

Discovery of M5049: A Novel Selective TLR7/8 Inhibitor for Treatment of Autoimmunity

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Non-standard abbreviations: 5-HT, 5-hydroxytryptamine; ADME, absorption, distribution, metabolism, and excretion; Alu, *Arthrobacter luteus*; ANOVA, analysis of variance; AOX, aldehyde oxidase; AUC, area under the curve; AUC_{0-τ}, area under the curve over 24 hours; c, cynomolgus monkey; CAMPKK2, calcium/calmodulin-dependent protein kinase kinase 2; CL, apparent total body clearance; C_{max}, maximum concentration; CMPD 2, Compound 2; CPS, counts per second; d, dog; DMSO, dimethyl sulphoxide; DOTAP, dioleoyl-3-trimethylammonium propane; F, bioavailability; h, human; HEK293, human embryonic kidney 293; hERG, human ether-à-go-go-related gene; hr, hour; IACUC, Institutional Animal Care and

Use Committee; IC₅₀, half maximal inhibitory concentration; IFN, type I interferon; IFN- α , interferon- α ; IL-6, interleukin-6; IRF, interferon regulatory factor; i.v., intravenous; KO, knockout; LC/MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; m, mouse; MCP-1, monocyte chemoattract protein-1; miRNA, microRNA; MMF, mycophenolate mofetil; MSD, Meso Scale Discovery; ND, not done; NF- κ B, nuclear factor-kappa B; NF- κ B Luc, nuclear factor-kappa B luciferase; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD, pharmacodynamic; pDCs, plasmacytoid dendritic cells; PK, pharmacokinetic; Poly I:C, polyinosinic: polycytidylic acid; PPB, percentage protein bound; QWBA, quantitative whole body autoradiography; r, rat; t_{1/2}, terminal half-life; TLR, toll-like receptor; veh, vehicle; V_{ss}, volume of distribution; WB, white blood cells.

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ABSTRACT

Toll-like receptors 7 and 8 (TLR7/8) are transmembrane receptors that recognize single-stranded RNA. Activation of these receptors results in immune cell stimulation and inflammatory cytokine production which is normally a protective host response. However, aberrant activation of TLR7/8 is potentially pathogenic and linked to progression of certain autoimmune diseases such as lupus. Thus, we hypothesize that an inhibitor that blocks TLR7/8 would be an effective therapeutic treatment. Prior efforts to develop inhibitors of TLR7/8 have been largely unsuccessful due to the challenge of producing a small molecule inhibitor for these difficult targets. Here, we report the characterization of M5049 and Compound 2, molecules which were discovered in a medicinal chemistry campaign to produce dual TLR7/8 inhibitors with drug-like properties. Both compounds showed potent and selective activity in a range of cellular assays for inhibition of TLR7/8 and block molecule synthetic ligands and natural endogenous RNA ligands such as microRNA and Alu RNA. M5049 was found to be potent in vivo and TLR7/8 inhibition efficaciously treated disease in several murine lupus models and, interestingly, was efficacious in a disease context where TLR7/8 activity has not previously been considered a primary disease driver. Furthermore, M5049 had greater potency in disease models than expected based on its in vitro potency and pharmacokinetic/pharmacodynamic properties. With preferential accumulation in tissues, and the ability to block multiple TLR7/8 RNA ligands, M5049 may be efficacious in treating autoimmunity and has the potential to provide benefit to a variety of patients with varying disease pathogenesis.

SIGNIFICANCE STATEMENT

We report discovery of a novel toll-like receptor 7 and 8 (TLR7/8) inhibitor (M5049), characterize its binding mode, potency/selectivity, pharmacokinetic and pharmacodynamic properties, and demonstrate its potential for treating autoimmune diseases in two mouse lupus models. TLR7/8 inhibition is unique in that it may block both innate and adaptive autoimmunity and thus we believe that M5049 has the potential to benefit patients with autoimmune diseases.

Introduction

Toll-like receptors 7 and 8 (TLR7/8) are receptors for single-stranded RNA that reside in the endosome. TLR7 is expressed primarily in B cells, monocytes, and plasmacytoid dendritic cells (pDCs) while TLR8 is found more in neutrophils, monocytes and myeloid dendritic cells. The two TLRs share some common downstream signaling, but TLR7 activation is more biased to signaling in the interferon regulatory factor (IRF) pathway resulting in production of type I interferon (IFN), while TLR8 activation results in greater nuclear factor-kappa B (NF- κ B) activation and production of other inflammatory cytokines (Gorden et al., 2005; Bender et al., 2020). Because of these differences in expression and downstream signaling, TLR7 and TLR8 activation can have very different outcomes and the two receptors likely play non-redundant roles in immunity.

Although TLR7 and TLR8 are involved in anti-viral defense, their aberrant activation can contribute to autoimmunity. Single nucleotide polymorphisms in both TLR7 and TLR8 have been associated with lupus (Lee et al., 2016). TLR7 has been implicated as a driver of lupus and data from mouse studies with TLR7 overexpression and TLR7 knockout (KO) have shown that TLR7 may promote disease (Christensen et al., 2006; Fairhurst et al., 2008; Lee et al., 2008; Savarese et al., 2008). Activation of TLR7 can trigger pDCs to produce large amounts of IFN, which is recognized as a significant contributor to lupus development. TLR7 can also activate B cells; the hallmark of TLR7-induced B cell activation is the presence of antibodies against RNA-containing proteins such as anti-SmRNP and anti-RiboP that contain TLR7 ligands.

The activity of TLR8 in mice is controversial and there is significantly less preclinical disease model data regarding its role in autoimmunity compared with TLR7. However, reports have suggested that TLR8 expression correlates with disease in BXS B -*Yaa* mice (Kimura et al.,

2014). Also, in normally healthy C57BL/6 mice, TLR8 overexpression can trigger disease development (Guiducci et al., 2013). Although the data implicating TLR8 is less extensive than for TLR7, it is a reasonable hypothesis that TLR8 may also play a role in human autoimmunity as some of the same RNA ligands that activate TLR7 can also activate TLR8 (Gorden et al., 2005). Given that TLR7 and TLR8 are implicated as drivers of autoimmunity, the two receptors may be considered valuable drug targets and dual inhibition may provide greater efficacy than inhibiting either receptor alone.

