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Curcuminoids from *Curcuma longa* and their inhibitory activities on influenza A neuraminidases

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ABSTRACT

The emergence of drug-resistant influenza viruses and the threat of pandemics highlight the need for new and effective antiviral agents. In this study, we describe the isolation of 3 new (**1**–**3**) and 10 known (**4**–**13**) curcuminoids from a methanol extract of *Curcuma longa* L. All compounds had strong inhibitory effects on the neuraminidases from two influenza viral strains, H1N1 and H9N2, as noncompetitive inhibitors with IC_{50} values ranging from 6.18 ± 0.64 to 40.17 ± 0.79 μg/ml and 3.77 ± 0.75 to 31.82 ± 1.33 μg/ml, respectively. Compounds **4**, **5**, and **13** also exhibited significant inhibitory activity against the neuraminidases from novel influenza H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y mutant) expressed in 293T cells. Our results suggest that the curcuminoids from *C. longa* may be potential supplemental molecules in the prevention and treatment of disease by influenza viruses.

1. Introduction

Influenza viruses (Orthomyxoviridae) cause annual epidemics and occasional pandemics that have claimed the lives of millions. The top of pharmacological strategies for dealing with influenza pandemic is now based on antiviral drugs, in which neuraminidase (NA) inhibitors are the most important (Colman, 2009; Xie, Gong, Li, Fang, & Xu, 2011). NA, also known as sialidase, is a surface glycoprotein of the influenza A virus, that plays a key role in not only in the release of virions from infected host cells but also in their movement through the upper respiratory tract (Beigel & Bray, 2008). When influenza viruses show deficient NA activity, particles of progeny viruses aggregate at the surface of infected cells, which severely impairs the further spread of viruses to other cells (Beigel & Bray, 2008; Zhang, Yu, Zhu, & Jiang, 2006).

To date, there are five well established anti-influenza drugs commercially available. These include NA inhibitors, oseltamivir, zanamivir and peramivir, which impair the efficient release of viruses from an infected host cell, and amantadine and rimantadine, which specifically inhibit viral proliferation by blocking the M_2 ion channel (Regoes & Bonhoeffer, 2006; Smee et al., 2010). Although antiviral chemotherapy with M_2 inhibitors reduces the duration of symptoms of clinical influenza, many side effects have been reported (Regoes & Bonhoeffer, 2006). While zanamivir (relenza) exhibits excellent antiviral activity, its bioavailability is low due to rapidly elimination by renal excretion. Some adults receiving oseltamivir (tamiflu) have also reported nausea and vomiting (Ryan, Ticehurst, & Dempsey, 1995). Furthermore, high-level drug resistance is associated with both inhibitors. In 2009, oseltamivir-resistant H1N1 viruses arose spontaneously and spread globally. This resistance was conferred by a single amino acid change in the viral neuraminidase (H274Y) (Hurt, Holien, Parker, & Barr, 2009). Therefore, continuous research for new antiviral compounds from natural products is needed to develop new therapeutic agents in the battle against the influenza viruses (De Clercq, 2006).

Curcuma longa is a plant belonging to Zingiberaceae family, which is found in south and southeast tropical Asia (Ammon & Wahl, 1991). Its rhizome is used as a spice (main ingredient of curry), a pigment dye of textiles, and in traditional medicine. Two fundamental groups of compounds from this plant are curcuminoids and sesquiterpenes (Nishiyama et al., 2005), in which curcuminoids with the pharmacological activities including cardiovascular protection, antitumour, antioxidant, anti-inflammatory, anti-Alzheimer, anti-hepatotoxic, and antiviral have been reported (Chen et al., 2010; Masuda, Jitoe, Isobe, Nakatani, & Yonemori, 1993; Miriyala, Panchatcharam, & Rengarajulu, 2007; Park & Kim, 2002; Ravindran, Prasad, & Aggarwal, 2009; Simon et al., 1998).

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As part of an ongoing anti-influenza screening programme from natural products (Dao et al., 2010; Nguyen et al., 2011), a methanol extract of *C. longa* was found to exhibit potential NA inhibitory properties. Although the antiviral activity of curcumin has been reported (Chen et al., 2010), the detailed relationships of structure and activity by curcuminoids are unclear. Moreover, there are no reports of the antiviral activities of *C. longa* on novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells. This prompted us to identify the active principles with the NA inhibitory activity of *C. longa* by bioactivity-guided fractionation. This paper describes the isolation, structural elucidation, and antiviral activity of these compounds on NAs from two influenza viral strains, H1N1 and H9N2, as well as from both novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) expressed in 293T cells.

2. Materials and methods

2.1. Plant material

The rhizomes of *C. longa* were collected in August 2009 at Jindo, Jeonnam province, Republic of Korea. Plant sample was identified botanically by Prof. Y.H. Moon. A voucher specimen (CU2009-06) was deposited at the Herbarium of Chosun University, Gwangju, Republic of Korea.

2.2. General experimental procedures

The UV spectra were recorded in MeOH on a JASCO V-550 UV/VIS spectrometer. The IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR (Thermo electron Corp.). The NMR spectra were obtained on a Varian Inova 600 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The EIMS and HREIMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63–200 μm particle size), silica gel (Merck, 40–63 μm particle size), and Sephadex LH-20 were used for column chromatography. TLC was carried out using silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). All solvents used for extraction and isolation were of analytical grade.

