Identification of geraldol as an inhibitor of aquaporin-4 binding by NMO-IgG

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Abstract. Neuromyelitis optica (NMO) is a severe neurological demyelinating autoimmune disease that affects the optic nerves and spinal cord. There is currently no effective cure or therapy. Aquaporin-4 (AQP4) is a known target of the autoimmune antibody NMO-IgG. Therefore, binding of NMO-IgG to AQP4, and subsequent activation of antibody-mediated and complement-dependent cytotoxicity (CDC), are thought to underlie the pathogenesis of NMO. In the present study, a cell-based high-throughput screening approach was developed to identify molecular inhibitors of NMO-IgG binding to AQP4. Using this approach, extracts from the herb Petroselinum crispum were shown to have inhibitory effects on NMO-IgG binding to AQP4, and the natural compound geraldol was purified from the herb extracts. Analytical high performance liquid chromatography, electrospray ionization-mass spectrometry and nuclear magnetic resonance analyses confirmed the identity of the isolated compound as geraldol, a flavonoid. Geraldol effectively blocked binding of NMO-IgG to AQP4 in immunofluorescence assays and decreased CDC in NMO-IgG/complement-treated FRTL-AQP4 cells and primary astrocytes. Geraldol exhibited low cytotoxicity, with no effect on proliferation or apoptosis of FRTL-AQP4 cells and primary astrocytes. Permeability assays indicated that geraldol did not alter the water transport function of AQP4 in either cell system. The present study suggests the potential therapeutic value of geraldol for NMO drug development.

Introduction

Neuromyelitis optica (NMO), also known as Devic's syndrome, is a severe demyelinating disease of the central nervous system that causes recurrent seizures. NMO mainly affects the optic nerves and the spinal cord, but it can also affect the brain, to a lesser extent (1). NMO is difficult to distinguish clinically from multiple sclerosis (MS) as both disorders have similar characteristics in the early stages of disease. NMO was once considered a subtype of MS, and was even termed optica-spinal MS in Japan. However, a previous study has suggested that NMO is an independent clinical entity that is distinct from MS (2). NMO is caused by an autoimmune antibody (NMO-IgG) targeting aquaporin-4 (AQP4), a plasma membrane water transport channel expressed in the glia (3-5). Binding of NMO-IgG to AQP4 on astrocytes can cause complement-mediated and cell-mediated injury to astrocytes, triggering a series of inflammatory cascades that result in the release of cytokines, activation of microglia and accumulation of leukocytes. Together, these responses result in neuronal injury and cause the clinical symptoms of NMO (6-8).

NMO remains to be an incurable disease. The goal of treating acute NMO events is to improve relapse symptoms and restore neurological functions, while the aim of long-term immunosuppression is to prevent further attacks (9-11). For long-term immunosuppression, patients usually receive either B cell-targeted therapies, such as intravenous rituximab or oral azathioprine, and/or prednisone (12-20). Other therapeutic options include mycophenolate mofetil (21), methotrexate (22) or mitoxantrone. Mitoxantrone can cause adverse effects, such as cardiotoxicity or leukemia, and is thus generally not considered for first-line treatment (11,23-28). A novel strategy for NMO treatment is blocking the binding of NMO-IgG to AQP4. Based on this strategy, a high-affinity, non-pathogenic monoclonal antibody (aquaporumab) was developed and exhibited positive effects in clinical trials (29). Aquaporumab can relieve NMO symptoms by competing with NMO-IgG to bind to AQP4 (29). However, monoclonal antibody drugs can also have disadvantages, for example, they may activate the complement system and cause pathological changes similar to those...
observed in NMO, or they may induce the production of auto-
immune antibodies, thus reducing their protective effects (10).

In this context, inhibitors that block binding between
NMO-IgG and AQP4 may represent potential drug candidates
for treating NMO. A previously established reporter cell-based
high-throughput screening system identified a small molecule
inhibitor (isotetrandrine, obtained from an extract of the
herb Mahonia japonica) that can block binding between
NMO-IgG and AQP4 in a dose-dependent manner (30). In
the present study, the natural compounds from another
herb (Petroselinum crispum) that exhibited similar inhibi-
tory effects in the screening system were purified. Another
compound from this herb, geraldol, inhibited binding of
NMO-IgG to AQP4.