TLR7 and TLR8 are both activated by single-stranded RNA molecules but have some differences in their ligand binding specificities. Studies have been conducted to identify the ligand features that drive TLR7/8 binding and activation. Interestingly, both small molecule agonists and larger RNA molecules have been shown to activate TLR7/8. It is accepted that RNA molecules that exist in immune complexes with RNA protein-binding autoantibodies (Hung et al., 2015) gain access to the endosome when they are taken up via B cell receptors or Fc receptors in autoimmunity (Avalos et al., 2010; Green and Marshak-Rothstein, 2011). RNA species that activate in this manner include U1 RNAs, Y1 RNAs and others. More recently, emerging data suggest that RNA molecules from retroelements such as Alu RNA (Mavragani et al., 2016) and microRNA molecules (miRNA) (Lehmann et al., 2012; Feng et al., 2017; Salvi et al., 2018) may also activate TLR7 and TLR8. Inhibiting TLR7/8 to effectively treat disease likely requires compounds that block ligands with varying structures from binding to the receptors.

Developing TLR7 and TLR8 selective inhibitors has been challenging for several reasons. TLR7/8 are membrane bound receptors that are difficult to recombinantly express and producing purified protein has been difficult, hindering binding studies and crystallographic

structural determination. Despite these difficulties, efforts have been made to produce TLR7/8 single and dual inhibitors and a few molecules have been developed. However, a molecule targeting these TLRs has never been tested in lupus patients, advanced to Phase III trials, or gained FDA approval. Because of the scarcity of drugs inhibiting these promising targets, we have endeavored to develop a dual TLR7/8 inhibitor for the treatment of autoimmunity. Herein we report the discovery of M5049 and Compound 2 (CMPD 2), which are potent and selective dual TLR7/8 inhibitors. Characterization of M5049 has found it to be a molecule with drug-like properties and a long duration of action that effectively reduces disease in lupus preclinical models.

Materials and Methods

Compounds. M5049 and CMPD 2, as well as selective agonists TLR7 (CL-087) and TLR8 (see Supplemental Fig. 1 for structures), were synthesized by EMD Serono (a business of Merck KGaA, Darmstadt, Germany).

Crystallography. The TLR8 protein used in this study was purchased from the lab of Professor Toshiyuki Shimizu at the University of Tokyo. The protein was produced as previously described (Tanji et al., 2013). Recombinant human TLR8 (aa 27–827) was crystallized by hanging-drop vapor diffusion by mixing 1 μ L of a reservoir solution (100 mM Tris, 11.5% PEG 8000, 200 mM NaCl, pH 8.0) with 1 μ L of concentrated protein solution (10.6 mg/mL protein in 10 mM Tris, 150 mM NaCl, 10 % Glycerol, 128 μ M poly-dT, 820 μ M CL075, pH 7.5). The resulting crystals of TLR8 were soaked with a solution containing 2 mM M5049 with 2% dimethyl sulphoxide (DMSO) for 24 hours. After soaking, the crystals were mounted on a cryo-loop for x-ray data collection and flash cryo-cooled by holding it in liquid nitrogen. Crystallographic data collection was done on the synchrotron radiation source at Swiss Light Source in Villigen, Switzerland. The wavelength of radiation used was 1.00000 Å and the data were collected using a Pilatus 6M area detector. Data processing and structure refinement were carried out according to standard crystallographic methods. Coordinates and structure factor have been deposited in the Protein Data Bank (PDB ID 6ZJZ).

Cell-based Assays. *Peripheral blood mononuclear cells (PBMCs)* – M5049 potency was tested using freshly isolated human PBMCs seeded in complete RPMI 1640 medium (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (Corning, Corning, NY). M5049 dilutions were added to cells and incubated at 37°C for 30 minutes prior to stimulation. The following final concentrations of ligands were added to the PBMCs: 5 μ M TLR7 ligand CL-087,

2 μ M TLR8 ligand (see Fig. S1 for structures), 4 μ g/mL TLR3 ligand Poly(I:C) (InvivoGen, San Diego, CA), 50 ng/mL TLR4 ligand lipopolysaccharide (*E. coli* 0111:B4, List Biologics, Campbell, CA), 75 μ g/mL TLR9 ligand ODN2006 (InvivoGen). RNA molecules (miRNA or Alu RNA) used for stimulation were synthesized with phosphodiester backbones (Sigma, St. Louis, MO) and were pre-incubated with dioleoyl-3-trimethylammonium propane (DOTAP; Roche, Basel, Switzerland) at room temperature for 15 minutes prior to adding to the cells. The final concentration of the RNA molecules was 50 nM and the final dilution of DOTAP was 1:200. After addition of M5049 and ligands, the cells were incubated at 37°C 5% and CO₂ overnight, and cytokines were measured in culture supernatants by AlphaLISA (PerkinElmer, Waltham, MA). All treatments were performed in duplicate or, in some instances, in triplicate.

Human embryonic kidney 293 (HEK293) cells – HEK293 cells were stably transfected with either TLR7 or TLR8 and an NF- κ B-luciferase reporter gene. For compound testing, cells were seeded in 384-well black, clear bottom plates (Corning) and after an overnight incubation at 37°C and 5% CO₂, M5049 dilutions were added in duplicate. The cells were stimulated with 10 μ M R848 (InvivoGen) or 30 μ M R848 for testing in HEK TLR7 or HEK TLR8 cells respectively. Following incubation for 5 hours at 37°C and 5% CO₂, SteadyGlo substrate reagent (Promega, Madison, WI) was added to each well and luminescence was measured using the Perkin Elmer Envision Multilabel Reader.

Whole blood – Blood was drawn from healthy donors into vacutainer tubes with EDTA as the anticoagulant (BD Biosciences, San Jose, CA) and used within 2 hours of collection. Four volumes of blood were diluted with one volume of phosphate-buffered saline (PBS) and 150 μ L/well of the diluted blood was dispensed per well of 96-well plates. M5049 dilutions were added in triplicate and plates were incubated at 37°C 5% and CO₂ for 30 minutes. DMSO, the

TLR7 ligand CL-087 (3 μ M) or the TLR8 selective agonist described above (0.5 μ M) were added to the cells for stimulation. The treated blood was incubated overnight at 37°C and 5% CO₂ and cytokines were measured in the plasma by AlphaLISA.

