Inhibition of HIV-1 Protease and RNase H of HIV-1 Reverse Transcriptase Activities by Long Chain Phenols from the Sarcotestas of Ginkgo biloba

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Abstract

Nine long-chain phenols: four cardanols (1–4), two bilobols (5, 6) and three alkylsalicylic acids (7–9) were isolated from the CH2Cl2 extracts of the sarcotestas of Ginkgo biloba as HIV-1 protease (PR) inhibitors. From these phenols, the bilobols (IC50, 2.6–5.8 μM) and alkylsalicylic acids (IC50, 10.2–24.9 μM) exhibited dose-dependent potent inhibitory activities on HIV-1 PR, while the cardanols did not. On the other hand, only the alkylsalicylic acids (IC50, 33.7–170.3 μM) inhibited the activities of RNase H of HIV-1 reverse transcriptase (RT), while all of the compounds failed to affect the RNA dependent DNA polymerase (RDDP) of HIV-1 RT. Therefore, we regard bilobols as a new class of inhibitors against HIV-1 PR; in addition, alkylsalicylic acids are elucidated as a new class of inhibitors against HIV-1 PR and RNase H of HIV-1 RT.

Key words

Ginkgo biloba - Ginkgoaceae - long-chain phenol - bilobol - alkylsalicylic acid - HIV-1 protease - RNase H of HIV-1 reverse transcriptase

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

While screening medicinal plants for HIV-1 PR, the CH2Cl2 extract of Ginkgo biloba sarcotestas displayed potent activity with 85.3% inhibition at 100 μg/mL. The long-chain phenols from G. biloba sarcotestas have been previously reported as potent inhibitors of phospholipase C/γ1 and cancer cell proliferation [5], but the compounds responsible for its HIV-1 PR inhibitory activity have not been reported. Therefore, through a bioactivity-guided fractionation and isolation technique, nine components were isolated from these sarcotestas for determination of the HIV-1 PR inhibitors (Fig. 1). Their structures were resolved as four cardanols (1–4), two bilobols (5, 6) and three alkylsalicylic acids (7–9) by comparison with the reported spectral data [5], [6], [7]. These phenolics are all known. Moreover, these phenolic compounds from G. biloba were evaluated for their ability to inhibit HIV-1 PR activities in vitro (Table 1). The bilobols and alkylsalicylic acids showed dose-dependent inhibitory activities against HIV-1 PR with IC50 values ranging from 2.6 to 24.9 μM. Meanwhile, cardanols (1–4), which have a hydroxy group, did not show any inhibitory activity. Bilobols (5 and 6, IC50, 2.6–5.8 μM), possessing two hydroxyl groups at the meta position, exhibited more potent activities toward HIV-1 PR than alkylsalicylic acids (7–9, IC50, 10.2–24.9 μM), possessing a hydroxy group and a carboxylic group. These results suggest the importance of additional hydroxyl or carboxyl groups for HIV-1 PR inhibition. As mentioned above, the HIV-1 RT may be an important target for AIDS chemotherapy. Only a few substance groups, such as naphthoquinones (IC50, 9.5 μM), polyphenolics (39.0 μM), illimaquinone (30.0 μM), novenamies, a degradation product of cephalosporin (≥ 50.0 μM), polyethylene sulfonic acid, azidothymidylate (50.0 μM) and sulphated polyanions (0.04–0.1 μg/mL) [3], [8], [9], have been reported as RNase H inhibitors of RT. Therefore, a new class of inhibitor against this enzyme would greatly contribute to combination AIDS chemotherapy. These compounds were also tested for their activities on RNA-dependent DNA polymerase (RDDP) and RNase H of HIV-1 RT. None of the compounds inhibited RDDP of HIV-1 RT in vitro. However, on the HIV-1 RNase H enzyme, the three alkylsalicylic acids (7–9) exhibited dose-dependent inhibitory effects with IC50 values ranging from 33.7 to 170.3 μM in vitro, while the other cardanols and bilobols did not. This fact also supports that the carboxylic group on the aromatic moiety of these long-
chain phenols might play key roles for the inhibitory action of HIV-1 RNase H. These long-chain phenols are involved in a broad range of biological activities. Not only are they therapeutically desired anti-microbial, anti-tumor, and phospholipase C inhibitors exhibited, but also undesired allergic and immunotoxic reactions can be seen [10], [11], [12]. Nevertheless, as mentioned above, the development of new therapeutic AIDS agents is urgently needed.

In our results, interestingly, these bilobols showed more selective inhibitory activities toward HIV-1 PR than either RDDP or RNase H of HIV-1 RT. In other words, bilobols could be presented as a new class of selective HIV-1 PR inhibitors. In addition, alkylsalicylic acids might also represent a new class of HIV-1 PR and RNase H inhibitors of HIV-1 RT. Therefore, bilobols and alkylsalicylic acids could be candidates for combination chemotherapeutic AIDS agents.

