713A, a New Metabolite Isolated from a Fungal Strain 713

Ying-shu Zou, Ke Wang, Yang Zhang, Yuan Li, Jie Meng, and Jian-bo Wu*

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College; Tiantanxili No. 1, Beijing 100050, P. R. China. Received March 19, 2008; accepted May 23, 2008; published online May 30, 2008

A new compound named 713A was isolated from the fermentation broth of a fungal strain 713. The structure of 713A was elucidated by spectroscopic methods. In the screening for interleukin-6 (IL-6) receptor antagonist, 713A exhibited inhibitory activity to the binding of IL-6 and IL-6 receptor with an IC_{50} value of 8.6 μM.

Key words 713A; interleukin-6; interleukin-6 receptor; antagonist

Interleukin 6 (IL-6) is a multifunctional cytokine participates in inflammation reaction and cell growth, elevated IL-6 concentrations have been associated with many disease states, such as inflammation, rheumatoid arthritis, myeloma and autoimmune diseases.[1,2] IL-6 plays its role on target cells through a double-stranded receptor complex consisting of IL-6R and a signal transduction subunit (gpl30).[3] Therefore, antagonism of IL-6 action at the receptor level has the potential to interfere the biological effect of IL-6. In the screening for IL-6 receptor antagonists, a novel compound, 713A (Fig. 1) was isolated from a strain of fungus. In the following paper we report the fermentation of the producing strain, the isolation, structural elucidation and bioactivity of the compound.

The fungal strain 713 has been deposited in the Center for Culture Collection of Pharmaceutical Microorganisms, Institute of Medicinal Biotechnology. The species however has not been identified. For maintenance on agar slants the strain was kept on YM medium. After incubation at 26 °C for 7 d, the slant was inoculated into 100 ml of a steril seed medium containing glycerin 1%, glucose 2%, sucrose 1%, soybean 0.2%, peptone 1%, KH_2PO_4 0.25%, PEG6000 0.3%, $NaNO_3$ 0.3%, $(NH_4)_2SO_4$ 0.3%, (pH 6.0 before sterilization), and cultured at 26 °C for 2 d on a rotary shaker (250 rpm). The second seed culture (5 ml) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a culture medium with the above composition. And the incubation was carried out at 26 °C for 4 d on a rotary shaker (250 rpm).

The culture filtrate (20 l) was absorbed on Amberlite XAD-5 column (2 l). After washed with water and 40% aqueous acetone, the column was eluted with 80% aqueous acetone. The eluted material was concentrated and subjected to sephadex LH-20 column with 70% methanol. The last yellow fraction was further purified firstly on ODS column (YMC Gel, ODS-A, 12 nm, S-150 μm), followed by medium pressure liquid chromatography (MPLC, Yamazen FMI-C pump, Ultrapack ODS, 15×300 mm, 30/50 μm), and both eluted with 50% methanol. A total of 5 mg of pure 713A was obtained. Purity was monitored by HPLC (Inertsil ODS-2, 4.6×150 mm, 5 μm) with CH_3CN–H_2O (60 : 40), 0.05% TFA at a flow rate of 1 ml/min. 713A showed a retention time of 8.7 min.

713A was isolated as yellow amorphous powder and not much stable when its methanol solution was exposed to air over 48 h, which would change from yellow to pink. The physico-chemical properties of 713A are summarized in Table 1. The molecular formula of 713A was determined to be $C_{23}H_{28}N_2O_8$, based on positive-ion HR-ESI-MS data ($(M+H)^+$ *m/z* Calcd 461.1924, Found 461.1913). The IR spectrum showed the presence of conjugated hydroxyl (3399 cm^{-1}), amide carbonyl (1638 cm^{-1}) and benzene rings (1587, 1557 cm^{-1}).[4]

The structure of 713A was elucidated using ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMQC and HMBC. The ^{13}C-NMR and DEPT spectra of 713A exhibited 23 carbon signals consisting of three methyls, four methylenes, six methines, and ten quaternary carbons. The ^{1}H-NMR, HMQC and ^{1}H–^{1}H COSY spectral data indicated the presence of a strongly chelated OH group (δ_H 15.15), an amide NH group (δ_H 7.93), four olefinic protons (δ_H 8.27, 6.34, 6.21, 6.13), an aromatic methoxyl (δ_H 3.77, δ_C 55.7) and a CH_3–CH_2–$CH(CH_3)$–CH– group. In the HMBC spectrum (Fig. 2), a 1,2,3,5-tetrasubstituted phenolic ring (C ring) was observed by the following correlations; from 9-OH (δ_H 15.15) to C-9 (δ_C 165.4), C-9a (δ_C 111.3) and C-8 (δ_C 98.2); from H-8 (δ_H 6.13) to C-9a (δ_C 111.3), C-9 (δ_C 165.4), C-7 (δ_C 164.1) and C-6 (δ_C 101.4); from 7-OCH_3 (δ_H 3.77) to C-7 (δ_C 164.1); and from H-6 (δ_H 6.34) to C-9a (δ_C 111.3). The HMBC couplings be-

