

# Generation of a unique small molecule peptidomimetic that neutralizes lupus autoantibody activity

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**Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the presence of pathogenic autoantibodies, many of which are directed against nuclear antigens, in particular double-stranded (ds) DNA. Both clinical studies and animal models have shown that anti-dsDNA antibodies contribute to kidney disease, which is present in 50% of lupus patients and is a major cause of mortality. We previously demonstrated that a subset of nephrotoxic anti-dsDNA antibodies also recognizes the pentapeptide consensus sequence D/E W D/E Y S/G (DWEYS) present in the NR2A and NR2B subunits of the *N*-methyl-D-aspartate receptor (NMDAR). Autoantibodies with this specificity are present in ~40% of lupus patient sera and are both nephrotoxic and neurotoxic. Elevated titers are present in cerebrospinal fluid of patients with central nervous system manifestations of SLE. Administration of the non-naturally occurring D form of the DWEYS pentapeptide prevents these antibodies from depositing in glomeruli and from mediating neuronal excitotoxicity. To craft a more useful therapeutic, we used the structural features of the DWEYS peptide to design a unique, selective, and potent small molecule peptidomimetic, FISLE-412, which neutralizes anti-dsDNA/NMDAR lupus autoantibodies and prevents their pathogenic interaction with tissue antigens. This compound, or others derived from it, may provide a unique strategy for the development of lupus therapeutics.**

autoimmunity | glomerulotoxicity | neurotoxicity | orally absorbable

Although multiple species of autoantibodies can contribute to systemic lupus erythematosus (SLE) pathogenesis, antibodies to dsDNA are diagnostic, correlate with disease activity, and mediate both systemic and local inflammation (1–7). Through direct binding of chromatin or through antigenic cross-reactivity, they deposit in tissue and activate complement and Fc receptors (FcRs) on FcR-bearing cells, thereby initiating inflammatory cascades. Some nephrotoxic anti-DNA antibodies bind to glomeruli even after DNase treatment of the tissue, indicating their ability to bind non-DNA cross-reactive tissue antigens. Alternatively, they bind cell surface molecules and alter cell function (8–11). Finally, DNA containing immune complexes can activate toll-like receptor 9 in dendritic cells and promote an inflammatory milieu.

Previously, we determined that a nephritogenic mouse monoclonal anti-dsDNA antibody, R4A, bound a consensus pentapeptide sequence, D/E W D/E Y S/G (DWEYS), that is present in both the NR2A and NR2B subunits of mouse and human NMDAR (12–15). The R4A autoantibody enhances the glutamate-induced excitatory postsynaptic potentials in a mouse hippocampal slice preparation and causes apoptosis-mediated neuronal death when microinjected in vivo into a mouse brain (16). Mice immunized with a multimerized configuration of DWEYS produce anti-dsDNA/NMDAR antibodies, display glomerular Ig deposition, and exhibit neuronal damage after a breach of the blood–brain barrier (BBB) that allows transit of these antibodies into the CNS (17). In humans, elevated titers of cross-reactive anti-dsDNA/NMDAR antibodies are present in the serum of 40% of lupus patients and their presence in CSF of lupus patients correlates with

CNS manifestations of neuropsychiatric lupus (NPSLE), which afflicts up to 80% of lupus patients (15, 18–22). Moreover, human anti-dsDNA/NMDAR antibodies have been eluted from post-mortem brain tissue, and such antibodies isolated from lupus patient sera can cause brain damage (e.g., cognitive or behavioral dysfunction) in mice (23, 24). Similarly, gestating mice that harbor anti-dsDNA/NMDAR antibodies in their circulation give birth to offspring with impaired brain development (25) because the BBB does not form until the end of gestation and the fetal brain is exposed in utero to the maternal antibody. Of therapeutic importance, the D-isoform of the DWEYS peptide can protect tissue against antibody binding: In vivo administration of D-DWEYS peptide blocks renal and brain deposition of anti-dsDNA/NMDAR antibodies and ameliorates ongoing disease in mouse lupus models (12, 23, 24). These studies underscore the principle that anti-dsDNA/NMDAR antibodies are pathogenic in lupus and suggest a possible clinical utility for a molecule with properties of the DWEYS peptide. The peptide itself is of limited utility because it is not orally absorbed. Here, we have used the structural features of the DWEYS peptide to design a unique small molecule that neutralizes anti-dsDNA/NMDAR antibodies both in vitro and in vivo.

