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Screening, Synthesis, and In Vitro Evaluation of Vinyl Sulfones as Inhibitors of Complement-Dependent Cytotoxicity in Neuromyelitis Optica

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Neuromyelitis optica (NMO) is a demyelinating autoimmune disease of the optic nerve and spinal cord triggered by binding of NMO-specific immunoglobulin G (NMO-IgG) auto-antibodies to the water channel aquaporin-4 (AQP4) in astrocytes. To find potential NMO therapeutics, a screening system was established and used to identify inhibitors of NMO-IgG-mediated complement-dependent cytotoxicity (CDC). The screening of approximately 400 compounds yielded potent hit compounds with inhibitory effects against CDC in U87-MG cells expressing human AQP4. Derivatives of the hit compounds were synthesized and evaluated for their inhibition of CDC. Of the small molecules synthesized, (*E*)-1-(2-((4-methoxyphenyl)sulfonyl)vinyl)-[4-[(3-trifluoromethyl)phenyl] methoxy]benzene (**5 c**) showed the most potent activity in both stably transfected U87-MG cells and mice-derived astrocytes. The results of this study suggest that **5 c**, which targets NMO-IgG-specific CDC, may be useful as a research tool and a potential candidate for therapeutic development for the treatment of NMO.

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Neuromyelitis optica (NMO) is an autoimmune disorder of the optic nerve and spinal cord of the central nervous system (CNS).[1] The symptoms of NMO include optic neuritis (inflammation of the optic nerve with a sudden decrease of vision) and acute myelitis (inflammation of the spinal cord). NMO was previously thought to be a variant of multiple sclerosis (MS) but in 2004, Lennon et al.[1b] reported that NMO immunoglobulin G (NMO-IgG) was present in patients with NMO but not in those with MS. Because NMO-IgG is detected with a high sensitivity and specificity in clinically defined NMO, this marker is now used as a major diagnostic criterion.[2] The etiology of NMO is elusive but recent studies have suggested that it may involve the binding of pathogenic NMO-IgG auto-antibody to the water channel aquaporin-4 (AQP-4), which is expressed by almost all CNS astrocytes, but it is particularly enriched in the spinal cord gray matter, the posterior optic nerve, and the floor of the fourth ventricle.[3] Several magnetic resonance imaging (MRI) results of patients with NMO showed that the associated brain lesions were extensively localized in high AQP4 expression sites.[4]

An in vitro assay showed that NMO-IgG was bound to the extracellular domain of AQP4, and in the presence of active complement, this binding leads to strong complement activation and rapid complement-dependent cytotoxicity (CDC).[5] Moreover, a recent study reported that passive transfer (intrathecal injection) of NMO-IgG and human activated complement into an animal model triggers symptoms similar to NMO.[6]

Considering that strong humoral responses are a central feature of NMO, common therapies include general immunosuppressants and plasma exchange to achieve a sustained depletion of NMO-IgG and complement. However, these therapies are associated with severe side effects. Several therapeutic strategies for perturbing complement proteins or interleukin (IL)-6 receptor, and depleting neutrophils, eosinophils, or B cells (CD19) are under clinical evaluation for the treatment of NMO.[7] Other therapeutic approaches have been developed to block the binding of NMO-IgG to AQP4 and decrease CDC. Among them, aquaporumab, a nonpathogenic human monoclonal antibody, competitively displaces NMO-IgG in the serum of patients with NMO. This direct blocker greatly decreased NMO-IgG-dependent cytotoxicity and NMO pathology in both in vivo and in vitro models of NMO.[7] In an alternative ap-

proach, small molecules were identified as possible blockers that decrease the binding of NMO-IgG to AQP4 by competitively binding to the extracellular surface of AQP4.[8] Compounds that specifically block NMO-IgG binding to AQP4 could provide an approach to inhibiting CDC and further inflammatory cascades. The small molecules identified so far exhibit relatively weak inhibitory activity against CDC and unfavorable pharmacological properties. Here, we developed a cell-based screening system and identified potent inhibitors of CDC caused by NMO-IgG binding to AQP4.

Our cell-based assay used stably transfected U87-MG cells expressing high levels of the M23 isoform of the human AQP4 channel. Research studies have revealed that the binding of NMO-IgG from NMO patients occurs more with transfected cells expressing the M23 isoform than those with M1 isoform.[9] AQP4 overexpression in the stable cell line was confirmed using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis (Figure S1 in the Supporting Information).