2.3. Extraction and isolation

The dried rhizomes of *C. longa* (3 kg) were extracted with MeOH (15 l× 3 times) at room temperature for a week. The combined methanol extract was then concentrated to yield a dry residue (380 g). This crude extract was suspended in distilled water (2.5 l) and partitioned successively with *n*-hexane (3 × 2 l), ethyl acetate (3 × 2 l), and butanol (3 × 2 l). The ethyl acetate and butanol fractions, which showed strong influenza NA inhibitory activity (Table S.1 in Supplementary data), were combined (130 g) and chromatographed over a silica gel column (10 × 30 cm; 63–200 μm particle size) eluting with gradient solvent $CHCl_3$/acetone (19:1,18:2,...,1:19, each 2.5 l), to yield six fractions (F1: 17.6 g; F2: 8.5 g; F3: 5.6 g; F4: 9.8 g; F5: 10.5 g; F6: 12.6 g; F7: 22.4 mg). The crystallisation of fraction F3 from n-hexane/EtOAc afforded compound **13** (curcumin, 4.2 g). Fraction F4 was further applied to a normal-phase silica gel column (5 × 40 cm; 40–63 μm particle size) with a stepwise gradient of $CHCl_3$/MeCN (9:1,8:2,...,1:9, each 2 l) to afford five subfractions (F4.1–F4.5). Crystallisation of subfractions F4.2 and F4.3 from $CHCl_3$ gave compounds **4** (demethoxycurcumin, 1.3 g) and **5** (bisdemethoxycurcumin, 2.5 g), respectively. Fraction F4.4 (90 mg) was separated by HPLC [Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeCN in H_2O containing 0.1% HCO_2H (0–40 min: 45% MeCN, 40–45 min: 100% MeCN, 45–55 min: 100% MeCN); flow rate 2 ml/min; UV detection at 205 and 254 nm] to give compounds **8** (t_R = 30.0 min, 5.5 mg) and **10** (t_R = 34.0 min, 14.0 mg).

Fraction F5 was chromatographed over a Sephadex LH-20 column (7 × 40 cm) using MeOH as the eluting solvent to afford three subfractions (F5.1–F5.3). Subfraction F5.2 (3.1 g) was further chromatographed over a silica gel column (5 × 40 cm; 40–63 μm particle size) with a gradient solvent of $CHCl_3$/MeCN (9:1,8:2,...,1:9, each 2.5 l) to yield five fractions (F5.2.1–F5.5.5). Subfraction F5.2.2 (150 mg) was purified by HPLC (0–45 min: 60% MeCN, 45–50 min: 100% MeCN) to yield compounds **9** (t_R = 35.0 min, 10.5 mg) and **11** (t_R = 38.5 min, 13.0 mg). Further separation of F5.2.3 (110 mg) by HPLC (0–65 min: 57% MeCN, 65–70 min: 100% MeCN) resulted in the isolation of compounds **6** (t_R = 42.0 min, 10.5 mg), **7** (t_R = 58.0 min, 7.5 mg), and **3** (t_R = 61.0 min, 6.0 mg). Finally, compounds **1** (t_R = 46.0 min, 4.5 mg), **2** (t_R = 48.5 min, 3.5 mg), and **12** (t_R = 52.0 min, 6.5 mg) were obtained from subfraction F5.2.4 (95 mg) by HPLC (0–60 min: 35% MeCN, 65 min: 100% MeCN).

2.4. Viruses, cells, cloning, and expression of neuraminidase

A full length cDNA encoding the neuraminidase of novel H1N1 influenza (A/California/08/2009(H1N1)) and oseltamivir-resistant neuraminidase (H274Y mutant) were constructed as previously reported method (Dao et al., 2010). The influenza strains A/Chicken/Korea/O1310/2001 (H9N2) and A/Sw/Kor/CAH1/04 (H1N1, KCTC 11165BP) were used in this study. 293T cells (human embryonic kidney cells) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. The 293T cells were counted and plated in 6-well plates at a density of 10^5 cells/well. After 24 h, the cells were transfected with the plasmids containing the cDNAs using a commercial transfection kit (Welfect EX-plus, Welgene, Daegu, Korea), according to the manufacturer's instructions.

2.5. Influenza A (H1N1 and H9N2) neuraminidase inhibition assays

The enzyme assay was performed as previously reported with a slight modification (Dao et al., 2010; Hung et al., 2009). Large-scale influenza virus suspension was prepared from the MDCK cells infected with the influenza viruses, H1N1 and H9N2. The virus suspensions were treated with formaldehyde at a final concentration of 0.01% at 37 °C for 30 min to inactivate the viral infectivity. The NA activity was measured using 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639) in an acetate buffer as the substrate.

2.6. Novel H1N1 (WT) and oseltamivir-resistant novel H1N1 (H274Y) neuraminidase inhibition assays

The 293T cells transfected with the plasmids were harvested by treatment with 0.02% EDTA in PBS. After washing with PBS, the cells (approximately 5 × 10^6 cells) were suspended in 250 μl PBS containing 3.5 mM $CaCl_2$. The suspensions were then divided into 50 μl aliquots and stored at −80 °C until needed. The NA inhibition assays were performed using 4-MU-NANA as the fluorescent substrate and dilutions of the samples with NA activity equivalent to 8–10 × fluorescence units compared to the background. The tested compounds were pre-incubated with 10 μl cell suspensions in 32.5 mM MES buffer (containing 4 mM $CaCl_2$, pH 6.5) at 37 °C in 30 min. After 30 min incubation, the substrate (30 μl) was added and the assays were incubated for a further 2 h at 37 °C, and finally