## Results

**Creation of a Small Molecule Peptidomimetic, FISLE-412.** To identify compounds that inhibit anti-dsDNA/NMDAR lupus antibodies from mediating tissue damage, we focused our approach on imitating the molecular topology of the DWEYS structure and designed molecular scaffolds predicted to have the desired DWEYS mimetic properties. Thus, the tryptophan and tyrosine residues were replaced, respectively, by 1,2,3,4-tetrahydroquinolin-3-ol and phenyl moieties mobilized on a polyamine scaffold (Fig. 1 *A* and *B*). FISLE-412 is a unique molecule that was synthesized in 12 steps and characterized by mass spectrometry and NMR. We screened this small molecule for its ability to mimic neutralizing activities of the DWEYS peptide by using in vitro, ex vivo, and in vivo assays. First, we asked whether FISLE-412 could block two well-described lupus anti-dsDNA/NMDAR monoclonal antibodies, R4A (derived from a mouse producing anti-dsDNA antibodies) and G11 (derived from a peripheral blood B cell of a lupus patient), from binding to their known antigens in a competitive ELISA. As expected, the DWEYS peptide inhibited in a dose-dependent manner the binding of both R4A and G11, to dsDNA and to the DWEYS peptide itself, at micromolar concentrations (Fig. 1 *C–F*, black circles). Indeed, as shown in Fig. 1 *C–F*, FISLE-412

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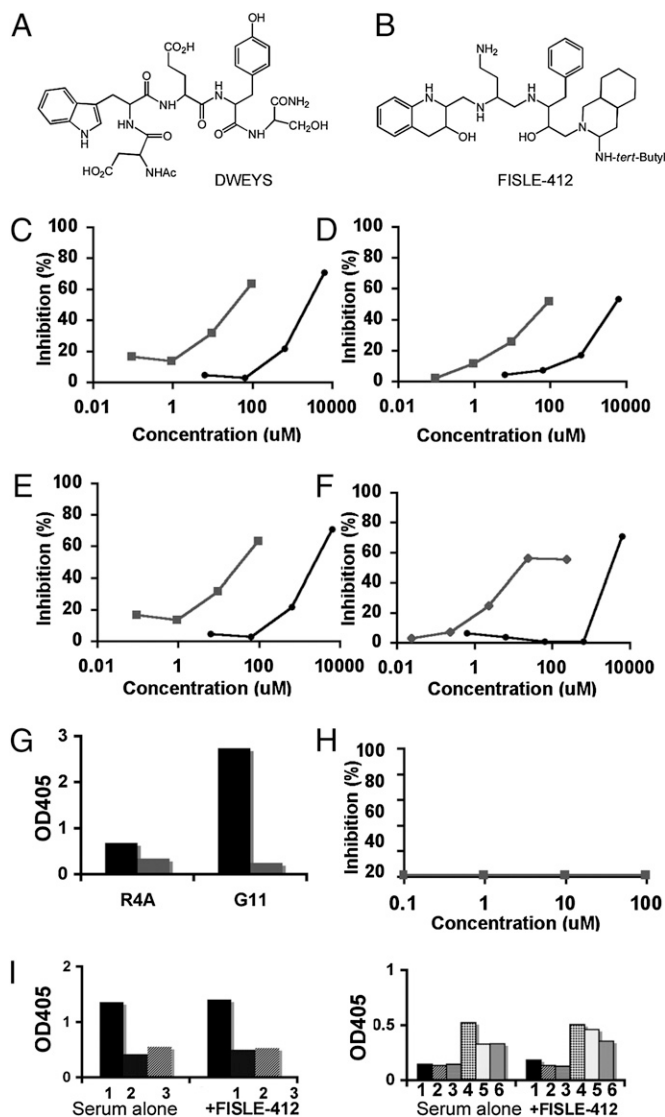
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**Fig. 1.** FISLE-412 is a unique peptidomimetic that is 30-fold more potent than DWEYS at inhibiting SLE anti-dsDNA/NMDAR autoantibodies in vitro. (A and B) Chemical structure of the DWEYS peptide (A) and FISLE-412 (B). (C and D) FISLE-412 inhibits mouse monoclonal anti-dsDNA/NMDAR antibody R4A binding to either DWEYS peptide (C) or dsDNA (D) in a dose-dependent manner. Representative assays ( $n = 4$ ) show inhibition of R4A binding in the micromolar range (FISLE-412, green) and in the millimolar range (DWEYS peptide, black). (E and F) Identical competitive ELISAs were performed with the human monoclonal anti-dsDNA/NMDAR antibody G11. FISLE-412 blocked G11 binding to both DWEYS peptide (E) and dsDNA (F). Representative assays are shown ( $n = 4$ ). (G) An ELISA for binding of R4A and G11 antibodies to cardioliopin was performed in the absence (black bars) and presence (green bars) of FISLE-412 (100  $\mu$ M). As shown, FISLE-412 blocked binding to cardioliopin (green squares). (H) A commercial ELISA for anti-BSA antibodies was performed in the presence and absence of FISLE-412. As shown, there was no inhibition of the anti-BSA antibody (16 ng/mL) binding to its substrate in the presence of FISLE-412 (0.1–100  $\mu$ M). (I) An ELISA for binding of human (Right, 1–3) and mouse (Left, 1–6) lupus sera to histones was performed in the absence (Left) and presence (Right) of FISLE-412 (250  $\mu$ M). As shown, FISLE-412 did not inhibit histone binding by lupus sera.

