A small-molecule screen yields idiotype-specific blockers of neuromyelitis optica-immunoglobulin G binding to aquaporin-4

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Running title: Idiotype-specific blockers of NMO-IgG binding to AQP4

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Background: A high-throughput, cytotoxicity-based screen was implemented to identify small molecule inhibitors of NMO-IgG / AQP4 binding.

Results: Pyrano[2,3-c]pyrazoles were identified as inhibitors that bind directly to the monoclonal NMO antibody used for screening but not to other NMO-IgGs.

Conclusion: Pyrano[2,3-c]pyrazoles are idiotype-specific blockers of NMO-IgG binding to AQP3.

Significance: Our results establish an antibody-targeted blocker strategy to prevent antibody-antigen binding.

ABSTRACT

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system caused by binding of anti-aquaporin-4 (AQP4) autoantibodies (NMO-IgG) to AQP4 on astrocytes. A screen was developed to identify inhibitors of NMO-IgG-dependent, complement-dependent cytotoxicity. Screening of 50,000 synthetic small molecules was done using CHO cells expressing human AQP4 and a recombinant monoclonal human NMO antibody (rAb-53). The screen yielded pyrano[2,3-c]pyrazoles that blocked rAb-53 binding to AQP4 and prevented cytotoxicity in cell culture and spinal cord slice models of NMO. Structure-activity analysis of eighty-two analogs yielded a blocker with IC₅₀ ~6 µM. Analysis of blocker mechanism indicated idiotype specificity, as: (i) pyrano[2,3-c]pyrazoles did not prevent AQP4 binding or cytotoxicity of other NMO-IgGs; and (ii) surface plasmon resonance showed specific rAb-53 binding. Antibody structure modeling and docking suggested a putative binding site near complementary determining regions. Small molecules with idiotype-specific antibody targeting may be useful as research tools and therapeutics.

INTRODUCTION

Antibodies are widely used as tools in cell biology and as therapeutics, and are implicated in the pathogenesis of various autoimmune diseases (1-3). The variable region of the Fab portion of an antibody confers idiotype specificity. A typical maneuver to reduce antibody-target interaction is the addition of excess, bystander target or target...
mimic to reduce the concentration of unbound antibody. Small molecules that bind to antibodies with idiotypic specificity could provide a potential alternative approach to block antibody binding. Small-molecule blockers could be useful as research tools in cell biology, as controls for antibody specificity, as therapeutics for autoimmune diseases or tumors associated with clonally expanded cell populations, or for neutralization of excess administered monoclonal antibodies.

We recently introduced candidate therapeutic approaches for the autoimmune disease neuromyelitis optica (NMO), in which inflammatory demyelinating lesions of the central nervous system are caused by binding of serum autoantibodies (NMO-IgG) to aquaporin-4 (AQP4) (4-6). AQP4 is a plasma membrane water channel expressed on astrocytes (7), which, when targeted by NMO-IgG, causes complement-dependent cytotoxicity and inflammation, resulting in secondary damage to oligodendrocytes and neurons (8-11). We developed a blocker antibody (aquaporumab) approach, in which a high-affinity monoclonal anti-AQP4 antibody was mutated to prevent its cytotoxicity effector functions (12). In an alternative approach, a small molecule screen based on NMO-IgG binding to AQP4 expressing cells yielded blockers that targeted the extracellular surface of AQP4 and reduced NMO-IgG binding (13).

In order to identify more potent small-molecule blockers for NMO therapy, here we implemented a cytotoxicity-based screen of NMO-IgG binding to AQP4. Interestingly, the screen yielded several idiotype-specific small molecule blockers, one of which was further characterized.

