TETRONOTHIODIN, A NOVEL CHOLECYSTOKININ TYPE-B RECEPTOR ANTAGONIST PRODUCED BY Streptomyces sp. NR0489

II. ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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A novel cholecystokinin type-B receptor antagonist named tetronothiodin has been isolated by column chromatography and preparative HPLC from the fermentation broth of Streptomyces sp. NR0489. Tetronothiodin inhibited the binding of CCK8 (C-terminal octapeptide of cholecystokinin) to rat cerebral cortex membranes (CCK type-B receptors) with an IC50 of 3.6 nM, whereas it did not inhibit CCK8 binding to rat pancreatic membranes (CCK type-A receptors). It also inhibited CCK8-induced Ca2+ mobilization in GH3 cells, a rat anterior pituitary cell line, but was without effect on the basal cytosolic Ca2+ concentration. This finding indicated tetronothiodin was an antagonist of CCK type-B receptors.

Cholecystokinin (CCK) is a hormonal regulator of pancreatic secretion10 as well as gallbladder contraction2) and gut motility3). It has also been proposed to act as a neurotransmitter in the central nervous system4). CCK type-B (CCK-B) receptors are suggested to be related to appetite5), pain6,7) and anxiety8,9). Some CCK-B receptor antagonists increased food intake5), enhanced morphine analgesia6,7) and reduced anxiety8,9) in rats. However, physiological and pharmacological roles of CCK-B receptors are not yet fully understood in part because of the shortage of potent and specific CCK-B receptor antagonists. To obtain structurally unique and specific CCK-B receptor antagonists, we screened microbial metabolites by employing a binding assay method in which rat cerebral cortex membranes and 125I labeled Bolton-Hunter CCK8 ([125I]-CCK8) were used as the receptors and the radioligand, respectively. In this screening program, we discovered a novel CCK-B receptor antagonist named tetronothiodin (1) from the culture broth of Streptomyces sp. NR0489, and determined the structure to be a macrocyclic compound containing an α-acyltetronic acid and a tetrahydrothiophene ring (Fig. 1). A preliminary communication of this work
has been reported\textsuperscript{10}). Details of the taxonomy and fermentation of 1 are reported in the preceding paper\textsuperscript{11}). The structural elucidation study of 1 is reported in the succeeding paper\textsuperscript{12)} in detail. In the present paper, we describe the isolation, physico-chemical characterization and biological activities of 1.

**Isolation**

Isolation of 1 was carried out by monitoring the inhibitory activity against \(^{125}\text{I}\)-labeled Bolton-Hunter CCK\(_8\) ([\(^{125}\text{I}\)]-CCK\(_8\)) binding to rat cerebral cortex membranes. The isolation procedure of 1 is outlined in Fig. 2. After cultivation of the producing organism for ten days in 50-liter jar fermenters by the procedure described in the preceding paper\textsuperscript{11)}, the mycelium was removed by centrifugation. The broth supernatant (181 liters) was adjusted to pH 7 with 6 N HCl and applied to a column (12 × 100 cm) of Diaion HP-21 (Mitsubishi Chemical Industries). The column was washed with water (25 liters) and 10% aqueous acetone (50 liters), and the active principle was eluted with 50% aqueous acetone (60 liters). The active eluate was concentrated to about 15 liters under reduced pressure and extracted with ethyl acetate (25 liters × 2) at pH 2. The organic layer was dried over anhydrous sodium sulfate and concentrated to 3.5 liters under reduced pressure. This solution was back-extracted with water (1.5 liters × 2) at pH 7.5. The water layer was concentrated to 1.5 liters under reduced pressure. The concentrate was applied to a column (5 × 16 cm) of QAE Sephadex A-25 (Pharmacia Fine Chemicals) which was developed stepwise with water (1 liter) and NaCl solutions (0.2, 0.3 and 0.5 M; 3.5 liters each). The active eluate (0.3 and 0.5 M NaCl fractions) was extracted with ethyl acetate (4 liters × 2) at pH 2. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give an oily residue, which was chromatographed on a Sephadex LH-20 column (3.2 × 120 cm) developed with MeOH. The active eluate was concentrated under reduced pressure and purified by preparative HPLC over a C\(_8\) reversed-phase silica gel column (YMC-Pack, Table 1. Physico-chemical properties of 1.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Pale brown powder</th>
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<tbody>
<tr>
<td>Molecular formula</td>
<td>C(<em>{31})H(</em>{38})O(_8)S</td>
</tr>
<tr>
<td>FAB-MS (positive ion)</td>
<td>593 (M + Na), 609 (M + K)</td>
</tr>
<tr>
<td>HRFAB-MS</td>
<td>569.2237 (M−H)</td>
</tr>
<tr>
<td>UV (MeOH)</td>
<td>233 (29,900), 273 (12,200)</td>
</tr>
<tr>
<td>UV (MeOH-HCl)</td>
<td>234 (31,000), 271 (13,500)</td>
</tr>
<tr>
<td>IR v(max) (KBr) cm(^{-1})</td>
<td>3700~2300 (br), 1760 (sh), 1728, 1638, 1600</td>
</tr>
<tr>
<td>[(\pi)](_D)</td>
<td>-56.9° (c 1.1, MeOH)</td>
</tr>
<tr>
<td>Rf (Silica gel 60 F(_{254}))</td>
<td>0.69 (CHCl(_3) - MeOH - 28% aqueous ammonia, 4:4:1)</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in DMSO, MeOH, THF</td>
</tr>
<tr>
<td></td>
<td>Insoluble in hexane, ether, CHCl(_3), H(_2)O</td>
</tr>
</tbody>
</table>
30 × 250 mm; YMC Co., Ltd.) with MeOH-0.1 M phosphate buffer (pH 2.2) (6:4) at a flow rate of 43 ml/minute. The active fraction (retention time, 13 minutes) was concentrated and extracted with ethyl acetate at pH 2.5. The organic layer was concentrated to dryness under reduced pressure to give 1 (240 mg) as a pale brown powder.