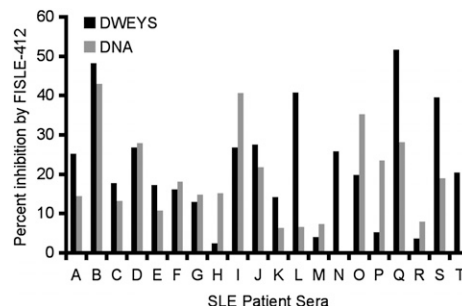
also inhibited the binding of R4A and G11 to these same antigens, but did so with markedly increased potency, at micromolar concentrations (Fig. 1 C–F, green squares). Cross-reactivity of anti-DNA antibodies with cardioliopin has been reported previously. R4A and G11 bind to cardioliopin (Fig. 1G, black bars), and this binding was also inhibited by FISLE-412 (Fig. 1G, green bars).

Control experiments underscored the specificity of these interactions in that neither a scrambled pentapeptide nor an altered FISLE scaffold small molecule could inhibit the binding of anti-dsDNA/NMDAR monoclonal antibody to dsDNA or DWEYS, even at high concentrations. FISLE-412 did not inhibit binding of an anti-BSA antibody to BSA (Fig. 1H), nor did it significantly inhibit binding to histone by human or mouse SLE sera (Fig. 1I). Thus, FISLE-412 showed specificity for DNA-reactive antibodies and did not nonspecifically inhibit binding to all SLE autoantigens (Fig. 1I).

Given its effectiveness in neutralizing mouse or human anti-dsDNA/NMDAR monoclonal antibodies, we next asked whether FISLE-412 would be effective in inhibiting the polyclonal auto-reactivity present in human lupus sera. Analogous to the studies of the monoclonal antibodies, we tested whether FISLE-412 inhibited autoantigen binding by SLE sera in an ELISA. FISLE-412 was able to inhibit a significant percent of DWEYS and DNA reactivity in most, but not all, lupus patient sera (Fig. 2), suggesting potential clinical utility in a serologically defined subset of patients.

**Effects of FISLE-412 on SLE Autoantibody Binding in Situ.** Because FISLE-412 was able to block DNA binding by SLE autoantibodies in vitro, we next assayed the ability of FISLE-412 to diminish or abrogate tissue binding by pathogenic anti-dsDNA, anti-DWEYS cross-reactive antibodies in a more complex tissue environment: the isolated kidney glomerulus. To test whether FISLE-412 could block glomerular binding, we incubated the R4A antibody with mouse glomeruli in the presence or absence of FISLE-412 and assessed binding with fluorescently conjugated secondary antibodies. As demonstrated previously, R4A bound to glomeruli before and after treatment with DNase (Fig. 3A, Left). In marked contrast, R4A pretreated with FISLE-412 showed greatly diminished binding to glomerular antigen (Fig. 3A, Right). Similarly, FISLE-412 mediated the loss of binding to glomerular tissue by the human monoclonal antibody G11 (Fig. 3B).