To establish a screening system for inhibitors of CDC caused by a human NMO-IgG autoantibody from patients with NMO, we measured lactate dehydrogenase (LDH) release from cells with damaged membranes. The binding of NMO-IgG to AQP4 recruited complement (C1q) and formed a membrane attack complex (MAC), which disrupted the plasma membrane followed by LDH release (Figure 1 a). To validate the efficacy of our CDC assay system, NMO-IgG from patients with NMO or control-IgG from healthy individuals was treated with increasing concentrations of human complement in either non-transfected or AQP4-overexpressing cells. NMO-IgG incubation with AQP4 overexpressing cells significantly increased LDH release dose-dependently whereas the control groups did not exhibit this effect (Figure S2 in the Supporting Information).

NMO-IgG acquired from the sera of patients with NMO was provided by Seoul National Hospital, Korea. Pathogenic waste sera from patients with severe relapsing NMO who had undergone plasmapheresis was stored at −80 °C and bio-banked for future reference. The use of NMO-IgG derived from patient sera is valuable for developing in vitro, in vivo, and ex vivo disease models of NMO.[10] This study was approved by the Institutional Review Board of the Seoul National University Hospital (IRB number: H-1012-023-317). All patients provided written informed consent. NMO-IgG was purified from sera, using the method outlined in the Supporting Information, because serum contains other IgGs, complement, cytokines, and plasma protein-bound toxins. The severity of disease progression of each patient differs, as does the concentration of IgG, and therefore, the purified NMO-IgG was subjected to activity testing prior to the screening experiments. The NMO-IgG binding affinity and CDC pathogenicity of the NMO-IgGs from different patients were tested using the same concentrations, and samples with optimal activity were selected (concentration of each NMO-IgG that induced 50% CDC).

To identify potent small-molecule inhibitors of NMO-IgG-dependent CDC, we screened ~400 synthetic small molecules from our central nervous system (CNS)-focused chemical library

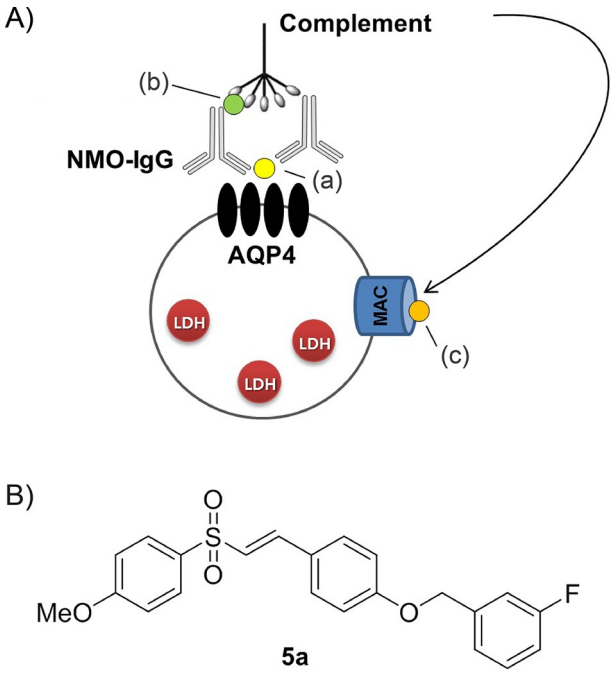
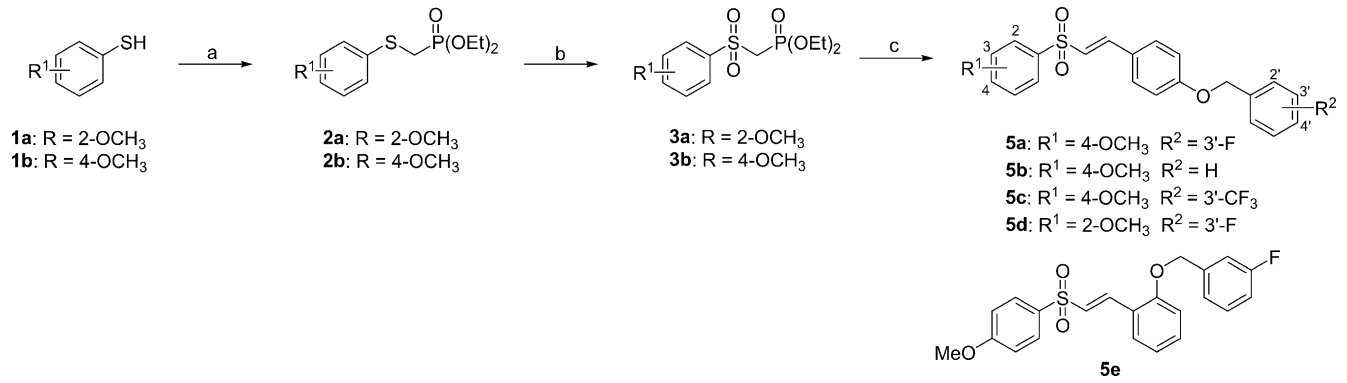