Physico-chemical Characteristics

The physico-chemical properties of 1 are summarized in Table 1. The isolation procedure of 1, extraction with ethyl acetate at pH 2 and back-extraction with water at pH 7.5, indicated its acidic nature. 1 was soluble in MeOH, THF, DMSO and alkaline water but insoluble in ether, chloroform, hexane and water. 1 was positive to FeCl₃, vanillin-H₂SO₄ and iodine reactions. The free form of 1 was unstable in solution; it gradually decomposed during NMR experiments for two weeks in DMSO-d₆ or CD₃OD. Its alkaline metal salts were stable for at least five months under the same experimental conditions. The IR (Fig. 3) absorption bands at 3000~2300 and 1728 cm⁻¹ suggested the presence of a carboxylic acid. A

Fig. 3. IR spectrum (KBr) of tetronothiodin.

--- MeOH, --- MeOH · NaOH, ----- MeOH · HCl.

Fig. 4. UV spectrum of tetronothiodin.
The γ-lactone function was also suggested by the shoulder band at 1760 cm\(^{-1}\) (KBr), which clearly separated from a large carbonyl band (1728 cm\(^{-1}\)) in THF. The UV spectrum (Fig. 4) in MeOH showed absorption maxima at 233 and 273 nm and hypochromic effect was observed in acidic methanol. These absorption maxima were attributable to an α-acyl tetronic acid chromophore\(^{13,14}\) with the former absorption maximum being partly due to a diene chromophore. The molecular formula (C\(_{31}\)H\(_{38}\)O\(_{8}\)S) determination was based on positive ion FAB-MS and negative ion HRFAB-MS data [569.2237, calcd for (M−H, C\(_{31}\)H\(_{37}\)O\(_{8}\)S)\(^−\) 569.2210]. The molecular formula was supported by the analyses of the \(^1\)H NMR spectrum (Fig. 5) and the \(^{13}\)C NMR spectrum (Fig. 6) showing 31 carbon signals, and by qualitative analysis for sulfur\(^{15}\). These physico-chemical properties indicated that the structure of 1 was different from known CCK receptor antagonists of microbial origin such as virginiamycin M\(_1\) analogues\(^{16}\), anthramycin\(^{17}\) and asperlicin\(^{18}\). The chromophore, α-acyl tetronic acid, is commonly contained in some antibiotics such as kijanimicin\(^{13}\), tetrocarcins\(^{14}\) and MM 46115\(^{19}\). However 1 was different from these antibiotics in terms of containing a sulfur atom in the molecule.

### Biological Activities

The inhibitory activities against the binding of \([^{125}\text{I}]-\text{CCK}_8\) to CCK-A and CCK-B receptors were observed by the following procedures. Test samples were incubated at 23°C with \([^{125}\text{I}]-\text{CCK}_8\) and rat pancreatic membranes (CCK-A receptors) or rat cerebral cortex membranes (CCK-B receptors) in a

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (nM)</th>
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<tbody>
<tr>
<td></td>
<td>CCK-A</td>
</tr>
<tr>
<td>Tetronothiodin (1)</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>L-365,260</td>
<td>2,700</td>
</tr>
<tr>
<td>Cl-988</td>
<td>Not done</td>
</tr>
<tr>
<td>CCK(_8)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Fig. 5. 400 MHz \(^1\)H NMR spectrum of the potassium salt of tetronothiodin in D\(_2\)O.

