CD45 Tyrosine Phosphatase Inhibitory Components from *Aspergillus niger*

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Two inhibitors of CD45 tyrosine phosphatase, dihydrocarolic acid (1) and penitricin D (2), were isolated from a fermentation broth of the fungus *Aspergillus niger* and purified by HSCCC (high speed countercurrent chromatography) followed by HPLC. The structures were determined by NMR. The inhibitory activities of both compounds were specific to tyrosine phosphatases.

Tyrosine phosphorylation is an important regulatory mechanism for modulating the activity of many proteins involved in signal transduction at the cell surface. The regulation of tyrosine phosphorylation is mediated by the reciprocal actions of protein tyrosine kinases (PTKases) and protein tyrosine phosphatases (PTPases)\(^1\). The PTKases are a large family of proteins that includes many growth factor receptors and potential oncogenes\(^2\). Many have been linked to initial signals required for induction of the cell cycle. PTKases are a growing family of proteins, probably equal in number to the PTPases, that also exist as either transmembrane or intracellular proteins\(^3,4\).

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\[\text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \]
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CD45 protein tyrosine phosphatase is a large molecular mass transmembrane glycoprotein expressed on all hematopoietic cells except erythrocytes. It is a member of the receptor-like transmembrane protein tyrosine phosphatase family. Expression of CD45 has been shown to be important for activation of both B and T cells via their antigen-specific receptors, and has generated considerable interest in the study of lymphocyte activation as well as a possible target for drug intervention in various auto-immune and/or inflammatory diseases.

Significant effort has been devoted to testing extracts derived from natural products for finding CD45 inhibitors. As a result, several CD45 inhibitors have been identified: anonaine, nornuciferine and roemerin isolated from a plant extract and pulchellalactam from the fungal Corollospora pulchella extract in our laboratory. In this communication we wish to report two additional CD45 inhibitors; dihydrocarolic acid (1) and penitricin D (2). Both compounds were isolated from the fungal Aspergillus niger extract.

Materials and Methods

Spectral Analysis

$^1$H and $^{13}$C NMR spectra were recorded at 300K on a Bruker AC 300 spectrometer operating at 300 MHz and 75 MHz, respectively. The samples for NMR characterization were dissolved in chloroform-d at a concentration of approximately 5–10 mg/ml. Mass and MS/MS spectra were obtained on a PE Sciex API III triple-quadrupole mass spectrometer interfaced with a Sciex Ion-Spray probe. Exact mass measurements were performed on a VG 70SEQ spectrometer at high resolution (HRFAB) using PEG300/thiogly as an internal standard. UV spectra were run on a Perkin-Elmer Lambda 6 spectrometer. IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer.

CD45 Tyrosine Phosphatase Activity

The enzyme activity was measured by monitoring residual phosphorylation of the substrate poly(glu-Na,Tyr, 4:1) bound to an ELISA plate as described earlier. Briefly, successive layering of biotinylated mouse anti-phosphotyrosine antibodies and streptavidin-linked β-galactosidase conjugate allowed fluorescent detection using fluorescein-di-β-galactopyranoside which was hydrolyzed to fluorescein and detected by a Cytofluor 2300 fluorescence plate reader (Millipore). Protein tyrosine phosphatase 1B (PTP1B) was purchased from Upstate Biotechnology and the activity was also measured employing the same procedure described for CD45 phosphatase activity.

Alamar Blue Cytotoxicity Analysis

The analysis was done using human foreskin fibroblast cells in the presence and absence of epidermal growth factor (EGF). Individual samples were added to the wells in DMSO (final assay concentration of DMSO did not exceed 0.5%). The cells were added to each individual well of the microtiter plates containing eight different sample concentrations. The resulting solutions were incubated for 45 hours. Alamar Blue reagent was added in an amount equal to 10% of the culture volume. Plates were then incubated at 30°C for an additional 2.5 hours. Fluorescence was measured with excitation wavelength at 560 nm and emission wavelength at 590 nm on a Millipore Cytofluor 2300 system.

Microbial Isolation and Cultivation

Strain AM410 was isolated from a soil sample collected near Perth, Western Australia. The strain was identified as Aspergillus niger var. niger. The microorganism was cultivated in a medium containing α-lactose 10 g/liter, soluble starch (Hayashi) 30 g/liter, fish meal (Sigma) 10 g/liter, CaSO$_4$·2H$_2$O 6 g/liter, and CaCO$_3$ 4 g/liter.