**EXPERIMENTAL METHODS**

**Cell lines and antibodies**—Chinese hamster ovary (CHO) cells expressing human M23-AQP4 were generated by stable transfection as described (14). CHO cells were cultured in F-12 Ham’s Nutrient mix medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Geneticin (200 µg/ml) was used as selection marker. Cells were grown at 37 °C in 5% CO2 / 95% air. Recombinant monoclonal NMO antibodies (NMO-rAb) were generated from clonally-expanded plasma blasts from cerebrospinal fluid (CSF) of NMO patients and purified as described (15). A non-NMO rAb (rAb-2B4) against measles virus nucleocapsid protein was used as a control isotype-matched antibody. NMO serum was obtained from NMO-IgG seropositive individuals who met the revised diagnostic criteria for clinical disease (16). Non-NMO human serum was used as control. For some studies IgG was purified from NMO or control serum and concentrated using a Melon Gel IgG Purification Kit (Thermo Fisher Scientific, Rockford, IL) and Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA).

**High-throughput screening**—Screening was done of 50,000 synthetic small molecules (ChemDiv, San Diego, CA). Screening was carried out using an integrated apparatus (Beckman Coulter, Fullerton, CA) consisting of a CO2 incubator, plate washer, Biomek FX liquid handling station and plate readers. M23-AQP4 expressing CHO cells were plated onto 96-well microplates (Costar, Corning; Corning, NY) at 15,000 cells/well and grown at 37 °C/5% CO2 for 18–24 h. Eighty wells contained test compounds, and the first and last columns of each plate were used for negative (no test compound) and positive (human complement only) controls. For screening, cells were washed with Hanks' balanced salt solution (HBSS) (without phenol red), leaving 45 µl HBSS. Test compounds were added (0.5 µl of 2.5 mM DMSO solution) to each well at 25 µM final concentration and incubated for 10 min at room temperature. A premixed solution (5 µl) of NMO-IgG (recombinant monoclonal antibody rAb-53, 30 µg/ml) and 20% pooled normal human complement serum (Innovative Research, Novi, MI) was added to each well to give a final volume of 50 µl. After 1 h incubation at room temperature, lactate dehydrogenase (LDH) release was measured by addition of 50 µl of CytoTox-ONE homogeneous membrane integrity assay solution (Promega, Madison, WI) according to the
manufacturer's protocol. LDH concentration was assayed from resorufin fluorescence (excitation/emission 560/590 nm). Percentage cytotoxicity was computed as (compound – negative control)/(positive control – negative control) × 100. For live/dead cell staining, cells were washed with Hanks’ BSS and then incubated with 1 μM calcein-AM (live cells, green fluorescence) and 2 μM ethidium homodimer-1 (dead cells, red fluorescence) (Invitrogen) in PBS for 15 min prior to imaging.

**NMO-IgG binding**—M23-AQP4 expressing CHO cells were plated onto 96-well microplate and grown for 18-24 h to confluence. Cells were washed twice with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. After removal of blocking solution, test compounds in PBS (90 μl, 1% DMSO) were added and incubated for 30 min at room temperature. A premixed solution (10 μl) of NMO-rAb (10 μg/ml) and horseradish peroxidase (HRP)-labeled goat anti-human IgG secondary antibody (1:500 dilution; Invitrogen) was then added. Following 1 h incubation at room temperature, cells were washed 3 times with PBS containing 0.05% Tween-20, and HRP activity was assayed by addition of 50 μl Amplex Red substrate (100 μM, Sigma) and 2 mM H2O2 as described (12). Fluorescence was measured after 10 min (excitation/emission 540/590 nm).

**Immunostaining**—Cells were grown on glass coverslips for 24 h. Cells were washed with PBS and incubated for 30 min in live cell blocking buffer (PBS containing 6 mM glucose, 1 mM sodium pyruvate, 1% BSA). Cells were incubated with compound or control (DMSO) for 30 min, and then for 60 min with NMO rAb at final concentration of 1 μg/ml in blocking buffer. Cells were then rinsed in PBS, fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100. Cells were incubated for 60 min with 0.4 μg/ml polyclonal, C-terminal specific rabbit anti-AQP4 antibody (H-80, Santa Cruz Biotechnology, Santa Cruz, CA) and then for 30 min with 4 μg/ml goat anti-human IgG-conjugated Alexa Fluor 488 and 4 μg/ml goat anti-rabbit IgG-conjugated Alexa Fluor 555 (Invitrogen) in blocking buffer, as described (17). Red and green fluorescence was imaged on a Nikon Eclipse TE2000S inverted epifluorescence microscope (Nikon, Melville, NY).