Sequences of RNA ligands used for stimulation:

Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGUGUGGUU
Let-7c	UGAGGUAGUAGGUUGUAUGGUU
Let-7e	UGAGGUAGGAGGUUGUAUAGUU
Let-7f	UGAGGUAGUAGAUUGUAUAGUU
miR-122	UGGAGUGUGACAAUGGUGUUUG
miR-223	CGUGUAUUUGACAAGCUGAGUU
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-574	UGAGUGUGUGUGUGUGAGUGUGU
Alu Motif B	UUUUUUUUUUUUUUUUUUUUUUUUUUUUUGAGACGGAGUCUCGCUCUGUCGCC

Absorption, Distribution, Metabolism, and Excretion (ADME). *Serum protein binding* – Compound serum protein binding was measured using rapid equilibrium dialysis (10 kDa) following the method of Banker et al. (Banker et al., 2003).

Caco-2 permeability/efflux – Caco-2 permeability and efflux ratio were determined at a concentration of 1 μ M following the method of Feng et al. (Feng et al., 2008).

Microsomal clearance – Microsomal clearance was determined at a concentration of 1 μ M in pooled liver microsomes following the method of Obach (Obach, 1999).

Hepatocyte clearance – Hepatocyte clearance was determined at a concentration of 1 μ M in pooled hepatocytes following the method of McGinnity et al. (McGinnity et al., 2004).

Human cytosol clearance (aldehyde oxidase, AOX) – Human cytosol clearance was determined at a concentration of 1 μM in pooled ($N = 200$) human cytosol in the presence/absence of hydralazine following the method of Zientek et al. (Zientek et al., 2010).

Cytochrome P450 inhibition – cytochrome P450 inhibition was determined in pooled human microsomes at concentrations ranging from 0 to 50 μM in a cocktail format following the methods of Kozakai et al. (Kozakai et al., 2012) for cytochrome P450s 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A.

Cytochrome P450 induction – Cytochrome P450 induction was determined at concentrations ranging from 0 to 50 μM in a HepaRG cell line following the method of McGinnity et al. (McGinnity et al., 2009).

Animal Studies. All procedures using animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and all local and national laws and regulations regarding animal care under protocols approved by the local Institutional Animal Care and Use Committee (IACUC).

Pharmacokinetic (PK) studies – Animals were dosed intravenous (i.v.) or oral gavage with M5049 or CMPD 2 formulated in 0.1 M Na-citrate buffer pH 4.5 (i.v.) pH 3 (p.o.). Blood was collected via different routes for each species and processed to obtain plasma that was subsequently analyzed for compound concentrations by liquid chromatography-mass spectrometry. Mouse (CD1; Charles River Labs, Margate, UK) blood was collected via submandibular bleeds and rat (Wister; Charles River Labs, Wilmington, MA) blood was collected via femoral catheter. Blood was collected from dogs (Beagle; Nuvisan Pharma Services, Neu Ulm, Germany) and monkeys (Cynomolgus; Aptuit, an Evotec Co., Verona, Italy) by femoral bleeds. For these studies, female mice, rats and dogs, and male monkeys were used.

Mouse pharmacodynamic (PD) studies – Female C57BL/6 mice (Charles River Labs) were dosed via oral gavage with M5049 formulated in 0.1 M Na-citrate buffer pH 3. At different timepoints after dosing, the mice were challenged with an intraperitoneal injection of 25 µg R848 dissolved in water. At 2 hours after R848 challenge the mice were euthanized and serum was collected by cardiac puncture. Mice were also challenged with miR-122 formulated in InvivoFectamine 3.0 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The miRNA was injected at 2 mg/kg i.v. via the tail vein and at 4 hours after injection blood was collected by cardiac puncture. Serum interleukin-6 (IL-6) and IFN- α (PBL) concentrations were measured by ELISA (BD, San Jose, CA) and the concentration of M5049 was measured by LC-MS.

BXSB-Yaa model – BXSB-Yaa studies were conducted as previously described (Bender et al., 2016). Male BXSB-Yaa mice were purchased from Jackson Labs (Jackson, MS) and at 7–8 weeks of age treatment was started. Mice were dosed once per day via oral gavage with M5049 formulated in 0.1 M Na-citrate buffer pH 3. After 14 weeks of treatment, mice were euthanized via CO₂ asphyxiation and blood was collected via the vena cava. Plasma was collected from a portion of the blood and 100 µL of blood was preserved in animal blood protect tubes (Qiagen, Hilden, Germany) for gene expression analysis. For monitoring of proteinuria in BXSB-Yaa mice, urine was collected by bladder massage in the morning on two consecutive days and the samples were pooled. The levels of albumin and creatinine in the urine were determined using the Advia 1800 clinical chemistry analyzer (Siemens, Berlin, Germany). The urinary albumin-to-creatinine ratio was calculated as the ratio of milligrams of albumin per gram of creatinine per deciliter of urine.

IFN- α NZB/W model – IFN- α NZB/W model studies were conducted as previously described (Haselmayer et al., 2019). Briefly, 10-week-old female NZB/W F1 mice (Jackson Labs) were given two i.v. injections on Day 0 and Day 1 of an adenovirus encoding for mouse IFN- α (BioFocus, Saffron Walden, UK) in saline, or were left untreated (sham). Treatments were initiated at 2 weeks after adenovirus injection and mice were treated for 10 weeks. Mice were dosed once per day p.o. with M5049 formulated in 0.1 M Na-citrate buffer pH 3 or mycophenolate mofetil (CellCept; Roche, Basel, Switzerland) at 300 mg/kg. After 10 weeks of treatment, mice were euthanized via CO₂ asphyxiation and blood was drawn for serum collection; 100 μ L of the blood was preserved in animal blood protect tubes (Qiagen) for gene expression analysis. Spleen cells were analyzed for B and T cell subsets on the final day using flow cytometry. Over the course of the study urine samples were collected and proteinuria was determined by measuring the urinary protein to creatinine ratio using the ADVIA 1800 clinical chemistry analyzer (Siemens).