Materials and methods

Cells, animals and antibodies. Chinese hamster lung fibroblast
cells V79 (American Type Culture Collection; cat. no. CCL-93)
were cultured in DMEM medium (D7777; Sigma-Aldrich; Merck KGaA). Fischer rat thyroid epithelial cells FrTL which
generously expressed M23-aQP4 were supplied by Liaoning
Merck KGaA). FrTL were cultured in F-12-modified coon's
Medical college and established from a plasmid transfec-
tion (30). FrTL were cultured in F12-modified Coon's
medium (Sigma-Aldrich; Merck KGaA). Both culture media
were supplemented with 10% (v/v) FBS (Sigma-Aldrich; Merck KGaA) and 2 mM glutamine. Cells were cultured at
37˚C under a humidified atmosphere containing 5% CO2.

In total, 30 female (age, 6-week-old; weight ~18 g) and
20 male (age, 8-week-old; weight ~23 g) AQP4−/− mice (Dalian
Medical University) were used in the present study, as previ-
ously described (31). Mice were housed in a 20-26˚C specific
pathogen-free animal facility on a 12 h light/dark cycle and
15 times per hour air exchange, with constant access to food
and water. Mice were mated until birth, then neonatal mice
were used for primary astrocytes isolation. Protocols for
mouse experiments were approved by the Laboratory Animal
Ethics Committee of School of Life Science, Jilin University
(Changchun, China; approval no. 2017-nsc049).

NMO-IgG was purified from the sera of patients with
NMO and concentrated using a Melon Gel IgG Purification kit
(Thermo Fisher Scientific, Inc.) and Amicon Ultra Centrifugal
Filter Units (EMD Millipore) according to the manufacturer’s
protocol. NMO serum and control (non-NMO) serum were
obtained from clinical patients whose sera were NMO-IgG
positive or negative, respectively (32). This study was
approved by Ethics Committee of The China-Japan Union Hospital,
Jilin University (approval no. 2017022238). Written informed
consent was obtained from all patients.

Primary screening procedures. The procedure for primary
screening of various herb fractions inhibiting the binding of
NMO-IgG to AQP4 was performed as previously described (30). Briefly, V79-AQP4 cells were plated in
black-walled and clear-bottomed 96-well tissue culture
plates (Costar; Corning, Inc.) and maintained in complete
DMEM at 37˚C for ~48 h until they reached 90% confluence.
Overall, 80 wells were used to test natural fractions or natural
compounds which were isolated and maintained in the lab of
the present study; the first row of each plate was used as a
negative control (V79-AQP4, no test compound) and the last
row of each plate was used as a positive control (V79-null, no
test compound). Each well was washed three times with PBS,
fixed with 4% paraformaldehyde (PFA) at room temperature
for 10 min and blocked with 3% BSA (Merck KGaA) at room
temperature for 30 min (100 µl/well). Test natural fractions
were added to each well (0.5 µl 20 mM solution in DMSO)
and incubated for 15 min at room temperature. After washing
twice with PBS, NMO-IgG (0.2 µg/ml) and horseradish perox-
dase (HRP)-labeled goat anti-human IgG secondary antibody
(1:1,000, Santa Cruz Biotechnology, Inc.; cat. no. sc-2907)
were added to each well and incubated at 37˚C for 1 h. After
washing three times with PBS, enhanced chemiluminescent
substrate (ECL-Plus; GE Healthcare) was added to measure
HRP activity. Chemiluminescence was measured after 5 min
using a plate reader (TECAN Infinite 200; Tecan Group Ltd.).

Isolation and identification of target natural compounds. P. crispum was purchased from a commercial herb supplier
(Hongjiang Herb Store). The isolation procedures were
performed as previously described (33). The aerial parts of
P. crispum were crushed and extracted using different
solvents (petroleum, ethyl acetate, chloroform, n-butanol;
all were 100%; purchased from Beijing Chemical Works)
and water. Mice were mated until birth, then neonatal mice
were used for primary astrocytes isolation. Protocols for
mouse experiments were approved by the Laboratory Animal
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NMO-IgG was purified from the sera of patients with
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approved by Ethics Committee of The China-Japan Union Hospital,
Jilin University (approval no. 2017022238). Written informed
consent was obtained from all patients.
Primary astrocyte cultures. Primary astrocytes were isolated from the cortices of 20 1-day-old wild-type and AQP4\textsuperscript{−/−} mice as previously described (34). Briefly, the cerebral hemispheres of the mice were separated, and the meninges, hippocampus, basal ganglia and olfactory bulb were removed. Cortical tissue was then isolated using forceps under a microscope, and digested in 0.25% trypsin-EDTA in DMEM at 37°C for 15 min. The digested cells were centrifuged at 300 x g for 10 min at room temperature, and cultured on a poly-L-ornithine hydro-bromide-coated (P3655; Sigma-Aldrich; Merck KGaA) 96-well plate or glass coverslips in DMEM supplemented with 10% FBS and 5% human complement (Sigma-Aldrich; Merck KGaA) at 37°C under a humidified atmosphere containing 5% CO\textsubscript{2}. The medium was changed every other day. To purify astrocytes, the culture plates were shaken in a rotator at 180 rpm overnight and then at 220 rpm for 4 h when confluency reached ~30%. To prevent proliferation of other cell types, cell mixtures were treated with 10 μM cytosine arabinoside for 48 h. The medium was replaced with DMEM containing 3% FBS and 0.15 mM dibutyryl cAMP to induce differentiation when confluency reached ~50%. Once astrocyte confluence had reached >90%, the complement-dependent cytotoxicity (CDC) assay was performed.