Given the success of FISLE-412 in blocking SLE monoclonal antibody binding in tissue *ex vivo*, and polyclonal binding in an ELISA, we next tested whether it could block the binding of polyclonal autoantibodies present in lupus sera to glomerular tissue. Not surprisingly, sera A and B, which displayed different profiles of inhibition by FISLE-412 in the ELISA (Fig. 2), were inhibited to different degrees in the glomerular binding assay (Fig. 3C). Specifically, serum A was only partially neutralized in the glomerular binding assay; in contrast, serum B was effectively neutralized, despite the fact that FISLE-412 inhibited only  $\approx 50\%$  of dsDNA binding by serum B in an ELISA. These data are consistent with previous data demonstrating a variable amount of cross-reactive antibody in patient sera and suggest that FISLE-412



**Fig. 2.** FISLE-412 inhibits lupus autoantibody-mediated antigen recognition by human SLE patient sera. FISLE-412 inhibited human SLE sera binding to DWEYS peptide (black) and dsDNA (gray) substrates in an ELISA. A representative assay ( $n = 2$ ) shows a range of inhibition of sera binding by FISLE-412 on both antigen substrates.





represents a significant therapeutic advance: It combines potent anti-dsDNA/NMDAR neutralization with increased molecular stability and oral availability in a compound that is not expected to elicit neutralizing antibodies. Given that current lupus therapies are inadequate and often introduce the additional risk of immunosuppression, our data provide hope for the development of more specific, less toxic therapy for lupus and provide a model for the development of customized therapeutics for a highly pathogenic subset of lupus antibodies, as well as other antibody-mediated symptoms or diseases.

## Materials and Methods

**Chemical Synthesis.** FISLE-412 was synthesized and characterized by the Laboratory of Medicinal Chemistry at the institute. MS-ES:  $m/z = 635$  [M+H]<sup>+</sup>. <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>):  $\delta$  7.4–7.2 (5H, multiplet, phenyl CH),  $\delta$  7.0–6.9 (2H, multiplet, isoquinoline CH),  $\delta$  6.65–6.55 (2H, multiplet, isoquinoline CH),  $\delta$  4.6 (1H, broad, isoquinoline NH),  $\delta$  4.05–3.9 (2H, multiplet, CH-OH),  $\delta$  3.9–3.3 (multiplet, CHx-NHx methine and methylene),  $\delta$  3.15–2.75 (5H, isoquinoline piperidine CHx),  $\delta$  2.55–2.45 (1H, multiplet, piperidine CH),  $\delta$  2.35–2.25 (2H, multiplet, piperidine CH<sub>2</sub>),  $\delta$  1.65–1.55 (broad, OH, NH, D<sub>2</sub>O exchangeable),  $\delta$  1.45–1.25 (multiplet, cyclohexane CH<sub>2</sub> and *t*-Butyl CH<sub>3</sub>). <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>):  $\delta$  145 (C1, isoquinoline)  $\delta$  139 (C1, benzene),  $\delta$  131–128 (CH, benzene),  $\delta$  121–115 (CH, isoquinoline),  $\delta$  67.5–67.0 (CH-OH),  $\delta$  65.5 (CH, isoquinoline),  $\delta$  59.5 (CH, benzyl),  $\delta$  56.5–56.0 (CH and CH<sub>2</sub>, piperidine),  $\delta$  50–43 (CH<sub>2</sub>-NH),  $\delta$  37.0 (CH<sub>2</sub>-NH<sub>2</sub>),  $\delta$  31.0–29.5 (CH<sub>2</sub>, piperidine and CH<sub>3</sub>, *t*-Butyl),  $\delta$  28.0–26.0 (CH<sub>2</sub>, cyclohexane).

**ELISAs.** The anti-dsDNA/DWEYS inhibition ELISAs were performed essentially as described (15, 24). Antigens were adsorbed overnight at 37 °C onto Costar plates (catalog no. 3690, Costar), either with calf thymus DNA (100  $\mu$ g/mL) in NaHCO<sub>3</sub> (0.1 M, pH 8.6) or D-DWEYS (20  $\mu$ g/mL) in PBS. The inhibition ELISA was performed as described above on R4A or G11 monoclonal antibodies (20  $\mu$ g/mL) (mouse and human, respectively), or a panel of deidentified human lupus sera (diluted 1:100), purchased from RDL. All were preincubated with dilutions of DWEYS peptide or FISLE-412 at varying concentrations for 1 h at 37 °C and then transferred to the 96-well plate for 1 h at 37 °C. The plates were washed and goat anti-mouse or anti-human IgG antibody was added for 1 h at 37 °C, essentially as described (12, 15, 24). The percent inhibition was calculated based on the OD at 405 nm: [(OD of test antibody, R4A, G11, or serum, minus OD of test antibody and FISLE-412 or DWEYS)/OD of test antibody alone]  $\times$  100. The D-DWEYS peptide was synthesized and purified by James I. Elliott at Yale University. An anti-cardiolipin ELISA was performed as follows: Cardiolipin (Sigma catalog no. C-0563, 75  $\mu$ g/mL in 100% ethanol) was adsorbed overnight at room temperature onto Immulon IIHB plates (catalog no. 3455, Thermo Scientific). Plates were blocked with 3% BSA/PBS for 1 h at 37 °C. The inhibition ELISA was performed on R4A or G11 monoclonal antibodies (20  $\mu$ g/mL), (mouse and human, respectively), as described above for the anti-dsDNA inhibition ELISA using FISLE-412 at 100  $\mu$ M. An anti-BSA antibody ELISA (Cygnus Technologies, catalog no. F030) was performed according to the manufacturer's recommendations in the absence and presence of FISLE-412. The anti-BSA antibody (final concentration of 16 ng/mL) was preincubated with FISLE-412 at varying concentrations, as shown in Fig. 1H, for 1 h at 37 °C and then incubated on a substrate coated 96-well plate for 90 min at room temperature. The plate was developed