Figure 1. Screening system for the identification of inhibitors of neuromyelitis optica-specific immunoglobulin G autoantibodies (NMO-IgG)/complement-mediated cytotoxicity. A) Schematic of the screening assay used for identifying potent inhibitors of (a) NMO-IgG binding to aquaporin-4 (AQP4), (b) complement binding to NMO-IgG, and (c) membrane attack complex (MAC) formation by measuring lactate dehydrogenase (LDH) release from AQP4-expressing U87-MG cells incubated with NMO-IgG and 5% complement. B) Chemical structure of hit compound **5a**.

and selected a hit compound (**5a**) that decreased the LDH release by >30% (Figure 1 b).

The analysis of the screening results revealed that the vinyl sulfone group is a potentially favorable motif for inhibitory activity against CDC. Accordingly, we synthesized five vinyl sulfone derivatives by introducing H or CF_3 instead of F (**5b** and **5c**) or by placing the OMe or PhOMe groups in different positions on the aryl group (**5d** and **5e**). Vinyl sulfone derivatives **5a**–**e** were prepared in three steps (Scheme 1).[11] First, substituted benzenethiols (**1**) were coupled with (diethoxyphosphoryl)methyl-4-methylbenzenesulfonate in the presence of a base to obtain the sulfides (**2**). In the next step, the sulfides (**2**) were oxidized with *meta*-chloroperoxybenzoic acid (mCPBA) at 0 °C to obtain the sulfones (**3**). The final compounds (**5a**–**e**) were obtained by Horner–Emmons olefination reactions with the desired substituted benzaldehydes (**4**; for synthetic methods, see the Supporting Information).

Using a CDC assay, we found that compound **5c** exhibited the most potent inhibitory activity (43.4% decrease in LDH release at 25 μM). The substitution of the trifluoromethyl group (CF_3) instead of the fluorine group (F) on the ring C significantly increased the inhibitory activity in the CDC assay. When the electron-withdrawing group (F or CF_3) was replaced with hydrogen (**5b**), the inhibitory activity was decreased (Figure 2). We placed a methoxy group at the 2-position on ring A instead of the 4-position and observed that the 2-methoxy deriv-



Scheme 1. Synthesis of vinyl sulfone derivatives **5a–e**. *Reagents and conditions*: a) (diethoxyphosphoryl)methyl 4-methylbenzenesulfonate, $CsCO_2$, DMF, RT, overnight, 69–73%; b) *meta*-chloroperoxybenzoic acid, CH_2Cl_2, RT, 4 h, 68%; c) [(substituted phenyl)methoxy]benzaldehydes (**4**), *n*BuLi, THF, −78 °C, 2 h, 73–90%.

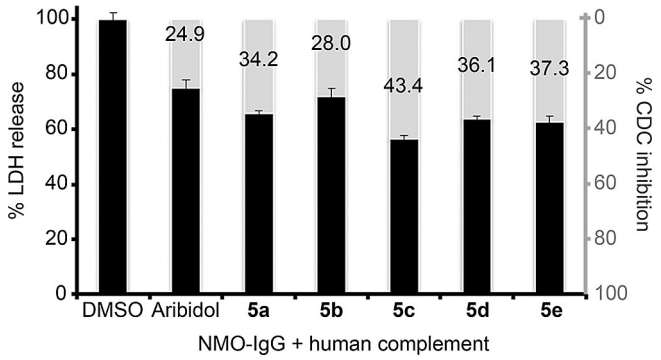


Figure 2. Potency of synthetic compounds (**5a–e**) against neuromyelitis optica-specific immunoglobulin G autoantibodies (NMO-IgG) and human complement-dependent cytotoxicity (CDC) in cell cultures Five vinyl sulfone derivatives (**5a–e**) were used at a final concentration of 25 μM before incubation with 100 μg mL^{-1} NMO-IgG and 5% pooled human complement in aquaporin-4 (AQP4)-expressing U87-MG cells. After 40 min, lactate dehydrogenase (LDH) release was measured. Arbidol was used as a positive control.[12] Black bars show LDH release (%) [data are the mean ± SEM $n = 4$] and grey bars show CDC inhibition (%) compared with dimethyl sulfoxide (DMSO)-treated group (no inhibition).

