 60</td>
<td>11.5 (1.5)</td>
<td>2.62 (0.3)</td>
</tr>
<tr>
<td>AP r HSV-1 53</td>
<td>8.43 (1.1)</td>
<td>4.57 (0.5)</td>
</tr>
<tr>
<td>Wild HSV-2 145</td>
<td>10.7 (1.7)</td>
<td>11.0 (0.7)</td>
</tr>
<tr>
<td>Wild HSV-2 125</td>
<td>6.37 (1)</td>
<td>16.6 (1)</td>
</tr>
<tr>
<td>Wild HSV-2 115</td>
<td>8.31 (1.3)</td>
<td>11.6 (0.7)</td>
</tr>
<tr>
<td>Wild HSV-2 80</td>
<td>14.3 (2.2)</td>
<td>7.02 (0.8)</td>
</tr>
<tr>
<td>Wild HSV-2 71</td>
<td>5.16 (0.8)</td>
<td>3.42 (0.2)</td>
</tr>
<tr>
<td>Wild HSV-2 64</td>
<td>12.1 (1.9)</td>
<td>2.69 (0.2)</td>
</tr>
<tr>
<td>Wild HSV-2 57</td>
<td>6.58 (1.0)</td>
<td>2.15 (0.1)</td>
</tr>
<tr>
<td>Wild HSV-2 48</td>
<td>8.64 (1.4)</td>
<td>3.06 (0.2)</td>
</tr>
<tr>
<td>Cell lysate 190</td>
<td>2.45 (0.5)</td>
<td>2.01 (0.4)</td>
</tr>
<tr>
<td>Cell lysate 105</td>
<td>5.13 (1)</td>
<td>5.04 (1)</td>
</tr>
<tr>
<td>Cell lysate 68</td>
<td>7.62 (1.5)</td>
<td>8.86 (1.8)</td>
</tr>
</tbody>
</table>

Percent of scanned total area. Asterisks (*) indicate the ratio of the percent of each protein to that of 155-kdalton protein for wild HSV-1 and AP r HSV-1, 125-kdalton protein for wild HSV-2 and 105-kdalton protein for cell lysate.
μg/ml, is more than 30 μg/ml, whereas Kishiwada et al. (1992) reported that it is 0.35 μg/ml. It is necessary to clarify that an antiviral agent should show direct antiviral activity by inhibiting virus replication cycle but not by cytotoxicity. Therefore the anti-HSV activity assay and cytotoxic assay should be done in the same assay conditions. Thus the culture cells and exposure time used by Kishiwada et al. (1992) may not be appropriate for anti-HSV activity assay, and the discrepancy in cytotoxicity may be caused by the difference in these assay conditions. In our experiments, eugeniin was not
cytotoxic in the condition that anti-HSV activity was examined, which indicates that the anti-HSV activity of eugenin was not caused by its cytotoxicity.

Eugenin inhibited the growth of AP HSV-1, TK- HSV-1 and wild HSV-2 strains as well as wild HSV-1 (fig. 2). This suggests that eugenin showed a different mode of anti-HSV action from ACV and PAA. In this respect, the relationship between eugenin and ACV may correspond to that between nucleoside analogs (zidovudine and didoxycyanosine) and a non-nucleoside inhibitor (Nevirapine) in the treatment of HIV-1 infection (Hammer, 1997; ACV and zidovudine as well as didoxycyanosine are strong chain terminators, whereas eugenin and Nevirapine are noncompetitive inhibitors of DNA polymerase and reverse transcriptase, respectively. Eugenin may be a possible candidate or mother compound of a promising anti-HSV agent with a different anti-HSV action from nucleoside analogs.

Eugenin exhibited anti-HSV activity by the inhibition of HSV DNA synthesis (figs 2 and 3). The synthesis of late HSV proteins were inhibited in the presence of 20 µg/ml of eugenin as well as PAA at 150 µg/ml, in which the growth of HSV and synthesis of HSV DNA were inhibited almost completely (figs 2, 3 and 4). Because PAA specifically inhibits the synthesis of HSV DNA but permits the synthesis of early HSV proteins (Hones and Roizman, 1973; Hones and Watson, 1977), eugenin was suggested to have anti-HSV action similar to PAA that inhibits the synthesis of HSV DNA selectively. Eugenin inhibited the partially purified HSV DNA polymerase in vitro (fig 5), and therefore, one of the major sites of its inhibitory action may be viral DNA synthesis.

Eugenin inhibited the activity of HSV-1 DNA polymerase more strongly than cellular DNA polymerases a and b (fig 5). The apparent Km values for HSV-1 DNA polymerase and cellular DNA polymerases a and b used in this study were within the range of those reported elsewhere (Allaudeen, 1985; Coen et al., 1982; Frank et al., 1985; Larder et al., 1983; Merta et al., 1990; Nishiyama et al., 1984), which indicates that they were successfully purified. The ratios of Km values of cellular DNA polymerases a and b for eugenin to that of HSV-1 DNA polymerase were 5.8 and 8.2, respectively. In contrast, the ratios for Ara-A ranges 3 to 4 (Coen et al., 1982). The higher specificity of eugenin for HSV-1 DNA polymerase was consistent with a higher therapeutic index (CC50/EC50: 13.2 to 15.4, table 1) of eugenin than that of Ara-A (1, De Clercq et al., 1980). Thus the inhibitory activity of eugenin on HSV-1 DNA polymerase was confirmed with higher specificity than that of Ara-A. Eugenin behaved as a noncompetitive inhibitor of both HSV-1 and cellular polymerases. Noncompetitive-type inhibition has been reported for the inhibition of DNA polymerase a by Evans blue (Nakane et al., 1988). Recently, Nevirapine, a unique noncompetitive-type and non-nucleoside inhibitor of reverse transcriptase of HIV-1, has been used for the treatment of HIV-1 infection (Carr and Cooper, 1996; Grob et al., 1997; Kilby and Saag, 1996; Kopp et al., 1991; Romero et al., 1991; Smerdon et al., 1994). The activity of these polymerases was mainly inhibited allosterically in the presence of those inhibitors. Because eugenin exerted differential inhibitory activity between HSV-1 and cellular DNA polymerases in the reaction mixtures with the same DNA template, nonspecific interaction between DNA and eugenin such as intercalation into DNA may not occur strongly. Because Km value of DNA polymerase b for eugenin in the presence of BSA at 500 µg/ml was similar to that of DNA polymerase a in its absence (fig. 5), eugenin may not interact with proteins nonspecifically. Thus eugenin may interact selectively with DNA polymerases and/or the template-primer itself. Biochemical analysis of the inhibitory mechanism is underway.

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References


Anti-herpesvirus Activity of Eugeniin


