SHORT COMMUNICATION

Inhibitory Effects of Antrodins A–E from Antrodia cinnamomea and Their Metabolites on Hepatitis C Virus Protease

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Antrodia cinnamomea is a highly valued folk medicine used for liver cancer, a disease often caused by the long term infection of hepatitis C virus (HCV). In the present study, the maleic and succinic acid constituents (antrodins A–E) of this medicinal fungus, the in vivo metabolites of antrodin C and the analogue of one of the metabolites were tested for their inhibitory activity on HCV protease. Most of the compounds showed potent inhibitory activity, with antrodin A being the most potent (IC50 = 0.9 μg/mL). Antrodin A was isolated as one of the constituents of A. cinnamomea and was also detected as an in vivo metabolite of the major constituent antrodin C. The mode of inhibition for antrodin A on HCV protease was revealed by a Lineweaver-Burk plot as competitive inhibition. These results strongly support the use of this folk medicine for liver cancer and HCV infection which is a global problem. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Antrodia cinnamomea; Basidiomycetes, antrodin, hepatitis C virus, HCV protease

INTRODUCTION

It is estimated that approximately 3% of the world’s population is infected with hepatitis C virus (HCV). In developed countries, chronic hepatitis C is the leading cause for cirrhosis, hepatocellular carcinoma and liver transplantation (Staub and Stadlbauer, 2006). The protease of hepatitis C virus is essential for the maturation of the virus by processing the viral nonstructural polyprotein to functional proteins and thus represents one of the attractive therapeutic targets for developing antiviral agents against HCV (Kakiuchi et al., 1999).

The fruiting body of Antrodia cinnamomea Chang & WN Chou (Basidiomycetes, synonym A. camphorate Wu) is a highly valued folk medicine in Taiwan. It is used as an antitumor and for diarrhea, abdominal pain, hypertension, itchy skin and liver cancer (Liu et al., 2004; Tsai and Liaw, 1982). Some polysaccharides, steroids, triterpenoids and sesquiterpene lactone have been isolated and characterized from the fruiting body of A. cinnamomea (Lin and Chen, 2007). In previous studies, five new maleic and succinic acid derivatives (antrodin A–E) were isolated from the mycelium of A. cinnamomea (Nakamura et al., 2004) (Fig. 1). The present study employed a SensoLyteTM 520 HCV protease assay kit to investigate the HCV-protease inhibitory activity of the isolated antrodins, the metabolites of antrodin C as well as a metabolite analogue. The assay method with a quenched-fluorogenic peptide substrate can be used to measure the activity of inhibitors as well as for continuous recording of the progress of the enzyme reaction. Using this assay method, the mode of inhibition of the most potent compound was studied by Lineweaver-Burk plot.

MATERIALS AND METHODS

Apparatus. NMR spectra were obtained on a Varian Unity Plus 500 (1H, 500 MHz; 13C, 125 MHz) spectrometer. The MS spectrum was measured on an electrospray ionization mass spectrometer (ESI-MS, Esquire 3000plus, Bruker Daltonik GmbH, Bremen, Germany).

Materials for protease assay. HCV NS3/4A protease (Lot 046-047 for the screening and Lot 046-079 for the mechanism study) and SensoLyte™ 520 HCV Protease Assay Kit *Fluorimetric* (Lot# AK 71147-1005) were purchased from AnaSpec, San Jose, CA, USA. The substrate was a 5-FAM/QXL™520 FRET peptide based on the sequence of Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ-COO] Ala-Ser-Lys (DABCYL)-NH2. The SensoLyte™ Green Protease Assay Kit *Fluorimetric* (Lot# AK 71124-1009) containing trypsin was purchased from AnaSpec, San Jose, CA, USA.

The assays were carried out on BD Falcon™ Microtest™ 384-well 120 μL black assay plates (Lot# 05391155). Fluorescence was measured by a TECAN GENios plate reader at excitation/emission of 485/530 nm.