**Surface plasmon resonance**—Surface plasmon resonance (SPR) measurements were done on a Biacore T100 instrument (GE Healthcare, Piscataway, NJ). NMO rAbs and control antibody (rAb-2B4) were diluted with 10 mM sodium acetate buffer (pH 5.5) to 20 μg/ml and immobilized onto the surface of a carboxymethylate-functionalized CM5 sensor chip (GE Healthcare) using standard carbodiimide coupling. Unreacted surface was blocked by injecting 1 M ethanolamine-HCl. The reference control flow cell was coupled with 1 M ethanolamine-HCl only. Binding was measured at 25 °C using pH 7.4 Hepes-buffered saline containing 5% DMSO as running buffer at a flow rate of 30 μl/min. Small molecules in identical buffer were injected for 25 s over sensor chip at the same flow rate. Sensorgrams were corrected by subtraction of responses of the unloaded reference flow cell. Equilibrium constants for small molecule-antibody binding were determined using a 1:1 Langmuir binding-affinity model.

**Ex vivo spinal cord slice model of NMO**—Wild type and AQP4 null mice in a CD1 genetic background were used, as generated and characterized previously (18). Transverse slices of cervical spinal cord of thickness 300 μm were cut from postnatal day 7 pups using a vibratome and placed in ice-cold Hank’s balanced salt solution (HBSS, pH 7.2), as described (19). Slices were placed on transparent membrane inserts (Millipore, Millicell-CM 0.4 μm pores, 30 mm diameter) in 6-well plates containing 1 ml culture medium, with a thin film of culture medium covering the slices. Slices were cultured in 5% CO2 at 37 °C for 7 days in 50% MEM, 25% HBSS, 25% horse serum, 1% penicillin-streptomycin, 0.65% glucose and 25 mM HEPES. On day 7, slices were incubated with blocker for 1 h and then rAb-53 (10 μg/mL) and human complement (5%) were added to the culture
medium on both sides of the slices. Slices were cultured for an additional 24 h, and immunostained for AQP4 and glial fibrillary acid protein (GFAP). Sections were scored as follows: 0, intact slice with normal GFAP and AQP4 staining; 1, mild astrocyte swelling and/or reduced AQP4 staining; 2, at least one lesion with loss of GFAP and AQP4 staining; 3, multiple lesions affecting > 30 % of slice area; 4, lesions affecting > 80 % of slice area.

Antibody structure modeling and molecular docking—The web-based antibody homology modeling tool PIGS (Prediction of Immunoglobulin Structure; http://www.biocomputing.it/pigs/) was used to create a structural model for rAb-53 antigen binding domain (20). Truncated sequences of the rAb-53 light chain (residues 1-117) and heavy chain (residues 118-249) were identified, based on alignment with conserved Fab sequences. Complementarity determining regions (CDRs) are indicated (underlined) of the truncated light chain sequence (EIVLTQSPGTLSLSPGERATLSCRASQTVRTNYLAWFQQKPGQAPRLLFAGSSRATGIPDRFSGSGTDFTLTISRLEPEDFAVYYQYGSSPWTFGQGTKVEIKT; CDR-L1, CDR-L2, CDR-L3) and heavy chain sequence (QVQLQESGAGLVKPSDELTLCTVSGGSISGHYNWIRQPGKGLEWIGYIHYGSTNYPKRSVTISVDTSKNQSLKLSVTAADTVYCYCARAEGRGWSAFYYMYEVWKGSTVSVS; CDR-H1, CDR-H2, CDR-H3). PIGS identified a template based on a structure of a Fab fragment of human IgM Cold Agglutinin (pdb 1DN0) with 90.1% sequence homology to the rAb-53 light chain and 72.6% homology to the heavy chain. Side chain conformations were modeled using SCWRL 3.0 (21). The rAb-53 structure was prepared for docking using the FRED-RECEPTOR (OpenEyes) utility. Initial docking computations were done using the rAb-53 Fab structure to identify potential small molecule binding sites on the antibody surface. The ligand structures for A-01 and A-72 (two enantiomers for each) were prepared using a protocol in Scitegic Pipeline Pilot (Accelrys) to generate the proper ionization state at pH 7.4, and the molecules were then converted into energy-minimized 3D structures. The ligand structures were passed through OMEGA (v2.4.6) (OpenEyes) to generate conformational libraries, which were subsequently assigned MMFF partial charges using MOLCHARGE (v1.5.0) (OpenEyes). The ligand structures were docked into the rAb-53 receptor model using FRED (v.2.2.5) (OpenEyes), free of pharmacophore restraint, and configured to employ consensus docking with its full suite of seven scoring functions. Partial charges were assigned to the rAb-53 receptor using the MMFF model with FRED, prior to ligand docking. The final protein-ligand complexes were visualized using PYMOL (Schrödinger, San Diego, CA).