according the manufacturer's protocol. An anti-histone ELISA was performed as follows: Calf thymus histones (H1, H2A, H2B, H3, and H4, Roche catalog no. 10223565001, at 10  $\mu$ g/mL in PBS) were adsorbed overnight at 4 °C onto Costar plates (catalog no. 9018, Costar). An inhibition ELISA was performed as described above in the presence and absence of FISLE-412 at 250  $\mu$ M. Human SLE sera (1–3, obtained for research, deidentified) were diluted 1:200, mouse sera were diluted 1:50 from C57BL/6J, 1, 2, and 3 mice (serum 1–3), and 3 from NZB/W mice (serum 4–6).

**Neurotoxicity.** These procedures were in compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training, the National Institutes of Health Guide for the Use and Care of Laboratory Animals, and the Research Animal Resource Center Users Guide. Adult male C57BL/6 mice (30–34 g) were anesthetized with 2.5% Avertin (i.p.) and placed in a stereotaxic frame, and injected stereotaxically (2  $\mu$ L) into the hippocampus, as described (15, 24, 26). Mice were euthanized 48 h later, and their brains were fixed and removed. Hippocampal sections (40  $\mu$ m) were cryoprotected and subjected to TACS-Tdt Apoptosis Detection (R&D Systems no. TA4625) and counterstained with methylene green, as described (25). TUNEL-stained tissue was visualized with an upright microscope (Axio-Imager; Zeiss). Images were captured digitally (12-bit camera, 25 MHz, 1388  $\times$  1040 resolution), processed (AxioVision 4.7, Zeiss), imported to Photoshop-CS4 as 8-bit TIFF files, and scale-adjusted equally for all frames.

**Glomerular Binding Assay.** Murine glomeruli were isolated, added to glass slides, and the glomeruli binding assay was performed essentially as described (26). Monoclonal antibodies R4A (mouse) or G11 (human) were applied at 20  $\mu$ g/mL or human lupus sera at 1:100 for 1 h at room temperature and visualized with anti-mouse or anti-human IgG antibodies conjugated to FITC or TRITC (Jackson ImmunoResearch Laboratories). For examination of antibody binding in the absence of DNA, some glomeruli were treated with DNase (100  $\mu$ g/mL, 45 min at 37 °C) before antibody incubation. To visualize the presence or absence of DNA, DAPI (Invitrogen) was applied to glomeruli for 1 min at room temperature (1  $\mu$ g/mL) before secondary antibody application. Slides were mounted by using Vectashield. For inhibition of antibody or sera binding, R4A, G11, or human lupus sera were preincubated with FISLE-412 (250  $\mu$ M) for 1 h at 37 °C, before incubation with glomeruli, as done for ELISA inhibition assays. Slides were washed with PBS and mounted with coverslips by using Vectashield Mounting Medium (catalog no. H-1000; Vector Laboratories). Images were acquired at room temperature on an upright microscope (Axioplan 2; Zeiss) with a Zeiss Plan-Neofluar 40 $\times$  lens using the OpenLab version 4.04 Software (Improvision). For each antibody or experimental condition, images were acquired with a Hamamatsu ORCA-ER Digital Camera (model no. C4742-80), at the same exposure for all conditions, imported into Adobe Photoshop-CS3 and then adjusted for contrast and brightness identically in all frames. Images were enlarged 2 $\times$  for the figures.

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