Chemical compounds. Antrodins A–E (1–5) were isolated from the mycelium of A. cinnamomea as reported (Nakamura et al., 2004). Embelin was isolated from a methanol extract of the fruits of Embelia schimperi using

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the method reported in a previous paper (Hussein et al., 2000). Compounds 6, 7a and 7b were detected as the metabolites of antrodin C in vivo and 6 was synthesized by treating 3 with 1 N KOH and 7a and 7b were synthesized by treating 3 with MeOH in the presence of 4-DMAP. Compounds 7a and 7b were unseparable due to the equilibrium of these two compounds through compound 1. It was thus used for the assay as a mixture of compounds 1, 7a and 7b and named 7. Compound 8 was an analogue of another metabolite whose glycine group was supposed to connect to the other carboxyl group in the structure.

Synthesis of 8. A pyridine (5 mL) solution of 1 (314 mg, 1 mmol), 4-(dimethylamino) pyridine (122 mg, 1 mmol) and glycine (113 mg, 1.5 mmol) was heated at 40 °C for 12 h and then kept at room temperature overnight. The product mixture was partitioned between EtOAc and 0.2 N HCl solution. The EtOAc layer was washed with water and concentrated to dryness. The residue was chromatographed over ODS eluted with CH₃CN–H₂O (30–100%) to obtain 8 from the 60% CH₃CN eluted part (200 mg, 51%).

1H NMR (DMSO-d₆, 500 MHz): δ 0.81, 0.82 (3H each, s, H-3′, 4′), 1.71, 1.75 (3H each, s, H-4″, 5″), 1.93 (1H, m, H-2′), 2.50 (overlapped with NMR solvent, H-1′), 4.20 (2H, s, H-α), 4.58 (2H, d, J = 6.5 Hz, H-1″), 5.45 (1H, m, H-2″), 7.07 (2H, d, J = 9.0 Hz, H-3″, 5″), 7.52 (2H, d, J = 9.0 Hz, H-2″, 6″). 13C NMR (DMSO-d₆, 125 MHz): δ 18.1 (5″), 22.5 (3″), 25.5 (4″), 27.5 (2″), 32.2 (1″), 39.5 (α), 64.5 (1″), 114.9 (3″, 5″), 119.6 (2″), 120.9 (1″), 130.9 (2″, 6″), 137.4 (3), 137.6 (3″), 137.9 (2), 159.5 (4″), 169.1 (4), 170.4 (β), 171.1 (1). ESI-MS (negative): m/z 370.0 ([M-H₂O-H]−, 100%).

**Assay procedure.** Compound 6 was dissolved in H₂O and other compounds were dissolved in DMSO for the assay. To each well of a 384-well plate were added 2 μL of respective compound solution and 8 μL of freshly diluted enzyme (0.5 μg/mL). The reaction was initiated by the addition of 10 μL of freshly diluted substrate (100 times dilution of a DMSO stock solution). After being incubated at room temperature (28 °C) for 30 min, the fluorescence intensities were measured at Ex/Em = 485 nm/535 nm. Inhibition percentages were calculated as 100 × (F_vehicle − F_sample)/F_vehicle = % inhibition, where F is the fluorescence value of the vehicle control or of the compound minus the fluorescence of the substrate control. The inhibitory activities on trypsin were evaluated following the procedure provided by the supplier of the assay kit.

**RESULTS AND DISCUSSION**

As shown in Table 1, of the five constituents (compounds 1–5) from the mycelium of A. cinnamomea, four of them (compounds 1 and 3–5) showed inhibitory activity on HCV protease. Compounds 6–8, the in vivo metabolites (or metabolite analogue) of a major constituent antrodin C (compound 3) showed activity too. Compound 1, which was isolated from A. cinnamomea and was also detected in vivo as one of the major metabolites of compound 3, showed the most potent activity with an IC₅₀ less than 1 μg/mL.