RESULTS
Small-molecule screen—A high-throughput screen was implemented to identify small-molecule inhibitors of complement-dependent cytotoxicity caused by a monoclonal human NMO autoantibody, rAb-53, which was characterized previously (12,17). Binding of complement protein C1q to rAb-53 initiates the classical complement activation pathway (Fig. 1A). Cytotoxicity was measured by LDH release in CHO cells stably expressing the M23 isoform of human AQP4. As diagrammed in Fig. 1A, the screen involved addition of rAb-53 and human complement in the presence of test compound, 60 min incubation, and enzymatic assay of LDH release. Maximum LDH release (100 % cytotoxicity) was determined by treatment of cells with 0.1% Triton X-100. Fig. 1B shows LDH release as a function of rAb-53 concentration at fixed, 2% complement. A rAb-53 concentration of 3 µg/ml (dashed vertical line) was used for screening. The screen was robust, with statistical Z’-factor generally greater than 0.6.

Screening of 50,000 small molecules gave 33 compounds that inhibited LDH release by more than 50 % at 25 µM. Secondary screening indicated many of the active compounds to be false positives, as they inhibited components of the LDH enzymatic assay. Chemical structures of
three verified classes of compounds with greatest potency are shown in Fig. 1C. Further analysis was done of the pyrano[2,3-c]pyrazole class of compounds (Class A) because of its interesting, idiotype-specific blocking mechanism (see below).

Structure-activity analysis of pyrano[2,3-c]pyrazoles—Fig. 2A shows a live/dead cytotoxicity assay in which AQP4-expressing CHO cells were exposed to rAb-53 and 2 % human complement in the presence of indicated concentrations of A-01, and then stained to reveal live cells as green and dead cells as red. Compound A-01 reduced cytotoxicity in a concentration-dependent manner. Eighty-two commercially available pyrano[2,3-c]pyrazoles were tested. Fig. 2B shows the concentration-dependence of LDH release for the most potent inhibitor, A-01, and for two other active analogs. Six compounds showed activity, with IC$_{50}$ from 6.6 to 32 µM (Table 1). Fig. 2C summarizes the structural determinants of activity, which revealed that pyrano[2,3-c]pyrazoles bearing a single ring at the R$^2$ position were inactive. Active analogs contained two rings, with compounds having 1,3-linkage between the rings being more potent than those with 1,2-linkage (eg. A-01 vs. A-48). For the R$^1$ substituent, small linear alkyl groups and substituted phenyls were tolerated, though a bulky alkyl group reduced activity (eg. A-01 vs. A-14).

Pyrano[2,3-c]pyrazole A-01 reduces NMO pathology in spinal cord slice culture model—A clinically important consequence of NMO-IgG-dependent cytotoxicity in the central nervous system is the development of lesions with loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity, as well as inflammation and demyelination. We previously developed an ex vivo model of NMO that recapitulates the major in vivo findings of NMO (19). As diagrammed in Fig. 3A, 300 µm-thick spinal cord slices were cultured for 7 days, then incubated for 24 h with NMO-IgG, complement and/or blocker, and then immunostained for AQP4 and GFAP as markers of NMO pathology. Fig. 3B shows marked loss of AQP4 and GFAP immunoreactivity in spinal cord slices incubated for 24 h with 10 µg/ml rAb-53 and 5 % complement. Fig. 3C summarizes lesion scores (0, no pathology; 4, extensive pathology). Inclusion of A-01 during the 24 h incubation with rAb-53 and complement significantly reduced lesion severity in a concentration-dependent manner. In control studies, rAb-53 or complement alone did not produce pathology, nor did A-01 alone.