Fig. 6. 100 MHz \(^{13}\)C NMR spectrum of the potassium salt of tetronothiodin in D\(_2\)O.
10 mM 2-(N-morpholino)ethanesulfonate buffer (pH 6.5) containing NaCl 130 mM, MgCl₂ 5 mM, bacitracin 0.02% and bovine serum albumin (0.2% and 0% for CCK-A and CCK-B receptors, respectively). After equilibrium was reached (120 minutes for CCK-A receptors or 20 minutes for CCK-B receptors), each mixture was filtered by a Durapore HVLP filter and the radioactivity of the filter was counted by an autorgamma counter.

1 inhibited the binding of [¹²⁵I]-CCK₈ to CCK-B receptors on rat cerebral cortex membranes in a concentration dependent manner with an IC₅₀ of 3.6 nM (Table 2). The affinity to CCK-B receptors of 1 was three or four times more potent than those of L-365,260 or CI-988 known as potent and selective CCK-B receptor antagonists, and only three times less potent than the natural ligand CCK₈ (IC₅₀ = 1.2 nM). However 1 did not inhibit the binding of [¹²⁵I]-CCK₈ to rat pancreatic membranes (CCK-A receptors).

The ratio of the affinity for CCK-A to CCK-B receptors of 1 was more than 27,000, which was 90-fold greater than the -A and -B affinity ratio of L-365,260 (300). 1 was thus revealed to be a highly selective binding inhibitor of CCK-B receptors.

GH3 cells were reported to express CCK-B receptors. This fact was corroborated by our results that 1 inhibited CCK₈ binding to GH3 cells with an IC₅₀ of 4.2 nM, which was of the same order as that for brain CCK-B receptors. It had also been demonstrated that the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in GH3 cells was increased by CCK₈ in a concentration dependent manner at 1 to 1,000 nM using the Fura-2 method. CCK₈ (100 nM) transiently increased [Ca²⁺]ᵢ from 448 nM (basal level) to 739 nM. This stimulation caused by 100 nM CCK₈ was 97% of the maximum stimulation obtained by the treatment with 1 μM CCK₈. The effect of 1 to this Ca²⁺ mobilization was investigated by measuring [Ca²⁺]ᵢ in GH3 cells. When GH3 cells were treated with 1 (1 μM) one minute prior to the treatment of 100 nM CCK₈ which induced a submaximum increase of [Ca²⁺]ᵢ, 1 inhibited this increase completely without affecting the basal level. This inhibitory activity was concentration dependent. At the concentration of 50 nM of CCK₈ which causes about 80% stimulation of the maximum [Ca²⁺]ᵢ increase, pretreatments of GH3 cells with 1, 10, 100, and 1,000 nM of 1 inhibited the [Ca²⁺]ᵢ increase by 12, 55, 70 and 93%, respectively. The IC₅₀ against the increase of [Ca²⁺]ᵢ induced by 50 nM CCK₈ was 26 nM. These results indicate that 1 acted as an antagonist of CCK-B receptors on GH3 cells.

1, at concentrations up to 9 μM, did not show cell growth inhibitory activity against HeLa cells. 1 was inactive against bacteria (Bacillus subtilis, Micrococcus luteus and Escherichia coli) and fungi (Candida albicans, Aspergillus fumigatus, Trichophyton mentagrophytes and Pyricularia oryzae) at concentrations up to 450 μM.

**Discussion**

The structure of 1 is completely different from natural CCK-B receptor antagonists (virginiamycin M₁ analogues and anthramycin produced by Streptomyces sp.) and a CCK type-A receptor antagonist (asperlicin produced by Aspergillus alliaceus) of microbial origin. It is also different from the other CCK antagonists: (1) cyclic nucleotides (dibutyryl cyclic GMP), (2) amino acids (proglumide, lorglumide andloxiglumide), (3) partial sequences and derivatives of the C-terminal heptapeptides of CCK (CCK-JMV-180), (4) benzodiazepines (devazepide and L-365,260) and (5) nonpeptide “peptoids” derived from fragments in the CCK molecule (CI-988). 1 is structurally related to some antibiotics such as kijanimicin, tetrocarcins and MM 46115 in terms of the macrocyclic molecule containing an α-acytetylic acid chromophore. In contrast to these antibiotics, 1 is inactive against Bacillus subtilis and Micrococcus luteus.

A CCK-B receptor antagonist, L-365,260, increased food intake in rats. An anxiolytic activity and
enhancement of morphine analgesia\textsuperscript{6-7} by CCK-B receptor antagonists in rats were also demonstrated by L-365,260 and CI-988. The possibility of clinical application of CCK-B receptor antagonists was suggested by these studies. However, physiological and pharmacological roles of CCK-B receptors are not yet fully understood partly because of the shortage of potent and specific CCK-B receptor antagonists. I is a novel, potent and highly selective CCK-B receptor antagonist. It will be a useful tool for the investigation of the physiological and pharmacological roles of CCK-B receptors. Full details of the biological activities will be reported elsewhere\textsuperscript{25}).

Acknowledgments

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References

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