CDC assay. For the CDC assay, cells were incubated with P. crispum fractions, isolated geraldol or DMSO for 15 min, followed by treatment of NMO-IgG (2.5 μg/ml) and 5% human complement (Sigma-Aldrich; Merck KGaA) for 1 h at 37°C. Control Human serum collected from patients without NMO (1:200) was added as a negative control. CellTiter-Glo\textsuperscript{®} Luminescent Cell Viability Assay (Promega Corporation) was used to measure cell viability and its decrease according to CDC. Briefly, after incubation at 37°C for 48 h, 100 μl of CellTiter-Glo\textsuperscript{®} reagent was added to each well and mixed by gentle vortexing, swirling or inverting to obtain a homogeneous solution. Luminescence was recorded after 5 min using a microplate reader (Fluostar Optima; BMG LabTech GmbH). All experiments were repeated twice with four replicates.

Immunofluorescence staining. V79 cells expressing M23-AQP4 were cultured on round glass coverslips in 24-well plate (Corning, Inc.) for 24 h. After washing three times with PBS, the cells were blocked with 3% BSA (Merck KGaA) at room temperature for 1 h. Geral dol or DMSO was added in 3% BSA and incubated at room temperature for 15 min, followed by the addition of NMO-IgG (20 μg/ml) or control serum from patients without NMO (1:200) at room temperature for 1 h. V79-AQP4 cells were washed with PBS three times and incubated with Alexa Fluor 555-conjugated goat anti-human IgG secondary antibody (1:400; Invitrogen; Thermo Fisher Scientific, Inc; cat. no. A-21433). For AQP4 immunofluorescence staining, V79-AQP4 cells were fixed in 4% PFA at room temperature for 10 min and permeabilized with 0.5% Triton X-100 at room temperature for 10 min. After blocking with 3% BSA at room temperature for 1 h, the cells were incubated with rabbit anti-AQP4 antibody (1:200; Santa Cruz Biotechnology; cat. no. sc-32739) at room temperature for 1 h. Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A32731) was used as the secondary antibody. Fluorescence images were obtained and observed using fluorescence microscopy (IX71; Olympus Corporation).

Cell viability assay. Cell viability was measured in 96-well plates with a colorimetric assay using the Cell Proliferation reagent (WST-1; Roche; cat. no. 11644807001) according to the manufacturer's protocol. Cells at 70% confluency were treated with serial dilutions of geraldol (2, 4, 8, 16, 32, 64, 128 or 256 μM) for 24 h. WST-1 reagent (10 μl/well) was added to cultured cells (100 μl/well) and incubated at 37°C for 1 h. Absorbance was measured at 440 nm. All experiments were repeated twice with 4 replicates.

Detection of apoptosis. Apoptosis was detected using a Cell Death Detection ELISA\textsuperscript{PLUS} kit (Roche) according to the manufacturer's protocol. This photometric enzyme immunoassay is used for quantitative in vitro detection of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) following programmed cell death. Cells at 70% confluency were treated with serial dilutions of geraldol (2, 4, 8, 16, 32, 128 or 256 μM) for 24 h. Cell lysates were then placed in a streptavidin-coated microplate and incubated with a mixture of biotinylated anti-histone and anti-DNA peroxidase antibodies that were included in the kit at room temperature for 2 h. The amount of peroxidase retained in the immuno-complex was photometrically determined after reaction with 100 μl 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt substrate at room temperature for 15 min. Absorbance was measured at 405 nm. All experiments were repeated twice with four replicates.