Autoantibody measurements – Autoantibodies were measured in mouse lupus plasma samples as previously described (Bender et al., 2016). Briefly, a custom manufactured 5-plex Meso Scale Discovery (MSD, Kenilworth, NJ) plate for assessment of five different autoantibodies (anti-dsDNA, anti-histone, anti-Ro/SSA, anti-Sm/RNP, and anti-RiboP) was used to test plasma samples collected at the start of treatment or at the end of the study. Plasma samples were diluted 1:100 or 1:200 with PBS containing 0.5% bovine serum albumin. Plasma samples from MRL/*lpr* mice with high autoantibody titers were pooled to create a standard curve that was used for quantitative comparison of the lupus study plasmas and values were expressed as autoantibody units per mL of plasma (U/mL).

Gene expression analysis – Gene expression analysis of lupus mouse blood samples was performed using the NanoString platform (NanoString, Seattle, WA). The RNeasy Protect Animal Blood System (Qiagen) was used for RNA isolation. A custom-designed gene panel for monitoring TLR7 and TLR8 activation along with general inflammation was used as previously described (Bender et al., 2020). For each study, all samples were normalized to the included housekeeping genes and a Log₂ fold change was calculated for the treatment groups relative to the vehicle group. IFN gene signature scores were calculated for all lupus mice relative to a healthy control group of DBA/1 mice.

Data Analysis. For in vitro assays testing M5049 against TLR7 and TLR8 in whole blood, or M5049 against RNA ligands in PBMCs, experiments were performed in duplicate or triplicate and half maximal inhibitory concentration (IC₅₀) values were calculated using a 4-parameter fit of data in Prism (GraphPad, San Diego, CA). The percentage inhibition was determined for stimulation in the presence of compound compared to stimulation in the absence of compound. Potency of compounds in HEK293 cells and PBMCs against TLR7 and TLR8 specific agonists was calculated with curve fitting in Genedata Screener and the Smart Fit function with the Neutral Controls normalization method. For mouse PD studies measuring inhibition of cytokine production the percentage inhibition was calculated for M5049 treated mice relative to mice dosed with R848 alone and the group means are reported with standard deviations. In lupus model studies, proteinuria and autoantibody data were analyzed with group medians and statistical significance was determined using a Kruskal-Wallis test or analysis of variance (ANOVA) for autoantibodies. Gene expression analysis for lupus studies was performed by calculating the Log₂ fold change for each mouse relative to the vehicle group and

statistical significance for M5049 dosed mice relative to the vehicle group was determined by t test.

Results

Discovery of TLR7/8 Selective Inhibitors. A screening campaign was performed to identify compounds that function as dual TLR7/8 inhibitors without activity against other TLRs. The campaign and additional structure-activity relationships optimization led to the discovery of two promising candidate drugs: M5049 and CMPD 2 (see Fig. 1A for structures). Both compounds were found to be competitive reversible inhibitors and the Schild plot for M5049 is shown in Supplementary Fig. 1 Z.

To characterize the molecular interactions of M5049 with its target TLRs, the three-dimensional structure of M5049 bound to recombinant human TLR8 was determined by single-crystal X-ray diffraction from a TLR8 crystal soaked in a solution containing M5049 (Fig. 1B, 1C). It was found that two molecules of M5049 bound in the mirrored hydrophobic pockets of the TLR8 dimer interface (herein referred to as TLR8 and TLR8* along with their corresponding amino acids). Similar to previous TLR8 inhibitors, M5049 has van der Waal interactions with Phe346, Val378, Ile403, Phe494*, Ala518*, and Tyr567* (Zhang et al., 2018). Further, H-bond interactions are seen between the M5049 N of the quinoline ring and backbone of Gly351, and a charged interaction between the aminopiperidine and the backbone carbonyl of Ser516*. Finally, off-set pi-pi stacking can be seen between the M5049 quinoline ring and Tyr348 and Phe495*. As has been seen with previous TLR8 antagonists, inhibition by M5049 is likely the result of the compound binding the open form of the dimer and thus stabilizing the receptor's resting (inactive) state, which prevents activation of the receptor. Supporting this idea, it was observed that the C-termini of the two monomers in our structure were 51 Å apart. This is in accordance with the observations of Zhang et al. who noted that the C-termini of TLR8 bound to inhibitor are 51 Å apart compared to a distance of 34 Å in the agonist-bound activated dimer (Zhang et al.,

2018). Although a structure could not be generated for either inhibitor complexed with TLR7, it is likely that the binding mode for M5049 to TLR7 is similar to that of TLR8.

M5049 and CMPD 2 were characterized for their inhibitory activity against different TLRs in a variety of cellular systems (Table 1). Both compounds were found to potently inhibit TLR7 and TLR8 in HEK NF- κ B-luciferase reporter cells with IC₅₀ values in the low nanomolar range. Additionally, both compounds were found to be potent against TLR7 and TLR8 in PBMCs as well as whole blood. There appears to be an increased selectivity for TLR7 inhibition relative to TLR8 for both M5049 and CMPD2 in whole blood compared with PBMCs and transfected HEK cells. Although different ligand concentrations were used for stimulation in the various systems, we think it is unlikely that this alone explains the increased TLR7 selectivity in whole blood. One possible reason for the selectivity shift is higher protein binding of the TLR7 agonist compared to the TLR8 agonist in whole blood, which could lead to less competition for M5049 or CMPD2 at TLR7. Another reason could be that neutrophils, which primarily express TLR8, are present in whole blood but not in PBMCs, so the activation of these additional cells may require more inhibitor activity in whole blood.