Table 1. Physico-chemical Properties of 713A

Molecular formula	$C_{23}H_{28}N_2O_8$
$[\alpha]_D$ (20 °C)	+155.3° (*c*=0.04, MeOH)
FAB-MS (*m/z*)	461 $[M+H]^+$, 483 $[M+Na]^+$, 499 $[M+K]^+$
HR-ESI-MS (*m/z*)	
Found	461.1913 $[M+H]^+$
Calcd	461.1924 $[M+H]^+$
UV λ_{max} nm (MeOH)	242, 298, 443
IR (KBr) γ cm^{-1}	3399, 2960, 2927, 1664, 1638, 1587, 1557, 1410, 1108

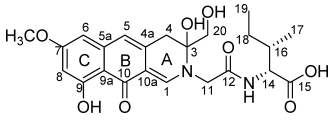

Fig. 1. Structure of 713A

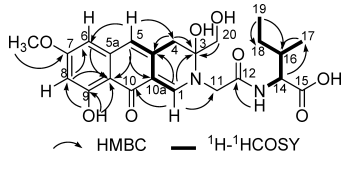

Fig. 2. Selected HMBC and COSY Correlations of 713A

* To whom correspondence should be addressed. e-mail: wujb0825@sohu.com

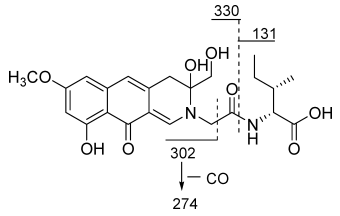

Fig. 3. Selected FAB Mass Fragments of 713A

tween H-5 (δ_H 6.21) and C-6 (δ_C 101.4), C-9a (δ_C 111.3) revealed the C ring adjacent to the C-5 vinyl of B ring. Other critical correlations were also shown as follows; from H-5 (δ_H 6.21) to C-4 (δ_C 39.4) and C-10a (δ_C 106.87); from H_2-4 (δ_H 3.02, 3.21) to C-3 (δ_C 87.2) and C-4a (δ_C 130.5); from H-1 (δ_H 8.27) to C-3 (δ_C 87.2), C-4a (δ_C 130.5), C-10 (δ_C 181.7), and C-11 (δ_C 53.4); from H_2-20 (δ_H 3.62) to C-3 (δ_C 87.2). The presence of a ketone group (δ_C 181.7) at C-10 of B ring was suggested by the ^{1}H chemical shift of the hydrogen-bonded 9-OH (δ_H 15.15).[5] And the chemical shifts of C-1 (δ_C 154.0) and H-1 (δ_H 8.27) indicated that C-1 methine was connected by a nitrogen atom. These findings enabled the establishment of the A—B ring structural segment except the substitution at methylene C-11. In the ^{1}H–^{1}H COSY spectrum (Fig. 2), the NH proton (δ_H 7.93) was coupled to the methine proton H-14 (δ_H 4.09) of $^{19}CH_3$–$^{18}CH_2$–$^{16}CH(^{17}CH_3)$–^{14}CH– group. Taking into account the molecular formula and two remaining carbonyl carbons (δ_C 173.1, 168.8), an isoleucine (Ile) residue should be located at C-11 by an amide bond. This structure was also supported by the FAB mass fragment patterns (Fig. 3). Finally, this compound was determined to be 2-(2-(3,4-dihydro-3,9-dihydroxy-3-(hydroxymethyl)-7-methoxy-10-oxobenzo[*g*]isoquinolin-2(10*H*)-yl)acetamido)-3-methylpentanoic acid. The ^{1}H- and ^{13}C-NMR assignments of 713A are summarized in Table 2.