Osmotic water permeability assay. Osmotic water permeability of the plasma membrane was assessed using a slightly modified version of a previously described calcein fluorescence quenching method (31). Briefly, cells were cultured on glass coverslips pre-coated for 24 h, incubated with 5 μM calcein-AM (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 15 min, and then transferred to a perfusion chamber designed for rapid solution exchange. The time course of cytoplasmic calcein fluorescence in response to cell shrinkage induced by exchange of perfusate between PBS (300 mOsml) and hypertonic PBS (500 mOsml containing 200 mM D-mannitol) was measured. A reciprocal exponential time constant (τ=1/s) was used as an indicator of cell shrinkage, where τ is the time from the initiation of the osmotic switch to the point where cytoplasmic calcein fluorescence quenching reaches its maximum. All experiments were repeated twice with four replicates.

Statistical analysis. Statistical analysis was performed using the unpaired two-tailed Student’s t-test in SPSS (version 17.0; SPSS, Inc.). Multiple comparisons between groups were performed using a one-way ANOVA followed by Sidak multiple comparisons test with GraphPad Prism software (version 7; GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Isolation and structural characterization of geraldol. Fractions from the herb P. crispum were isolated by solvent extraction and subject to a screening as reported in a previous
study (30). During the primary screening, four continuous fractions isolated from ethyl acetate extraction were identified to have inhibitory effects on NMO-IgG binding to AQP4 (Fig. 1A). These fractions also exhibited inhibitory effects on NMO-IgG-dependent complement cytotoxicity in secondary screens (Fig. 1B).

This bioactivity-guided phytochemical study led to the isolation of the flavonoid geraldol from all the four fractions. The purity of the compound was assessed using analytical HPLC. As presented in Fig. 2, only a single peak with a retention time of 47.74 min was observed. The purity of the peak was determined to be 99.5%. These results indicated that >99% pure geraldol could be obtained successfully using HSccc separation under optimized conditions. The HSccc-purified fraction was structurally characterized using ESI-mass spectrometry (ESI-MS) and NMR as follows:

Molecular formula: C_{16}H_{12}O_{6}. ^1H NMR (500 MHz, CDCl3): δ (ppm) 7.93 (1H, d, J=8.8 Hz, H-5), 6.91 (1H, dd, J=2.2 Hz, 8.8 Hz, H-6), 6.97 (1H, d, J=2.2 Hz, H-8), 7.77 (1H, d, J=2.1 Hz, H-2'), 6.94 (1H, d, J=8.5 Hz, H-5'), 7.70 (1H, d, J=8.5 Hz, H-6'), 9.66 (1H, s, OH-3), 10.74 (1H, s, OH-7), 9.08 (1H, s, OH-4') and 3.83 (1H, s, OCH3-3'). ^13C NMR (100 MHz, CD3OD): δ (ppm) 144.9 (C-2), 137.3 (C-3), 172.0 (C-4), 126.4 (C-5), 114.7 (C-6), 162.3 (C-7), 102.1 (C-8), 156.3 (C-9), 114.2 (C-10), 122.6 (C-1'), 111.7 (C-2'), 147.4 (C-3'), 148.4 (C-4'), 115.5 (C-5'), 121.4 (C-6') and 55.6 (OCH3-3').

Geraldol decreases CDC in both NMO-IgG/complement-treated FRTL-AQP4 cells and primary astrocytes. Next, the inhibitory effects of geraldol on CDC were evaluated. FRTL cells stably expressing human AQP4 were incubated with NMO-IgG for 60 min in the presence of human complement, along with either geraldol or DMSO. The cytotoxicity of geraldol was also measured using this system. As presented in Fig. 4A, geraldol increased the viability of NMO-IgG/complement-treated FRTL-AQP4 cells in a concentration-dependent manner. The half maximal inhibitory concentration (IC_{50}) of geraldol was 24.6 µM. A group of FRTL cells without AQP4 expression were used as the negative control. In order to confirm the effects of geraldol purified from a natural source, a commercial geraldol compound was also purchased and its inhibitory effects were tested in the same CDC assay with similar results (data not shown).

The inhibitory effect of geraldol on CDC was also assessed in cultured primary astrocytes from wild-type and AQP4−/− mice (Fig. 4B). Geraldol increased the viability of cultured NMO-IgG/complement-treated primary astrocytes from wild-type mice in a concentration-dependent manner. The IC_{50} of geraldol was 30.3 µM. No inhibitory effect was observed in cultured primary astrocytes from AQP4−/− mice.

Geraldol does not affect the viability of FRTL-AQP4 cells and primary astrocytes. To determine whether geraldol affects cell viability, its effects on cell proliferation were analyzed using the WST-1 assay and cell apoptosis using the cell death assay. As presented in Fig. 5A and B, no significant differences in cell proliferation or apoptosis were observed within the geraldol dose range used in the present study.