The compounds showed good selectivity and were found to be inactive against TLR3, TLR4 and TLR9 in vitro (Table 1) and in vivo (Supplemental Figure 2). Selectivity was also established by testing for activity against a panel of 389 kinases as off-target profiling (Reaction Biology, Malvern, PA) and at 1 μ M M5049 only inhibited the activity of one kinase more than 50% (CAMKK2) and CMPD 2 inhibited none. M5049 was also tested for off-target effects in the CEREP Extended Receptor Profile assay (Eurofins, Saint Etienne, Luxembourg) and 5-HT uptake was the only activity affected out of the many tested. Lack of off-target activity was confirmed by profiling using the BioMAP[®] Diversity PLUS panel of primary human cell co-

culture systems using a wide concentration range (10 nM to 10 μ M) (Berg et al., 2010). No significant activity of M5049 was observed in the panel and no cytotoxic activity was detected either (Supplemental Fig. 3).

Evaluation of M5049 Drug-like Properties and Duration of Action in vivo. Studies were conducted to characterize the ADME properties of M5049 and CMPD 2 and the compounds showed drug-like features. They both showed favorable in vitro characteristics such as low microsomal clearance, good Caco-2 permeability, and no relevant P450 inhibition or induction liabilities. However, M5049 showed much lower clearance in the human cytosol assay, indicating greatly reduced AOX-mediated clearance compared to CMPD 2 (Table 2). Preclinical PK assessments were conducted in CD1 mice and Wistar rats for M5049 and CMPD 2, and additional studies were conducted for M5049 in beagle dogs and cynomolgus monkeys (Fig. 2A and Supplemental Table 1). The compounds both showed high bioavailability with moderate half-life and clearance (Table 2). Neither compound was positive in human ether-à-go-go-related gene (hERG) or Ames tests, which are considered important safety features. The compounds both have high bioavailability, but a relatively short half-life. As M5049 was found to have superior PK properties in mice due to its much greater exposure (6-fold higher area under the curve (AUC)), and CMPD 2 was found to be a human AOX substrate, it was determined that M5049 is a more promising drug candidate and was advanced for further in vivo testing.

M5049 was tested in mice to characterize its PD properties and duration of action. The TLR7/8 agonist R848 can effectively stimulate both interferon- α (IFN- α) and IL-6 production in mice in vivo and the ability of M5049 to block an R848 challenge was used as a PD assessment. When M5049 was administered 1 hour prior to R848 challenge it dose dependently decreased IFN- α and IL-6 production (Fig. 2B). Inhibition of cytokine release was significant even at

dosages of M5049 where the resulting plasma levels of M5049 were very low (i.e. 0.1 and 0.3 mg/kg).

The duration of action of M5049 was determined by dosing M5049 at 1 mg/kg at different timepoints before R848 challenge (-1, -4, -8, -12, -24 hours). Despite the relatively short half-life of M5049 in mice (<3 hours, Fig. 2A), the compound was found to have a long duration of action as IL-6 production was inhibited more than 50% when the compound was dosed 24 hours prior to challenge and more than 80% when dosed 12 hours prior to challenge. In both cases the plasma concentrations of M5049 were low: 6 nM and 33 nM plasma M5049 were found at the time of blood collection for the -24 hour and 12-hour groups respectively (Fig. 2C). Although these plasma concentrations are low, the compound will still likely provide TLR7/8 inhibitory activity as the IC_{50} for M5049 in mouse blood in vitro has been found to be 1.9 nM against R848. If the values for the plasma concentrations of M5049 versus the percentage inhibition of IL-6 from the PK/PD timecourse study (Fig. 2C results) are overlaid on the in vitro dose response curve, there is a nearly perfect overlap (Fig. 2D), suggesting that the in vitro and in vivo potencies are highly similar and a very good PK/PD correlation exists for M5049. These results suggest that M5049 has a long duration of action and is capable of substantial target inhibition with once per day dosing in mice.

Effects of M5049 on Disease in Mouse Lupus Models. The ability of M5049 to reduce disease was tested in two different mouse lupus models, the BXSB-*Yaa* model and the IFN- α accelerated NZB/W model. In the BXSB-*Yaa* model male mice have a Y-linked autoimmune accelerator locus (*Yaa*) on their Y chromosome that normally resides on the X chromosome, which results in a duplication of genes such as *TLR7* that drive autoimmunity and the mice spontaneously develop a lupus-like disease (Pisitkun et al., 2006; Deane et al., 2007). Treatment

of BXS B - Yaa mice with M5049 led to an improvement in survival (Fig. 3A) and reduction in kidney disease as evidenced by lower proteinuria (Fig. 3B, 3C). M5049 was also tested in the IFN- α accelerated NZB/W F1 model and again showed significant efficacy. The pathogenesis of the IFN- α accelerated NZB/W model is different from that of the BXS B - Yaa model. The model uses treatment with an adenovirus expressing IFN- α at the beginning of the study to induce a transient elevation of IFN- α , accelerating disease development in NZB/W F1 mice, which normally require many months to spontaneously manifest disease (Dubois et al., 1966). TLR7 has not been demonstrated to drive disease in this model (or in the standard NZB/W model without disease acceleration) in contrast to the BXS B - Yaa model. In the IFN- α accelerated NZB/W model M5049 treatment also improved survival (Fig. 3D) and reduced kidney damage as evidenced by reduced proteinuria (Fig. 3E, 3F) and lower histopathological glomerulonephritis scores.

The dosages that were efficacious in the BXS B - Yaa and IFN- α NZB/W models resulted in low 24-hour plasma concentrations of M5049 in lupus mice (Supplemental Fig. 4) and are ~~below the concentration~~ at the low end of the dose response curve where inhibition of R848 in vitro or in vivo is seen (Fig. 2D). This suggests that 24-hour target inhibition is not needed for efficacy, or that the disease-driving TLR7/8 ligands naturally existing in lupus mice are different from R848 in their levels or nature. It is also possible that the primary site of action of M5049 is localized in tissues instead of in the circulation. Supporting this idea are results from a whole-body radiography study with ^{14}C -M5049 (Supplemental Fig. 5). When rats were dosed with ^{14}C -M5049 it was found that there was preferential accumulation of the compound in tissues as evidenced by the high AUC and C_{max} ratios for different tissues compared to blood. Tissue

accumulation of M5049 could be the reason for the disconnect between plasma concentrations and efficacy in lupus mice.

