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Iritectol G, a novel iridal-type triterpenoid from *Iris tectorum* displays anti-epileptic activity *in vitro* through inhibition of sodium channels

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ABSTRACT

Iritectol G, a novel iridal-type triterpenoid containing an uncommon tetrahydrofuran moiety, was isolated from the rhizomes of *Iris tectorum*. The structure was elucidated by comprehensive spectroscopic analysis. Iritectol G inhibited spontaneous and 4-aminopyridine-evoked calcium oscillations in primary cultured neocortical neurons with IC_{50} values of 8.2 μM and 12.5 μM, respectively. Further electrophysiological study demonstrated that iritectol G preferred to interact with inactivated state of voltage-gated sodium channel with an IC_{50} value of 7.0 μM. These data demonstrated that iritectol G was a novel sodium channel inhibitor.

1. Introduction

Iridals, a class of structurally unique metabolites featured by one highly substituted cyclohexane ring connected with a homofarnesyl side chain, were first reported from *Iris germanica* L. by Marner et al. [1]. Iridals were proposed to be biosynthesized through cyclization of epoxysqualene to a bicyclic intermediate followed by an opening of ring A between C-3 and C-4, together with a methyl and hydride shift [2]. Iridals have been reported to display many biological activities including cytotoxicity [3,4], ichthyotoxicity [5], antiplasmodial activity [6], and activation of PKC [7,8]. The plant *Iris tectorum* is a rich source of iridals with unique structures. Many iridal-type triterpenoids [9–12] and several other constituents [13] have been obtained from *I. tectorum* in previous work.

Primary cultured neocortical neurons form neuronal network as demonstrated by synchronized spontaneous Ca^{2+} oscillation (SCOs) and electric spike activity (ESA) [14,15]. These SCOs occur simultaneously with action potential generation and depend on the neuronal excitable neurotransmission [16,17]. Pharmacological alteration of neuronal excitatory/inhibitory inputs influences both SCO and ESA [18]. Seizurogenic agents such as picrotoxin, 4-aminopyridine (4-AP) and kainate which enhance neuronal excitatory inputs increase the frequency or the amplitude of SCOs while anti-epileptic drugs have the opposite effects [19]. Therefore, measurement of SCO in primary cultured neurons can reflect the neuronal excitability.

As part of our ongoing neuroprotectants discovery program, we discovered a novel iridal-type triterpenoid termed iritectol G, from *I. tectorum*. We found that iritectol G inhibited spontaneous and 4-AP-evoked Ca^{2+} oscillations in primary cultured neocortical neurons with IC_{50} values of 8.2 μM and 12.5 μM, respectively, suggesting its anti-epileptic activity *in vitro*. Further pharmacological evaluation demonstrated that iritectol G was a novel sodium channel inhibitor.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-2000 polarimeter. The UV spectrum was recorded on a JASCO V-650 spectrophotometer. The IR spectrum was measured on a Nicolet 5700 FT-IR spectrometer. NMR measurements were recorded in $CDCl_3$ on a VNS-600 spectrometer, and HRESIMS data were acquired using a Micromass Autospec-Ultima ETOF spectrometer. HPLC separations were performed using a Shimadazu instrument equipped with an SPD-20A detector, LC-6AD

Abbreviations: 4-AP, 4-aminopyridine; 95% CI, 95% Confidence Intervals; $[Ca^{2+}]_i$, intracellular Ca^{2+}; AUC, area under curve; ARA-C, cytosine arabinoside; BSA, bovine serum albumin; DIV, days *in vitro*; DNase I, Deoxyribonuclease I; ESA, electric spike activity; FLIPR, Fluorescence Imaging Plate Reader; IC_{50}, half maximal inhibitory concentration; SCO, spontaneous Ca^{2+} oscillation; VGSC, voltage-gated sodium channel

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pumping system, in a YMC-Pack ODS-A column (250 × 20 mm, 5 μm). HPLC analyses were conducted on an Agilent 1260 instrument equipped with a DAD detector. TLC experiments were conducted on GF254 silica gel plates (Qingdao Haiyang Chemical Factory, Qingdao, China). Column chromatography experiments were carried out on Sephadex LH-20 (GE), ODS (50 μm, YMC, Japan), and silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China).

2.2. Materials

The rhizomes of *I. tectorum* were purchased from Lotus Pond Chinese Medicinal Herbs Wholesale Market in Chengdu, Sichuan province, China, and were authenticated by Professor Lin Ma from the Institute of Materia Medica, Peking Union Medical College. A voucher specimen (ID-S-2469) was deposited at the Herbarium of the Department of Medicinal Plants, the Institute of Materia Medica, Peking Union Medical College, Beijing. Trypsin, soybean trypsin inhibitor, glutamine, neurobasal medium and heat-inactivated fetal bovine serum were obtained from Life Technology (Grand Island, NY). D-glucose, glycine, phenol red, HEPES, cytosine arabinoside (ARA-C), bovine serum albumin (BSA), veratridine, 4-AP, and all the inorganic salts were purchased from Sigma-Aldrich (St. louis, MO, USA). Deoxyribonuclease I (DNase I) was purchased from Worthington Biochemical Co. (Lakewood, NJ, USA). Poly-L-lysine was purchased from Peptide Institute Inc. (Osaka, Japan). Fluo-8/AM was obtained from TEFlabs (Austin, TX, USA).