**Materials and Methods**

The fresh sarcotestas of *Ginkgo biloba* L. (Ginkgoaceae) were collected from Ginkgo trees grown around the Kwanak Campus of the Seoul National University, Seoul, Korea, in October, 1999. A voucher specimen (SNUPH-0053) has been deposited in the herbarium of Seoul National University. Nine long-chain phenols were isolated as pure compounds from the sarcotestas of *G. biloba* using the method described previously [5]. Briefly, the fresh sarcotestas (0.5 kg) were extracted with CH2Cl2 in an ultrasonic apparatus for 3 hours to yield the CH2Cl2 extract (10.5 g) upon removal of the solvent *in vacuo*. The CH2Cl2 extract was subjected to silica gel, Sephadex LH-20, semipreparative HPLC to isolate compounds 1 (9 mg), 2 (20 mg), 3 (11 mg), 4 (5 mg), 5 (8 mg), 6 (9 mg), 7 (120 mg), 8 (28 mg) and 9 (13 mg). The HIV-1 protease (PR) assay was performed as described by Ma et al. [13]. The proteolytic activity against HIV-1 PR of the test samples was determined using HPLC with the synthetic heptapeptide [His-Lys-Ala-Arg-Val-Leu-(pNO2-Phe)-Glu-Ala-Nle-Ser-NH2] as substrate, which was purchased from BACHEM Feinchemikalien AG. The fused recombinant HIV-1 PR was obtained as reported before. To a reaction mixture (25 μL) containing 50 mM acetate buffer (pH 5.0), 2.5 μg of a substrate and 2.5 mL of a DMSO solution of test compound, and 2.5 μL of recombinant HIV-1 PR (0.175 μg protein) were added and the reaction was incubated at 37°C for 15 minutes. The reaction was stopped by addition of 2.5 μL of 10% trifluoroacetic acid. The hydrolysate and remaining substrate were quantitatively analyzed by HPLC. The HPLC system was composed of an LC9A liquid chromatograph, SPD-6A UV spectrophotometric detector, SLC-6B autoinjector and an integrator C-R6A Chromatopac (Shimadzu Corporation). Five microliters of the reaction mixture were injected into an RP-18 column (4.6 × 150 mm, YMC), eluted with a gradient of acetonitrile (15–40%) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The elution profile was monitored at 280 nm. The substrate and p-NO2-Phe-boryl hydrolysate were eluted at 11.55 and 6.6 minutes, respectively. Acetyl-pepstatin, which was widely used as positive control (Bachem), showed an IC50 of 0.09 μM under these conditions.

The RNase H of HIV-1 reverse transcriptase (RT) assay was performed as described by Roya et al. [3], [8]. HIV-1 RT (Eiken) was adjusted to 3.3 units/μL with a solution of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl2 and 2.5 mM dithiothreitol (DTT). The reaction mixture (20 μL) containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl2, 2.5 mM DTT and approximately 7.2 nM of [3H] poly(rA)poly(dT), and 1.0 μL of a test compound dissolved in dimethyl sulphoxide (DMSO, final concentration of 5%) were preincubated at 37°C for 5 minutes. Then 3.3 units of HIV-1 RT were added and the mixture was incubated for 2 hours at 37°C. A blank reaction was carried out under the same conditions without adding enzyme and a control reaction was included without test compound. The reaction was terminated by addition of 20 μL of 0.02 M EDTA. The mixture was applied onto a DEAE-cellulose disc, which was washed batchwise with 3 mL of 5% Na2HPO4, distilled water three times, ethanol once and ether once. The paper disc was then dried and immersed in 3 mL of scintillation fluid. RNase H activity was measured as the inhibition of the degradation of RNA in a hybrid in the presence of a test compound. Illimaquinone (Sigma) was used as a positive control, which inhibited RNase H activity with an IC50 of 50 μM under the above conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HIV-1 Protease (HIV-1 PR)</th>
<th>IC50 value (μM)a</th>
<th>RNA dependent DNA polymerase (HIV-1 RT)</th>
<th>RNase H (HIV-1 RT)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;200</td>
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<td>2</td>
<td>&gt;100</td>
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<td>&gt;100</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>5</td>
<td>2.6 ± 0.3</td>
<td>&gt;100</td>
<td>&gt;200</td>
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</tr>
<tr>
<td>6</td>
<td>5.8 ± 1.1</td>
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<td>&gt;200</td>
<td>&gt;200</td>
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<tr>
<td>7</td>
<td>24.9 ± 2.2</td>
<td>&gt;100</td>
<td>33.7 ± 1.5</td>
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</tr>
<tr>
<td>8</td>
<td>17.8 ± 1.1</td>
<td>&gt;100</td>
<td>58.5 ± 0.6</td>
<td>&gt;200</td>
</tr>
<tr>
<td>9</td>
<td>10.2 ± 0.5</td>
<td>&gt;100</td>
<td>170.3 ± 0.7</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Acetyl-pepstatin</td>
<td>0.09 ± 0.01</td>
<td>NTb</td>
<td>0.28 ± 0.02</td>
<td>NTb</td>
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<tr>
<td>Nevirapine</td>
<td>NTb</td>
<td>50.0 ± 1.6</td>
<td>NTb</td>
<td>50.0 ± 1.6</td>
</tr>
</tbody>
</table>

a Data are expressed as mean ± SE of three experiments.
b Not tested.

* Table 1 Inhibitory activities against HIV-1 protease, RNA dependent DNA polymerase of HIV-1 reverse transcriptase (RT) and RNase H of HIV-1 RT in vitro by compounds 1–9 from *G. biloba*.
previously [14]. Briefly, 20 μL of the reaction mixture containing a homogenous template/ primer hybrid, (αT)n(dT)15 and a triphosphate substrate, dUTP/dTTP, were added to the wells of a streptavidin-coated microtiter plate that contained 20 μL of test sample solution and 4 ng of the HIV-1 RT in 20 μL of lysis buffer. Final concentrations of the template/ primer hybrid and triphosphate substrate (dUTP/dTTP) were 750 μM at 260 nm/mL and 10 μM, respectively. The reaction was carried out at 37°C for 1 hour and was followed by the addition of each 200 μL solution of anti-digitoxigenin-peroxide, and ABTS [2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt] substrate for a coloring reaction. The absorbance of each well was recorded at 405 nm with the reference wavelength at 490 nm. Nevirapine (Viramune, Boehringer-Ingelheim Pharma KG) was used as a reference compound with an IC50 of 0.28 μM under the above conditions.

Supporting information

The spectral data of compounds 1–9 are available as Supporting Information.

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Bibliography

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