Idiotype specificity of pyrano[2,3-c]pyrazoles—To determine whether the pyrano[2,3-c]pyrazoles target rAb-53 or AQP4, binding and cytotoxicity was measured with other monoclonal NMO antibodies and with polyclonal NMO antibodies in NMO patient sera. Fig. 4A shows a binding assay in which AQP4-expressing cells were incubated with NMO-rAb and then a red-fluorescent anti-human secondary antibody; AQP4 was immunostained green. Whereas rAb-53 binding was reduced by A-01, the binding of a different NMO recombinant antibody, rAb-58 (as characterized previously, ref. 17), was not affected. Fig. 4B summarizes the A-01 concentration-dependence data for rAb-53 and rAb-58 binding to AQP4 using a HRP-based Amplex Red fluorescence assay. Binding of rAb-53 to AQP4 was reduced by up to 75 %, whereas binding of rAb-58 was not affected.

Fig. 4C summarizes complement-dependent cytotoxicity for several monoclonal NMO antibodies and human NMO sera. While A-01 greatly reduced cytotoxicity produced by rAb-53, it did not protect for the other monoclonal antibodies or for NMO patient sera, including serum of the patient (serum 4) from which rAb-53 was isolated. The lack of cytoprotection for serum 4 indicates that rAb-53 is a minor component of total NMO-IgG.

Surface plasmon resonance shows specific rAb-53 binding—The rAb idiotype-specificity data above suggest that A-01 targets rAb-53 rather than AQP4. SPR was done to investigate A-01 binding to rAb-53. For SPR measurements, rAb-53, rAb-58 and control antibody rAb-2B4 were covalently immobilized by standard primary amine coupling to the carboxymethylated dextran matrix of a CM5 sensor chip. Fig. 5A shows binding curves for A-
01 with rAb-53, rAb-58 and 2B4. A-01 produced a concentration-dependent increase in the SPR signal for rAb-53, showing characteristic fast binding and dissociation for small molecule-protein interactions. A-01 showed no binding signal to rAb-58 or 2B4. As another control, an inactive pyrano[2,3-c]pyrazole analog, A-72, showed no binding interaction with rAb-53 (Fig. 5B). The dissociation constant K_d for A-01 binding to rAb-53 was estimated as 51 ± 20 µM (Fig. 5C).

**Computational analysis of the pyrano[2,3-c]pyrazole binding site on rAb-53**—Fig. 6A shows the relatively large size of rAb-53 compared with AQP4, which is assembled in membranes as tetramers that form higher-order aggregates. Antibody modeling and molecular docking computations, as described under Experimental Methods, indicated a putative binding site for A-01 in the vicinity of highly variable CDR-H3 and CDR-L2 regions. Docking was also done for the inactive analog A-72 (see Fig. 5B). Because A-01 and A-72 each possess a single chiral center, both enantiomers of A-01 and A-72 were examined individually. Both enantiomers of A-01 showed significantly better docking scores compared to A-72, using consensus scoring across seven independent scoring functions implemented in the docking software (consensus scores 6-7 vs. 14-15 for A-01 vs. A-72, respectively). Close examination of the docking pose of A-01 (R enantiomer) shows that both the lipophilic propyl and para-methyl phenyl groups make significant complementary hydrophobic contacts with the rAb-53 rAb surface (Fig. 6B).