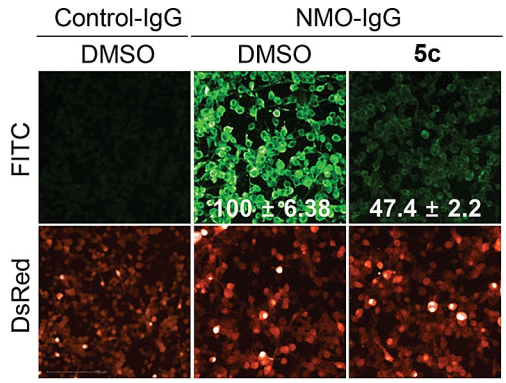


Figure 3. Analysis of inhibition of neuromyelitis optica-specific immunoglobulin G autoantibodies (NMO-IgG) binding to AQP4 by compound **5c** using immunofluorescence in aquaporin-4 (AQP4)-expressing U87-MG cells, which expressed DsRed were treated with compound **5c** (50 μM) before incubation with neuromyelitis optica-specific immunoglobulin-G autoantibodies (NMO-IgG, 6 μg mL^{-1}). Binding of NMO-IgG or control-IgG to AQP4 was detected using fluorescein isothiocyanate (FITC)-conjugated antihuman secondary antibody. Binding affinities were determined using Harmony software programmed to automatically calculate nonlinear regression of background-subtracted green/red fluorescence intensity ratios. Values shown are the relative binding affinity (%); data are the mean ± SEM ($n = 5$).

ative (**5d**) had a similar inhibitory activity with the 4-methoxy derivative (**5a**). Next, we changed a methylphenyl group attached to the *para* position on ring B to the *ortho* position (**5e**) but did not observe a clear change in activity.

Next, we evaluated the ability of **5c** to block the binding of NMO-IgG to AQP4 using immunofluorescence. An NMO-IgG polyclonal antibody against the three-dimensional epitopes on the extracellular surface of AQP4 was incubated with the AQP4-expressing U87-MG cells (DsRed positive) in the absence or presence of **5c** for 30 min. Then, the cells were washed with phosphate-buffered saline (PBS), fixed, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated antihuman secondary antibody. The NMO-IgG but not the control-IgG efficiently bound to the AQP4-overexpressing U87-MG cells. The immunofluorescence micrographs showed a substantial decrease in NMO-IgG binding to AQP4 following **5c** treatment (Figure 3). This result suggests that **5c** physically interferes with the binding of polyclonal NMO-IgG to AQP4 (Figure 1a).

The highest potency compound (**5c**) was further evaluated for its ability to decrease CDC in primary astrocytes highly expressing AQP4. It has been suggested that NMO-IgG binding to AQP4 is accompanied by complement-dependent astrocyte cytotoxicity, which produces NMO lesions in the disease pathogenesis.[13] CDC in astrocytes was tested using a two-color staining method in which live and dead cells were stained blue and green, respectively. Mouse primary astrocytes were incubated with NMO-IgG in the presence of human complement with or without **5c**. Compound **5c** decreased cytotoxicity dose-dependently in the NMO-IgG/complement-treated AQP-4-expressing astrocytes (Figure 4). We found that **5c** decreased cell death of primary astrocytes by 46.4% at 10 μM, which indicated a higher CDC inhibition than was shown in U87-MG cells (43.4% decrease at 25 μM). This result may be