The mode of inhibition was kinetically analysed by plotting the enzyme activity at different concentrations of the substrate (10, 50, 100 and 200 times dilution of...
Table 1. IC_{50} values of antrodins and the metabolites against HCV protease

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (µg/mL)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9</td>
<td>C, M</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100.0</td>
<td>C, M</td>
</tr>
<tr>
<td>3</td>
<td>2.9</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>20.0</td>
<td>C</td>
</tr>
<tr>
<td>5</td>
<td>20.1</td>
<td>C</td>
</tr>
<tr>
<td>6</td>
<td>6.6</td>
<td>M</td>
</tr>
<tr>
<td>7 (7a:7b:1 = ca 5:8:4)</td>
<td>1.2</td>
<td>M</td>
</tr>
<tr>
<td>8</td>
<td>57.5</td>
<td>A</td>
</tr>
</tbody>
</table>

Emboline 4.1

C, constituent of the mycelium of A. cinnamomea.
M, in vivo metabolite of antrodin C.
A, analogue of one of the in vivo metabolites of antrodin C.

Figure 2. Lineweaver-Burk plot (1/V vs 1/[S]) for the inhibition of HCV-NS3 protease by 1 in the presence of various concentrations of substrate (● 10 µg/mL, ■ 5 µg/mL and ▲ 0 µg/mL).

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REFERENCES


Lin ES, Chen YH. 2007. Factors affecting mycelial biomass and antrodin C, metabolite of A. cinnamomea has been shown to has been used for liver cancer (Lin and Chen, 2007). Poly saccharides of A. camphorata has been shown to have a hepatoprotective effect (Han et al., 2006b) and anti-hepatitis B virus activity (Lee et al., 2002). Of the maleic and succinic acid derivatives, antrodin C (3) showed protective activity in Propionibacterium acnes and lipopolysaccharide treated mice (Hirakawa et al., 2004). Quantitative analysis showed that 3 was the most abundant compound of this chemical type in the mycelium with a content of ca. 5% of the dry weight of mycelia (Han et al., 2006a). Our recent investigation on the in vivo metabolism of 3 revealed that this compound was converted to six metabolites, i.e. compounds 1, 2, 6, 7a, 7b and an analogue of 8 (data not shown). The results suggested that the metabolites are also responsible for the in vivo pharmacological activities of 3 and consequently of the folk medicine. Interestingly, in the present study, all those in vivo metabolites except for 2 showed potent inhibitory activity on HCV protease. These results strongly support the use of this folk medicine for liver cancer which is often caused by long term infection of hepatitis C virus. The active compounds found in the present study may serve as leading compounds for the development of potent anti-hepatitis C agents through the mechanism of inhibition against the virus protease.

The substrate stocking solution) with (5 µg/mL and 10 µg/mL) and without [0 µg/mL (DMSO)] compound 1. As shown in Fig. 2 the mode of HCV PR inhibition by compound 1 was found to be competitive. These compounds were also tested for their inhibitory activity on trypsin, a serine protease found in the digestive system. It was found that only compound 3 showed a moderate activity with an IC_{50} of 41.0 µg/mL, while other compounds showed no inhibition on trypsin at a concentration as high as 100 µg/mL. The result suggested that these compounds selectively inhibited HCV protease and implied the relatively low toxicity of the compounds and of the folk medicine to human beings.

Traditionally, the fruiting body of A. cinnamomea has been used for liver cancer (Lin and Chen, 2007). Polysaccharides of A. camphorata has been shown to have a hepatoprotective effect (Han et al., 2006b) and anti-hepatitis B virus activity (Lee et al., 2002). Of the maleic and succinic acid derivatives, antrodin C (3) showed protective activity in Propionibacterium acnes and lipopolysaccharide treated mice (Hirakawa et al., 2004). Quantitative analysis showed that 3 was the most abundant compound of this chemical type in the mycelium with a content of ca. 5% of the dry weight of mycelia (Han et al., 2006a). Our recent investigation on the in vivo metabolism of 3 revealed that this compound was converted to six metabolites, i.e. compounds 1, 2, 6, 7a, 7b and an analogue of 8 (data not shown). The results suggested that the metabolites are also responsible for the in vivo pharmacological activities of 3 and consequently of the folk medicine. Interestingly, in the present study, all those in vivo metabolites except for 2 showed potent inhibitory activity on HCV protease. These results strongly support the use of this folk medicine for liver cancer which is often caused by long term infection of hepatitis C virus. The active compounds found in the present study may serve as leading compounds for the development of potent anti-hepatitis C agents through the mechanism of inhibition against the virus protease.

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