**DISCUSSION**

A cytotoxicity-based small molecule screen yielded blockers of the binding of a human monoclonal NMO autoantibody to its cell surface target, AQP4. We previously reported small-molecule and antibody blockers of NMO-IgG binding to AQP4 that targeted the extracellular surface of AQP4 and blocked the binding of polyclonal NMO-IgG to AQP4 and downstream cytotoxicity (12,13). Here, we identified a class of small molecules that exclusively blocked AQP4 binding of the monoclonal NMO antibody used for screening. The compounds did not interfere with AQP4 binding of other monoclonal or polyclonal (from NMO patient sera) NMO-IgG, and were found to bind directly to the monoclonal antibody used for screening. We conclude that the pyrano[2,3-c]pyrazoles identified here are idiotype-specific for NMO rAb-53, demonstrating for the first time, to our knowledge, the development of idiotype-specific small-molecule blockers.

The monoclonal NMO antibody used here, rAb-53, is a recombinant monoclonal antibody of IgG1 isotype that was generated from sequence analysis of a clonally expanded plasma blast population in the cerebrospinal fluid of an NMO patient (15). The rAb contains an antigen binding site composed of a VH4-59 heavy chain and VK3 light chain. Fluorescence cell binding assays gave a dissociation constant of 44 nM for binding of rAb-53 to AQP4 (17). Slow dissociation of rAb-53 from AQP4 over many hours was found in unbinding assays in live cells and surface plasmon resonance measurements in AQP4-reconstituted proteoliposomes (13). The Fe portion of rAb-53 has been mutated to generate aquaporumab blocking antibodies that lack effector functions (13). The target of rAb-53 binding, AQP4, functions as a water-selective channel in astrocytes where it is involved in a variety of biological functions, including brain water balance, neuroexcitation, astrocyte migration, glial scarring and neuroinflammation (22). AQP4 monomers of ~30 kDa molecular size assemble at the cell plasma membrane as tetramers that form supramolecular aggregates called orthogonal arrays of particles (23,24). Binding of rAb-53 to AQP4 arrays has ~5-fold higher affinity than to individual AQP4 tetramers (17). NMO patient serum consists of a polyclonal mixture of autoantibodies that bind to various 3-dimensional epitopes on the AQP4 extracellular surface. The inability of A-01 to prevent binding of serum NMO-IgG of the patient from which rAb-53 was isolated indicates that rAb-53 is at most a minor component in that patient serum.
Pyrano[2,3-c]pyrazoles, as identified here, are reported to have a broad range of biological activities. A pyrano[2,3-c]pyrazole was found to bind to Bcl-2 protein and induce apoptosis in human acute myeloid leukemia cells with EC₅₀ ~18 µM (25). Virtual screening against the ATP-binding site of Chk1 kinase identified a pyrano[2,3-c]pyrazole as an inhibitor with IC₅₀ ~20 µM (26). Certain pyrano[2,3-c]pyrazoles were reported to exhibit molluscicidal activity towards Biomphalaria alexandrina snails at low nanomolar concentration (27). Another study reported anti-inflammatory effects of pyrano[2,3-c]pyrazoles in carrageenan-challenged activity towards Biomphalaria alexandrina snails at low nanomolar concentration (27). Synthetically, racemic pyrano[2,3-c]pyrazoles are prepared via a four-component, one-pot reaction (29). Preparation of a computationally designed focused library of analogs might produce compounds with nanomolar potency.

Idiotype-specific small molecules have potential applications as research tools and as therapeutics. Small-molecule blockers of antibody-target interaction could be used to modulate or terminate the action of an administered monoclonal antibody therapeutic. For example, natalizumab is a humanized recombinant monoclonal IgG4 antibody that binds the α₄ subunit of the α₄β₁ integrin and is highly effective for the treatment of relapsing forms of multiple sclerosis. However, natalizumab therapy is complicated by the risk of a potentially lethal infection with the human polyoma JC virus, progressive multifocal leukoencephalopathy (PML) (30). While PML is untreatable, an important initial step in management of natalizumab-induced PML is rapid depletion of circulating antibody with plasma exchange (31). Early treatment with an idiotype-specific small molecule blocker of natalizumab could displace bound antibody, restore T cell migration, and expedite immune reconstitution.