Extraction and Purification

The fermentation broth (3 liter) was extracted with ethyl acetate (4.5 liter). The extract was dried over Na$_2$SO$_4$ and concentrated in vacuo to dryness to yield a brown material (610 mg). The extract was first fractionated using our standard dual mode high speed countercurrent chromatography (HSCCC) protocol. The activity was concentrated in two chromatographic peaks that eluted at 156–162 (fractions 51–54) and 192–204 minutes (fractions 63–68). Final purification was achieved using semi-preparative HPLC. The chromatography system consisted of a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector. Separation was achieved on two semi-preparative C$_{18}$-cartridges connected in series (25×100 mm, Nova-pak 6 micron). The mobile phase was pumped as a binary system at a rate of 10 ml/minute and consisted of a 35 minutes linear gradient, starting with 80% H$_2$O, 20% CH$_3$CN and ending with 100% CH$_3$CN. Dihydrocarolic acid (50 mg) and penitricin D (10 mg) were isolated from CCC fractions 51–54 and fractions 63–68, respectively.

Dihydrocarolic acid (1) was identified by direct comparison of its spectroscopic properties (UV, MS, and
Table 1. Inhibition of phosphatase activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CD45 IC₅₀ (µg/ml)</th>
<th>PTP1B IC₅₀ (µg/ml)</th>
<th>Calcineurin IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrocarolic acid (1)</td>
<td>1.2</td>
<td>38.0</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Penitricin D (2)</td>
<td>2.3</td>
<td>15.8</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>16.8</td>
<td>15.5</td>
<td>183.0</td>
</tr>
</tbody>
</table>

NMR) with those reported in the literature\(^{(1)}\).

Penitricin D (2) was isolated as a white powder; m.p. 82°C and \([\alpha]_D -20\) (CHCl₃). The UV spectrum showed maxima at 250 and 275 nm, and the mass spectrum (API) gave a molecular ion at \(m/z\) 85 (M+H)\(^{1}\). The molecular formula was determined to be \(C_4H_4O_2\) by HREI-MS analysis (\(m/z\) 84.1012 calcd for \(C_4H_4O_2\), found 84.1004). The IR spectrum (KBr) exhibited bands at 3345 (OH), 1840 (C=O) and 1610 (C=C) cm\(^{-1}\). The latter two absorptions are characteristic for cyclopropenone\(^{(4,15)}\). The \(^1\)H NMR spectrum (DMSO) showed just three signals at \(\delta\) 5.05 (brs), 5.07 (brs) and 5.21. Only four signals were observed in the \(^{13}\)C NMR spectrum: \(\delta\) 168.5, 150.8, 91.4 and 89.5. The latter two absorptions are characteristic for cyclopropenone\(^{(4,15)}\). The \(^1\)H NMR spectrum (DMSO) showed just three signals at \(\delta\) 5.05 (brs), 5.07 (brs) and 5.21. Only four signals were observed in the \(^{13}\)C NMR spectrum: \(\delta\) 168.5, 150.8, 91.4 and 89.5. The DEPT 135 spectrum revealed only two peaks: a methine peak at \(\delta\) 91.8 and a methylene peak \(\delta\) 89.5, indicating that the remaining two signals belong to quaternary carbons. Only three protons were accounted for from the \(^{13}\)C NMR data suggesting the presence of one exchangeable proton in the molecule. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum. Two protons at \(\delta\) 5.05 and 5.07 showed correlations to a single carbon atom at \(\delta\) 89.5. This and a quaternary carbon resonance at \(\delta\) 150.8 are suggestive of an exocyclic double bond. An additional unsaturation was denoted by a \(^{13}\)C NMR signal at \(\delta\) 168.5 indicative of a \(\alpha, \beta\) unsaturated carbonyl carbon. One degree of unsaturation remained which was assigned to one ring. Based on these observations, this compound was considered to be hydroxycyclopropenone (2).

The name penitricin D was proposed for compound 2 because of its close structural resemblance to penitricin A (3).

Inhibition of CD45 Tyrosine Phosphatase Activity

Dihydrocarolic acid and penitricin D exhibited a dose dependent inhibition of CD45 tyrosine phosphatase activity. Both compounds were more potent in inhibiting CD45 phosphatase activity than sodium orthovanadate, a known inhibitor of the enzyme (Table 1). The inhibitory activity of 1 and 2 was specific to tyrosine phosphatases as both CD45 and PTP1B were inhibited with IC₅₀ values similar to that observed for the inhibition of these enzymes by sodium orthovanadate, a known phosphatase inhibitor. However, neither compound inhibited the activity of calcineurin, a serine threonine phosphatase. Further, Alamar Blue cytotoxicity analysis was carried out in order to determine the probable cytotoxicity of these compounds. This was done by studying EGF dependent cellular proliferation of human foreskin fibroblasts in the presence of penitricin D and dihydrocarolic acid. We have determined earlier that these cells show enhanced proliferation in response to EGF, presumably via activation of the EGF receptor tyrosine kinase following binding of EGF to receptors on the cells. Both compounds were found to be non-toxic as the EGF mediated proliferation of the human foreskin fibroblasts which is a tyrosine phosphorylation dependent process was not altered (data not shown).

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References

3) Krueger, N. X.; M. Streuli & H. Saito: Structural