The absolute configuration of the isoleucine residue was determined by the Marfey method[6] using 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA) and a chiral HPLC analysis of the acid hydrolysate of 713A. The Marfey method clarified the existence of D-Ile or D-*allo*-Ile in the hydrosate of 713A, and the chiral HPLC analysis successfully defined the absolute configuration of isoleucine as D-*allo*-Ile.

Thus, the 713A structure was characterized as shown in Fig. 1, except for stereochemistry at the C-3 position of ring A moiety.

713A was detected for the IL-6 receptor antagonistic activity using an immobilized ligand-binding assay method. The experimental procedures were briefly described as follows; 100 μl of 1.0 μg/ml IL-6 in PBS (0.05 M phosphate buffer, pH 7.2, 0.15 M NaCl) was coated onto a 96-well immunoplate overnight at 4 °C, then with 250 μl/well of 3.0% BSA (bovine serum albumin) in PBS for 8 h, followed by washing with PBS. The samples that dissolved in 50 μl PBS with 1.0% BSA, were mixed with 50 μl sIL-6R. And the mixture was added to the plate with 100 μl/well and incubated overnight at 4 °C. After the plate was washed with PBST (0.1% Tween 20 in PBS), 100 μl/well of mouse monoclonal anti-human IL-6R (1 : 1000 dilution in PBST) were added, incubated for 1 h at 4 °C. Then 100 μl/well of horseradish peroxidase (HRP)-labeled horse monoclonal anti-mouse IgG (1 : 5000 diluted in PBST) were added and incubated at 4 °C for 1 h. After the final wash with PBST, 100 μl/well TMB solution (3,3′,5,5′-tetramethyl benzidine dihydrochloride) were added to cause a color reaction. And the reaction was ended by the addition of 100 μl/well 2 N HCl, after it took place for 1 h at room temperature. The absorbance at 450 nm was recorded as measurement of the reaction. 713A exhibited the IL-6 receptor antagonistic activity with IC_{50} value of 8.6 μM. In addition, the MTT assays didn't indicatew this compound has any cytotoxicities on the HCT-8 (human coloncancer), Bel-7402 (human hepatocarcinoma) and BGC823 (human gastric carcinoma) cell lines when tested at 10 μmol/l. A further investigation of other bioactivities of 713A is in progress.

Table 2. NMR Data of 713A in DMSO-d_6 (400 MHz for ^{1}H and 100 MHz for ^{13}C)

No.	^{13}C	^{1}H mult, *J* (Hz)
1	154.0	8.27 (1H, s)
3	87.2	4.2 (OH, br s)
4	39.4	3.21 (1H, d, 17.2)
		3.02 (1H, d, 17.2)
4a	130.5	
5	112.4	6.21 (1H, br s)
5a	141.6	
6	101.4	6.34 (1H, br s)
7	164.1	
7-OCH_3	55.7	3.77 (3H, s)
8	98.2	6.13 (1H, br s)
9	165.4	15.15 (OH, s)
9a	111.3	
10	181.7	
10a	106. 87	
11	53.6	3.56 (2H, br s)
12	168.8	
13		7.93 (NH, br d, 6.0)
14	57.4	4.09 (1H, br s)
15	173.1	11.5 (OH, br s)
16	36.8	1.88 (1H, m)
17	16.0	0.88 (3H, m)
18	25.2	1.42 (1H, m)
		1.14 (1H, m)
19	11.7	0.85 (3H, m)
20	60.5	3.62 (1H, dd, 5.3, 10.4)
		3.58 (1H, br d, 10.4)
		4.2 (OH, br s)

Acknowledgement The study was supported by the National Science Foundation of China (NSFC, 30370038).

References

1) Horváth B. V., Falus A., Tóth S., Szalai C., Lázár-Molnár E., Holub M. C., Buzás E., Nagy A., Fulop A. K., *Immunol. Lett.*, **80**, 151—154 (2002).
2) Gentiletti J., Fava R. A., *Arthritis Rheum.*, **48**, 1471—1474 (2003).
3) Varghese J. N., Moritz R. L., Lou M. Z., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 15959—15964 (2002).
4) Mukai A., Fukai T., Matsumoto Y., Ishikawa J., Hoshino Y., Yazawa K., Harada K., Mikami Y., *J. Antibiot.*, **59**, 366—369 (2006).
5) Shu Y. Z., Cutrone J. Q., Klohr S. E., Huang S., *J. Antibiot.*, **48**, 1060—1065 (1995).
6) Marfey P., *Carlsberg Res. Commun.*, **49**, 591—596 (1984).