Geraldol does not affect the water permeability of FRTL-AQP4 cells and primary astrocytes. Whether geraldol influences the osmotic water permeability of AQP4 was further investigated in the present study. Using a calcein-AM fluorescence quenching-based method as previously described (31), geraldol did not significantly influence osmotic water permeability in either FRTL-AQP4 cells or astrocytes (Fig. 6).
Figure 2. (A) Representative HPLC chromatogram, (B) mass spectrum and (C) structure of the isolated compound, geraldol. Reverse-phase HPLC analysis was performed using the C18 Octadecyl-silica chromatographic column (5 µm, 4.6x200 mm) at a flow rate of 1.0 ml/min. HPLC, high performance liquid chromatography; AU, absorbance unit.

Figure 3. Immunofluorescence images exhibiting the effect of geraldol on NMO-IgG binding to AQP4. V79-AQP4 cells were incubated with 32 µM geraldol or DMSO prior to antibody addition. Non-AQP4-expressing V79 cells were used as a negative control. First row, staining with NMO-IgG and an Alexa Fluor 555-conjugated anti-human secondary antibody (red). Second and fourth row, there were, approximately, an equal total number of cells in the different groups. Third row, staining with rabbit anti-AQP4 antibody and an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (green). Scale bar, 50 µm. AQP4, aquaporin 4; NMO, neuromyelitis optica; Ab, antibody.
Discussion

Serum NMO-IgG targets astrocytic AQP4, which is the most highly expressed aquaporin in astrocytes (6,35-37). NMO-IgG/AQP4 antibodies are present in up to 80% of patients with NMO (6,38-40). Although drugs and therapies are available for NMO, including methylprednisolone, immunosuppression and plasma exchange,
relapse may still occur, making NMO an incurable disease (9-11).

Inhibitors of NMO-IgG binding to AQ4 are potential therapeutic candidates for NMO. An ideal inhibitor should effectively block binding of NMO-IgG to AQ4 without harming other cells. In a previous study, inhibitors of NMO-IgG binding to AQ4 were identified (30). The activity of isotretinadrine, an alkaloid compound purified from fractions of Mahonia japonica, was demonstrated using a CDC experiment. It was also demonstrated that isotretinadrine had low cytotoxicity and did not affect the water transport function of AQ4 (30). In the present study, the inhibitory effect of a flavonoid compound (geraldol, purified from a fraction of P. crispum) on NMO-IgG binding to AQ4 was examined.

P. crispum, commonly known as parsley, is a culinary herb that was originally found in the Mediterranean region, but now grown worldwide. Its main constituents include coumarins, furanocoumarins (bergaptin and imperatorin), ascorbic acid, carotenoids, flavonoids, apiole, various terpenoid compounds, phenylpropanoids, phthalides and tocopherol (41,42). P. crispum has been used in a number of different medicinal applications, acting as an antimicrobial, antianemic, anti-inflammatory, antiinflammatory, antihypertensive, diuretic, hypoglycemic, hypouricemic, antioxidative and estrogenic agent (43). Geraldol was purified from P. crispum fractions via HPLC and it was demonstrated that this molecule inhibited binding of NMO-IgG to AQ4.

Geraldol is a flavonoid. Flavonoid compounds have broad biological properties, including antioxidative, free-radical scavenging, as well as anti-inflammatory/infecive effects (44). These compounds usually have low cytotoxicity, and thus, may be safe for potential therapeutic use. Geraldol has been reported to show milder inhibitory effects against the interaction of human papillomavirus-16-E6 with caspase-8 (45). In addition, geraldol is a 3'-methoxylated metabolite of the flavonoid fisetin, which has distinct antioxidant, anti-inflammatory and antiangiogenic properties (46).

Geraldol blocked NMO-IgG binding to AQ4 in immunofluorescence assays and reduced cytotoxicity in NMO-IgG/complement-treated FRTL-AQ4 cells and primary astrocytes. Furthermore, it did not affect the viability of FRTL-AQ4 cells and primary astrocytes, or water permeability in either cell system. These data suggest that geraldol is an effective inhibitor of NMO-IgG binding to AQ4, and could be a candidate molecule for use in NMO therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and MS designed the experiments and analyzed the experimental results. JW and SW carried out the experiments and wrote the manuscript. HX, WL, DW, LZ, YL, JC and FL also performed the experiments, and helped write and review the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Protocols for mouse experiments were approved by The Laboratory Animal Ethics Committee of School of Life Science, Jilin University (Changchun, China; approval no. 2017-nsfc049). The use of patient blood samples was approved by Ethics Committee of The China-Japan Union Hospital, Jilin University (approval no. 2017022238). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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