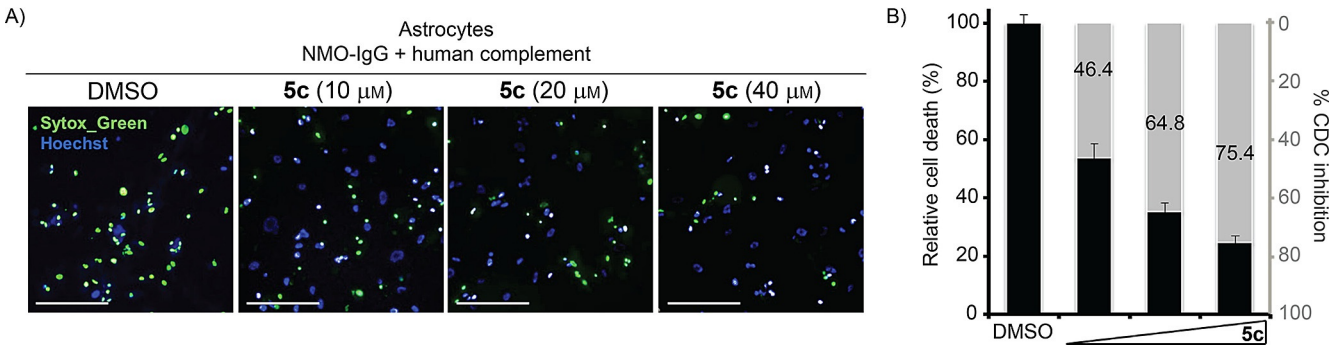


Figure 4. Inhibitory effect of compound **5c** against neuromyelitis optica-specific immunoglobulin G autoantibodies (NMO-IgG)-dependent complement-dependent cytotoxicity (CDC) in primary mouse astrocytes. Primary astrocytes from cortices of wildtype mice were induced with CDC with NMO-IgG in the presence of compound **5c** at the indicated concentration. A) Cell staining (dead cells: green; total cells: blue) and B) relative % cell death (Sytox green staining index/Hoechst staining index). Compound **5c** blocked cell death in a concentration-dependent manner. Black bars show Sytox green index (%) [data are the mean ± SEM $n = 5$] and grey bars show CDC inhibition (%) compared with dimethyl sulfoxide (DMSO)-treated group (no inhibition).

due to overexpression of the M23 isoform of AQP4 in U87-MG cells, which probably enhances the binding efficiency of NMO-IgG to AQP4 compared with endogenous AQP4 in astrocytes.

Compound **5c** was identified as a potent inhibitor of CDC induced by NMO-IgG binding to AQP4. For a compound to be used as a drug candidate, a number of pharmacokinetic requirements must be met. To assess the metabolic stability of **5c**, we determined its degree of degradation using liver microsomes from four different animal species (human, dog, rat, and mouse) and plasma from two different animal species (human and rat). Compound **5c** exhibited favorable liver microsomal stabilizing effects in the human, rat, and mouse with 43.9, 69, and 62.5%, respectively, remaining after 30 min incubation with nicotinamide adenine dinucleotide phosphate (NADPH). In addition, it exhibited complete resistance to metabolism in dog liver microsomes (~100%; Table 1). Compound **5c** was also stable in human and rat plasma during 120 min incubation. The favorable stability of **5c** against the human enzymes suggests that the compound may have a high bioavailability when administered to humans.

Metabolism-related drug–drug interactions in vivo might cause adverse reactions or severe side effects, and this was evaluated using a cytochrome 450 (CYP) inhibition test, particularly in subtypes 1A2, 2C9, 2C19, 2D6, and 3A4.[14] As shown in Table 1, for the subtypes evaluated, the IC_{50} values of compound **5c** were all over 10 μM except for 2C19 (slightly below 10 μM), indicating that **5c** is not likely to cause drug-drug interaction-related side effects.

Table 1. In vitro profile of compound **5c** determined using metabolic stability and cytochrome P450 (CYP) inhibition assays.

Species[a]	Stability[b]		CYP Inhibition	
	Microsomes	Plasma	Subtype	% Inhibition at 10 μM
Human	43.9	~100	1A2	<5
Dog	~100	–	2C9	<5
Rat	69.0	86.7	2C19	43.4
Mouse	62.5	–	2D6	<5
			3A4	<5

[a] All microsomes were purchased from BD Gentest (Woburn, MA, USA); [b] % Remaining was determined after 30 min (microsomes) or 120 min (plasma).

In conclusion, we report on a novel compound **5c**, which has inhibitory effects against NMO-IgG/CDC and may be a potential therapeutic candidate for NMO treatment. Further evaluations in ex vivo and in vivo animal models and drug optimization are necessary to evaluate its desired drug properties.

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Keywords: aquaporin-4 · complement-dependent cytotoxicity · neuromyelitis optica · NMO-specific immunoglobulin G autoantibodies · vinyl sulfones

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