As another example, IgM, IgG or IgA monoclonal gammopathies may be found in B cell lymphoproliferative disorders (Waldenstrom macroglobulinemia, B cell lymphoma, multiple myeloma) or in normal individuals (monoclonal gammopathy of undetermined significance). Some individuals with monoclonal gammopathies have neuropathic syndromes driven by antibody binding to protein or ganglioside components of peripheral nerve myelin (32). In these disorders, treatment of the underlying malignancy may be augmented by anti-idiotype therapy to remove pathogenic antibody from its peripheral nerve target. Similarly, in rare paraneoplastic disorders such as NMDA encephalitis (33), idiotype-selective small molecule therapy may dissociate pathogenic antibody from its CNS target and reduce seizure activity.

In conclusion, a cytotoxicity-based small molecule screen identified pyrano[2,3-c]pyrazoles as idiotype-specific inhibitors of binding of a human monoclonal NMO antibody to astrocyte water channel AQP4. Idiotype-specific small molecules have potential as therapeutics for diseases that are caused by binding of monoclonal pathogenic antibodies to their target antigen, such as natalizumab-induced multifocal leukoencephalopathy, and certain monoclonal gammopathies and paraneoplastic disorders. The identification of idiotype-specific pyrano[2,3-c]pyrazoles provides proof of concept for an antibody-targeted, small molecule blocker strategy to prevent specific antibody-antigen binding.
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FIGURE LEGENDS

Figure 1. High-throughput screen for identification of inhibitors of NMO-rAb/complement-mediated cytotoxicity. A. (left) Schematic of the screening assay, which measures LDH release from M23-AQP4 expressing CHO cells treated with recombinant monoclonal NMO antibody rAb-53 and 2% complement. (right) Flow diagram of the screening assay. B. Dependence of CDC on rAb-53 concentration in CHO cells in presence of 2 % complement (S.E., n = 4). The dash line indicates rAb-53 concentration used for screening. C. Chemical structures of three classes of inhibitors identified from screening. MAC = membrane attack complex.

Figure 2. Small-molecule blocker inhibits rAb-53 and complement-dependent cytotoxicity. A. Fluorescence micrographs showing live/dead (green/red) staining of AQP4 expressing CHO cells after 1 h incubation at room temperature with 3 µg/ml rAb-53 and 2 % complement in the presence of indicated concentrations of inhibitor A-01. B. Concentration-dependence of LDH release as a function of inhibitor concentration after 1 h incubation at room temperature with 3 µg/ml rAb-53 and 2 % complement. C. Structural determinants of inhibitor activity.

Figure 3. Inhibitor A-01 reduces pathology in an ex vivo spinal cord slice model of NMO. A. Schematic of spinal cord slice model of NMO. Mouse spinal cord slices were cultured for 7 days, followed by 24 h in the presence of rAb-53 antibody (10 µg/ml) and/or human complement (HC, 5 %), without or with A-01 (10 and 25 µM). B. Immunofluorescence of AQP4 (red) and GFAP (green). C. NMO lesion scores (S.E., n = 4-6, * \( P < 0.01 \)).

Figure 4. Idiotype specificity for A-01 inhibition of rAb-53 binding to AQP4 and complement-dependent cytotoxicity. A. Binding of monoclonal NMO antibodies rAb-53 and rAb-58 (red), shown with AQP4 immunofluorescence (green) in M23-AQP4 expressing CHO cells. Cells were incubated with indicated concentrations of A-01 for 30 min before addition of NMO antibody (1 µg/ml) and anti-AQP4 antibody (0.4 µg/ml). B. (top) Schematic of HRP fluorescence assay. (bottom) A-01 concentration-dependent inhibition of binding of rAb-53 and rAb-58 (each 1 µg/ml) to AQP4 (S.E., n = 4). C. CDC
measured for indicated NMO-rAbs (left), NMO patient sera (center), and the serum of the patient from rAb-53 was identified (right, tested at 3 dilutions) (S.E., n = 4, * P < 0.001).

Figure 5. **A-01 binding to rAb-53 measured by surface plasmon resonance.** A. Sensorgram showing concentration-dependent binding of A-01 to rAb-53 (left) but not to rAb-58 (center) or rAb-2B4 (right). Antibodies were ligated onto a CM5 sensor chip using standard primary amine coupling chemistry. A-01 was injected for 25 s over the antibodies at a flow rate of 30 µl/min. B. Control study showing that inactive analog A-72 does not bind to rAb-53. C. Binding affinity of A-01 to rAb-53 (Kd 51 ± 20 µM).