In the BXS B -*Yaa* model there is a robust increase in multiple autoantibodies and M5049 significantly reduced both DNA-containing reactivities such as anti-dsDNA and anti-histones (Fig. 4A, 4B) as well as RNA-binding protein reactivities such as anti-RiboP and anti-SmRNP (Fig. 4C, 4D). At high dosages of M5049 the effect on autoantibodies is so profound that titers are reduced below the baseline levels. In contrast, there is much less development of autoantibodies in the IFN- α NZB/W model and titers are not largely increased over the course of the study from baseline (Fig. 4E, 4F, 4G, 4H). Titers of anti-dsDNA and anti-SmRNP autoantibodies were not increased with disease progression while anti-histones and anti-SmRNP levels were only slightly increased, and the increase was much less than observed in the BXS B -*Yaa* mice. The lack of an increase in autoantibody titers in the IFN- α NZB/W mice may indicate that the disease pathogenesis is more localized in the tissues and less systemic, and autoreactive antibody-producing B cells play a smaller role in disease pathogenesis. M5049 had a negligible effect on autoantibody titers in this model, which is consistent with the hypothesis that the compound may be efficacious in these mice due to effects in tissues.

Effects of M5049 on Gene Expression in Mouse Lupus Models. Gene expression analysis was performed using NanoString on blood samples collected from the BXS B -*Yaa* and IFN- α NZB/W lupus mice treated with M5049 at the end of the studies to try and better characterize the effects of compound treatment on disease. A custom NanoString panel of 97 genes was used for the analysis and the genes selected were previously identified to be regulated downstream of TLR7/8 signaling and also disease relevant (Bender et al., 2020). It was found that there were 66 genes expressed above the lower limit of detection in both models and these

genes were analyzed in detail. A Log₂ fold change in gene expression was calculated for the compound-treated groups in each model compared to the vehicle control group to determine the effect of M5049 treatment. In both models M5049 impacted many of the same genes, although there were some differences in the magnitude of the effect (Fig. 5). Volcano plots were constructed for the M5049 10 mg/kg group of each study to best identify the most significantly affected genes (Fig. 6A, 6B). In the IFN- α NZB/W model the most highly affected genes were markers for myeloid and granulocyte populations such as *CEACAM1*, *CLEC4E*, *FCER1G*, and *TLR8*. In the BXSB-*Yaa* model some of the same markers for those subsets were also decreased such as *CLEC4E*, *TLR8*, and *FCER1G* along with other myeloid markers such as *LILRA5* as well as *TLR7*. However, in the BXSB-*Yaa* mice there was a much more significant effect of treatment on IFN-regulated genes such as *IFI44*, *OAS1*, *OAS2*, *OAS3*, *IFI27*, *IFIH1*, and *IRF7*. Given the high degree of efficacy at the 10 mg/kg dose, it is a little surprising that larger effects on gene expression were not observed and more genes were not seen to be affected significantly and to a larger degree in the volcano plot. It is possible that the mechanism of action of M5049 leading to efficacy was more at work in tissues and less in the circulation, and this could be the reason for the modest effect on gene expression in the blood.

To more fully compare the strength of IFN signaling in these models and the effect of M5049 on IFN activity, a panel of 10 IFN-regulated genes (*OAS1*, *OAS2*, *OAS3*, *CMPK2*, *GBP5*, *IFI44*, *IFIH1*, *ISG15*, *IFI27*, *IRF7*) that were significantly increased with disease in both models were used to calculate an IFN gene signature score for each mouse (Supplemental Fig. 6). The IFN- α NZB/W model mice had higher IFN gene signature scores (vehicle group median of 4.6) compared to the BXSB-*Yaa* mice (vehicle group median of 1.2). M5049 had no effect on IFN scores in the IFN- α NZB/W model but did significantly reduce scores in the BXSB-*Yaa* model.

The source of IFN in the IFN- α NZB/W model leading to the robust upregulation of IFN-regulated genes at the end of the study is unclear. We have observed that adenovirus injection of these mice results in a very high, but transient, peak in IFN- α protein which is greatly reduced within days, but it is possible that the early disease triggering adenovirus injection is the reason for the strong IFN gene signature in these mice at the end of the study and the reason why M5049 failed to suppress it since it would not be TLR7 dependent. Taken together, these results indicate that M5049 efficacy may be achieved in the IFN- α NZB/W model by a mechanism other than inhibition of IFN production.

Activation of TLR7/8 by Natural RNA Molecules and Inhibition by M5049. To better understand how effectively M5049 may inhibit natural TLR7/8 ligands activating the receptors in vivo, the ability of the compound to block miRNAs and Alu RNA was determined. Human PBMCs were treated with miR-122, Let7c, Alu RNA, or R848 in a dose response, and after an overnight incubation the production of IL-6 and IFN- α were measured and the EC₈₀ was determined. A concentration of roughly the EC₈₀ was chosen for each ligand and then used to stimulate PBMCs after a pre-treatment of the cells with M5049 to determine if the compound could inhibit the different ligands with equal potency. All the ligands tested were found to induce IL-6 and IFN- α production (Fig. 7A, 7B) but with slight differences for the two cytokines. The miRNAs and Alu RNA induced less IL-6 but more IFN- α compared to the small molecule agonist R848. M5049 inhibited production of IL-6 stimulated by all the ligands nearly equally, with IC₅₀ values ranging from 35 to 45 nM (Fig. 7C). The M5049 IC₅₀ values for inhibition of IFN- α by the miRNAs were similar (210 nM for miR-122, 161 nM for Let7c) while the value for Alu RNA was slightly lower (68.5 nM) (Fig. 7D). The IC₅₀ value for R848 stimulated IFN- α production was the lowest (8.9 nM) and this may be due to its much weaker activity compared to

the other ligands for stimulation of IFN- α . Several other miRNAs have been reported to be disease associated (Wang et al., 2012) and their ability to stimulate PBMCs and their sensitivity to inhibition by M5049 was also tested (Supplemental Fig. 7). It was found that a variety of miRNA molecules could induce IL-6 and IFN- α production, and all were susceptible to inhibition by M5049. The differences in M5049 IC₅₀ values for natural ligands and synthetic small molecule agonists highlights the importance of using endogenous stimuli whenever feasible to evaluate candidate inhibitors of these targets.