2.3. Extraction and isolation

The air-dried powder of the rhizomes of *I. tectorum* (100 kg) were exhaustively extracted with 95% EtOH under reflux (3 × 100 L). The extracts were combined and concentrated under vacuum to give a residue, which was suspended in water and partitioned with EtOAc and n-BuOH, successively. The EtOAc soluble fraction was applied to a macroporous resin column eluted with 40%, 70%, and 85% EtOH in ddH_2O, successively. After removing the solvent, the 70% EtOH eluate (480 g) was dissolved in 5 L methanol and the suspension was filtrated using waterman No. 1 filter paper and the supernatant was subjected to Sephadex LH-20 chromatography. After removing residual flavones in the filtrate using Sephadex LH-20 ($MeOH/CHCl_3$, 1:1), an iridals-enriched portion (100 g) was obtained, which was further chromatographed on a reversed-phase C_{18} silica gel column (80 × 6 cm) eluted with 50%, 60%, 70%, 80%, 90%, and 100% MeOH in H_2O to afford 6 fractions (F1–F6). F6 (5 g) was purified using Sephadex LH-20 ($CHCl_3$-MeOH, 1:1), followed by preparative HPLC (MeCN-H_2O, 55:45, 5 mL/min), to afford iritectol G (23 mg, t_R = 105.9 min).

Iritectol G: glassy soild; $[\alpha]_D^{20}$ + 1.59 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 266 (4.29) nm; IR ν_{max} 3406, 2967, 2936, 1709, 1655, 1609, 1451, 1376 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data were listed in Table 1; HRESIMS *m/z* 491.3744 $[M + H]^+$ (calcd. for $C_{30}H_{51}O_5$, 491.3731).

Table 1
^1H and ^{13}C NMR spectroscopic data for iritectol G in $CDCl_3$.[a]

Position	δ_H	δ_C
1	10.13, s	190.1
2		133.1
3	3.57, t (6.0)	62.9
4	1.35, m	32.6
	1.25, m	
5	2.02, m	26.6
	1.76, m	
6	3.27, br d (10.2)	43.3
7		163.2
8	2.58, br td (13.2, 4.8)	23.8
	2.50, br d (13.2)	
9	1.82, m	36.9
	1.64, m	
10		75.0
11		44.7
12	1.27, m	36.6
	1.16, m	
13	1.92, m	21.8
	1.88, m	
14	5.29, t (6.6)	126.2
15		134.9
16	4.42, dd (8.4, 6.6)	81.1
17	1.99, m	39.1
	1.88, m	
18	4.02, br s	77.0
19		84.9
20	1.46, m	40.8
21	2.03, m	22.9
22	5.08, t (7.2)	124.3
23		131.6
24	1.66, s	25.6
25	1.80, s	10.9
26	1.06, s	17.8
27	1.12, s	26.2
28	1.49, s	11.3
29	1.19, s	18.8
30	1.59, s	17.6

[a] ^1H NMR data (δ) were measured in $CDCl_3$ at 600 MHz; ^{13}C NMR data (δ) were measured in $CDCl_3$ at 150 MHz.

2.4. Primary cultures of neocortical neurons

All animal experiment protocols were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) approved by the China Pharmaceutical University Institutional Animal Care and Use Committee. Animals were treated humanely and with regard for alleviation of suffering. Neocortical neuron cultures were obtained from embryonic day 16 C57 BL/6 mice. Briefly, neocortices were collected, stripped off meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37 °C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase I-containing isolation buffer, centrifuged, and resuspended in neurobasal complete medium (neurobasal medium supplemented with 2% NS21 [20], 1 mM L-glutamine, 1% HEPES with 5% fetal bovine serum), Cells were plated onto poly-L-lysine-coated clear-bottomed, black-wall, 96-well plates (Corning Life Sciences, Acton, MA) at a density of 5.6 × 10^5 cells/cm^2 and incubated at 37 °C in a 5% CO_2 and 95% humidity atmosphere. A final concentration of ARA-C (10 μM) was added to the culture medium after plating for 24–36 h to prevent the astrocytes proliferation. The culture medium was half-replaced on day 5 and 7 with serum-free growth medium containing Neurobasal medium supplemented with 2% NS21, 1% HEPES and 0.2 mM L-glutamine.

2.5. Intracellular Ca^{2+} measurement

Neocortical neurons at 8–9 days *in vitro* (DIV) were used to investigate the alteration of iritectol G on intracellular Ca^{2+} dynamics. Briefly, the growth medium was removed and replaced with dye loading buffer (100 μL/well) containing 4 μM Fluo-8/AM and 0.5% BSA in Locke's buffer (in mM: HEPES 8.6, KCl 5.6, NaCl 154, D-glucose 5.6, $MgCl_2$ 1.0, $CaCl_2$ 2.3, and glycine 0.1, adjust pH to 7.4). After 45 min incubation in dye loading buffer, the neurons were washed four times with fresh Locke's buffer (200 μL/well) and placed in a Fluorescent Imaging Plate Reader (FLIPR^{Tetra}; Molecular Devices, Sunnyvale, CA) incubation chamber. Basal fluorescence levels were acquired in Locke's buffer for 5 min at a sampling rate of 1 Hz followed by an addition of vehicle or iritectol G using a programmable 96-channel pipetting robotic system, and the fluorescent signals were recorded for an additional 7 min. To investigate influence of iritectol G on 4-AP or