Figure 6. **Putative binding site of A-01 on rAb-53.** A. Zoomed-out view of rAb-53 showing A-01 (R enantiomer) binding as determined by docking computations. Structure and size of AQP4 (pdb 3GD8) shown for comparison. B. Zoomed-in view of A-01 docked to the surface of rAb-53, showing proximity to CDR-H3 and CDR-L2 regions.
Table 1. Inhibition activity of pyrano[2,3-c]pyrazoles.

![Chemical structure](image)

| Compound | R<sup>1</sup> | R<sup>2</sup> | IC<sub>50</sub> (µM) |
|----------|--------------|--------------|-------------------|
| A-01     |              | ![Structure](image) | 6.6               |
| A-13     | ![Structure](image) | ![Structure](image) | 11                |
| A-14     | ![Structure](image) | ![Structure](image) | 16                |
| A-48     | ![Structure](image) | ![Structure](image) | 22                |
| A-52     | ![Structure](image) | ![Structure](image) | 32                |
| A-63     | ![Structure](image) | ![Structure](image) | 30                |
**Figure 1**

A. Schematic representation of the complement activation pathway and cytotoxicity release involving AQP4, C1q, test compound, and rAb-53.

B. Graph showing the relative LDH release against [rAb-53] (µg/ml) concentration.

C. Structures of test compounds classified into Class A, Class B, and Class C.
Figure 2

A complement +

control rAb

0 µM A-01

5 µM A-01

10 µM A-01

50 µM A-01

rAb-53

B

% LDH release

A-13

A-14

A-01

[compound] (µM)

C

R1

Best: propyl, alkyl

Moderate: aryl

R2

Best:

Moderate:

Inactive:

Figure 2
Figure 3

A

slices ▶ 7 days → add A-01 ▶ add HC + rAb-53 + A-01 ▶ fix and stain

B

| control | HC | rAb-53 | 50 μM A-01 | control | 10 μM A-01 | 25 μM A-01 |
|---------|----|--------|-------------|---------|-------------|-------------|
| ![control](image1) | ![HC](image2) | ![rAb-53](image3) | ![50 μM A-01](image4) | ![control](image5) | ![10 μM A-01](image6) | ![25 μM A-01](image7) |

C

| control | HC | rAb-53 | 50 μM A-01 | HC + rAb-53 |
|---------|----|--------|-------------|--------------|
| ![control](image8) | ![HC](image9) | ![rAb-53](image10) | ![50 μM A-01](image11) | ![HC + rAb-53](image12) |

Score 0 1 2 3 4

* *
Figure 4

Panel A: Images of AQP4 and rAb-53 and rAb-58 under different concentrations of [A-01] (0, 10 µM, 50 µM).

Panel B: Diagram illustrating the antibody binding to AQP4 with HRP-conjugated secondary antibody.

Panel C: Graph showing the concentration of [A-01] (µM) and the corresponding % cytotoxicity for different sera and antibodies.

Figure 4
Figure 5

(A) Graph showing the response units for rAb-53 and rAb-58, with concentrations of 50 µM, 25 µM, and 12 µM A-01. The control rAb shows a similar but smaller response.

(B) A diagram of rAb-53 with a chemical structure and a graph showing the response units at 50 µM A-72 and 0 µM A-72.

(C) A graph showing the response units against [A-01] (µM) with a curve and data points indicating the concentration response relationship.
Figure 6

AQP4 tetramer

8 nm

Fab

rAb-53

Fc

A-01

CDR-H3

CDR-L2

Heavy chain; Light chain

Figure 6
A small-molecule screen yields idiotype-specific blockers of neuromyelitis optica-immunoglobulin G binding to aquaporin-4
Puay-Wah Phuan, Marc O Anderson, Lukmanee Tradtrantip, Hua Zhang, Joseph Tan, Chiwah Lam, Jeffrey L Bennett and Alan S. Verkman

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