We expanded our testing of miRNA to the in vivo setting and found that miR-122 can also stimulate IL-6 and IFN- α production in mice. When mice were dosed with miR-122, increased IL-6 and IFN- α were both detected in the plasma (Fig. 7E, 7F). Pre-treatment with M5049 before miR-122 injection dose-dependently inhibited the production of IL-6 and IFN- α . These results indicate that miRNA may be TLR7 or TLR8 activators in vivo and are inhibitable by M5049 in such a setting as they were in vitro.

Discussion

Despite the challenges associated with developing antagonists of TLR7 and TLR8 we have successfully produced two compounds that are potent and selective dual TLR7/8 inhibitors. The question of which endosomal TLRs to target for most effective autoimmune disease treatment is not easy to answer. Prior studies using KO of TLR7, TLR8, or TLR9 in various combinations have suggested that there is crosstalk between them and KO of one may unexpectedly exacerbate disease by increasing the activity of TLR7 as this has been observed with TLR9 KO (Christensen et al., 2006; Jackson et al., 2014; Nundel et al., 2015) and TLR8 KO (Demaria et al., 2010). However, dual KO of TLR7 and TLR8 or TLR7 and TLR9 was found to be protective, suggesting that dual TLR KO is a safer approach to reduce disease. We believe that inhibition of TLR8 may be important for achieving efficacy in lupus patients and other autoimmune diseases as it is expressed in a number of abundant immune cell types and its activation can lead to robust inflammatory cytokine production (Gorden et al., 2005; Bender et al., 2020). It is unclear how much TLR8 contributes to disease in our mouse models and how much of the efficacy observed for M5049 is due to TLR7 vs TLR8 inhibition, and this assessment is out of the scope of this publication. The activity of TLR8 in mice is controversial, but we did observe that TLR8 mRNA is detected and elevated with disease in the mice and that potential TLR8 ligands such as miRNA are present in their plasma. Moving forward, we believe that the dual TLR7/8 inhibitory activity of M5049 may allow it to block multiple aspects of autoimmunity in a variety of cell types to provide efficacy.

M5049's novel mechanism of inhibiting TLR7/8 may make the compound valuable for patients as it provides differentiation from existing standard of care and other emerging novel treatments for lupus. Many currently used treatments have weaknesses; for example, steroids and

mycophenolate mofetil are associated with untoward side effects and anti-malarials are weakly efficacious. Other available lupus treatments such as belimumab and rituximab target only the B cell component of disease, while newer anti-IFN treatments such as anifrolumab will inhibit only one disease driver downstream of TLR activation and not NF- κ B-stimulated cytokines or direct B cell activation. TLR7/8 inhibition is unique in that it may block both innate and adaptive autoimmunity and inflammation. M5049 may inhibit TLR7/8 activation in pDCs, neutrophils, and monocytes thus reducing IFN and inflammatory cytokine production. In addition, M5049 may also block adaptive inflammation by inhibiting autoreactive B cells. Because of this broad inhibitory profile but non-depleting nature, we believe that TLR7/8 inhibition by M5049 is a promising therapeutic approach.

Based on the characteristics reported here, M5049 may have advantages over most existing TLR7/8 inhibitors. Synthetic oligodeoxynucleotide antagonists specific for TLR7 (IRS661) or TLR7/9 (IRS954) were produced and tested preclinically (Pawar et al., 2007) and an oligonucleotide-based TLR7/8/9 antagonist, IMO-8400, has been developed and tested in early clinical trials (Balak et al., 2017). However, oligo-based inhibitors require dosing regimens not very practical for clinical usage with frequent subcutaneous injections required; in contrast to M5049 which can be administered p.o. Small molecule TLR8 (Zhang et al., 2018) and TLR7 (Bou Karroum et al., 2019) specific inhibitors have also been reported, but the *in vivo* properties of these molecules have not been characterized and their potency may not be sufficient for clinical use. More recently, Phase I trial results in healthy volunteers have been reported for a TLR7/8 inhibitor (E6742) (Nakai et al., 2018) but there is only a minimal amount of published information on its potential. Some other previously characterized small molecule TLR inhibitors have been found to accumulate inside cells in a compartment such as the endosome due to

protonation at low pH. This has previously been observed for hydroxychloroquine, which is used clinically, and other endosomal TLR7 and TLR9 inhibitors (Lamphier et al., 2014). Part of the mechanism of action of these compounds may be general interference of lysosomal pH and function and consequently inhibition of multiple endosomal TLRs (Ohkuma and Poole, 1978). Given that M5049 does not inhibit TLR3 and TLR9 and was demonstrated by crystallography to directly bind TLR8, it is unlikely that the compound is sequestered in lysosomes in the same way and thus it has a different mechanism of action than hydroxychloroquine and similar lysosomotropic compounds and an advantageously more targeted mechanism of action.

The detailed selectivity and potency characterization of M5049 along with its ADME and PK properties suggest that it is a strong drug candidate. In our studies M5049 not only showed a long duration of action in PK/PD experiments after a single dose, but once per day dosing in lupus mice was sufficient to provide efficacy even at unexpectedly low dosages where plasma levels are below the IC_{50} predicted by in vitro and in vivo PK/PD studies in healthy mice. We hypothesize that the long duration of action of M5049 in disease model studies may be due to its high potency and in part the result of sustained presence of the compound in tissues and this localization could be beneficial for treating organ inflammation in lupus patients with disease manifestations in tissues such as skin, joints, or the kidney. Supporting this hypothesis, it has recently been reported that L1 mRNA transcripts are increased in lupus kidneys and Sjogren's patient's salivary glands and these RNAs can stimulate TLR7 (Mavragani et al., 2016). Our in vitro results demonstrate that M5049 effectively inhibits these natural endogenous TLR7/8 ligands and in vivo activity against them may reduce disease.

The observed efficacy for M5049 was expected in the BXS B -*Yaa* model as this model is recognized to be driven by TLR7, but the effectiveness of M5049 in the IFN- α NZB/W model

was not anticipated. To date, TLR7 has not been reported to be a driver in the IFN- α NZB/W model and these mice do not show high titers of canonical RNA-containing autoantibodies such as anti-SmRNP and anti-RiboP which are hallmarks of TLR7 driven disease such as in the BXSB-*Yaa* and pristane models (Reeves et al., 2009; Perry et al., 2011). Previously it has been noted that NZB/W mice show an elevated IFN gene signature although the mechanism for the increase is unclear (Zhuang et al., 2015). It is possible that TLR7 is activated in the IFN- α NZB/W model by a previously not recognized mechanism such as circulating miRNA or Alu RNA molecules or more localized TLR7 ligands. However, the presence of the IFN gene signature in these mice could also be due to persistent effects of the adenovirus injection.

The unexpected observed efficacy in the IFN- α NZB/W model may indicate that TLR7/8 inhibition could be effective in a large variety of lupus patients and not only those with elevated RNA autoantibody reactivities. It is also notable that IFN activity is not reduced in the IFN- α NZB/W model by M5049 and we have also observed efficacy for CMPD 2 in the pristane model without a reduction in IFN activity (data not shown). It has previously been demonstrated that TLR7 activation by R848 in NZM2328 lupus-prone mice can drive disease in an IFN-independent manner (Wolf et al., 2018). These observations along with the observation that the elevation of autoantibody titers in our NZB/W studies is modest may further support the idea that M5049's activity is a consequence of inhibiting inflammation in tissues and less of a systemic TLR7-IFN driven effect. Although we have extensively tested M5049 selectivity, and have shown it to have very targeted activity against TLR7/8, we cannot completely rule out the possibility that some off-target effects contribute to its efficacy in vivo.

Overall, these results indicate that M5049 is a compound with characteristics that make it a promising drug candidate. Additionally, our studies provide new insight into the consequences of TLR7/8 activation by a variety of ligands and how the TLRs may contribute to disease.

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Authorship Contributions

Participated in research design: Vlach, Bender, Haselmayer, Sherer.

Conducted experiments: Bender, Przetak, Pereira, Zimmerli, Deshpande, Johnson, Reissig, Haselmayer, Musil, Okitsu, Tzvetkov, Morse, Walsky.

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Financial Disclosures

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Footnotes

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Numbered footnotes: ¹Tested in Reaction Biology 389 kinase panel; ²CEREP receptor profiling assay.

Figure Legends

Fig. 1. TLR7/8 inhibitor structures and crystallography. (A) The structures of M5049 and CMPD 2 are shown. (B) The crystal structure of M5049 in complex with human TLR8 shows the bound inhibitor at the interface between the two dimers presented as a ribbon diagram. (C) The TLR8 residues contacting M5049 at the binding site are shown with the two different monomers colored as pink or magenta (PDB ID: 6ZJZ).

Fig. 2. M5049 pharmacokinetic/pharmacodynamic activity in mice. (A) To determine the PK properties of M5049, mice were dosed with M5049 at 1 mg/kg via oral gavage or i.v. and blood samples were collected over time for the measurement of plasma concentrations. (B) To test the PD activity of M5049, mice were given a single oral injection of M5049 and 1 hour later were dosed i.p. with 25 μ g/mouse of R848. After 2-hour, blood samples were collected and plasma concentrations of M5049, IFN- α , and IL-6 were measured. The percentage inhibition of cytokine release was calculated relative to mice dosed with R848 alone. (C) The duration of inhibition of M5049 was determined by dosing mice with M5049 at 1 mg/kg oral and at 24, 12, 8, 4, and 1 hour after dosing an i.p. injection of 25 μ g R848 was given. After 2-hour, blood samples were collected and plasma concentrations of M5049, IFN- α , and IL-6 were measured. (D) A plot was constructed overlaying the IC_{50} curve for M5049 inhibition of R848 stimulation in mouse blood in vitro and M5049 inhibition of R848 stimulation in vivo (from B). Groups of five mice were used and each study is representative of two separate experiments. Group means \pm SD are shown.

Fig 3. The effect of M5049 on disease in mouse lupus models. BXSB-*Yaa* mice were dosed starting at 7-8 weeks of age with M5049 via oral gavage. NZB/W mice were treated with an injection of an IFN- α adenovirus to trigger disease development and 14 days later treatment was started with M5049 or with MMF at 300 mg/kg. Survival was tracked over time (A, D) and kidney disease was assayed by measuring proteinuria and the data is plotted as a timecourse (B, E) or the AUC for individual mice (C, F). For each study 10-12 mice per group were used and results are representative of two separate studies for each model. The proteinuria timecourse graph presents means \pm SEM and AUC data are shown as medians. Statistical significance was determined by Kruskal-Wallis ($*P < 0.05$; $**P < 0.01$). Survival statistical significance was determined using a Mantel-Cox test ($**P < 0.01$).

Fig. 4. The effect of M5049 on autoantibodies in mouse lupus models. At the beginning of treatment (baseline) and at the end of the BXSB-*Yaa* and IFN- α NZB/W studies plasma samples were collected, and autoantibodies were measured using a custom MSD multiplex assay for various reactivities. Titers of anti-dsDNA (A, E), anti-Histones (B, F), anti-SmRNP (C, G), and anti-RiboP (D, H) were measured. Bars show group medians and statistical significance was determined by one-way ANOVA ($*P < 0.05$; $**P < 0.01$). For each study, 10–12 mice were used per group and results are representative of two separate studies for each model.

Fig. 5. The effect of M5049 on gene expression in mouse lupus models. Blood samples were collected from IFN- α accelerated NZB/W or BXSB-*Yaa* model mice at the conclusion of each study. RNA was extracted and analyzed by NanoString using a custom-designed panel to measure gene expression. The Log₂-fold change in expression was calculated relative to the

vehicle group (V) for each study and the results are presented in a heat map. For each study, 10–12 mice were used per group and results are representative of two separate studies for each model.

Fig. 6. Genes significantly affected by M5049 in mouse lupus models. Blood samples were collected from IFN- α accelerated NZB/W (A) or BXSB-*Yaa* (B) model mice at the conclusion of each study and gene expression was determined by NanoString. A standard t test was performed to determine statistical significance for difference relative to the vehicle group and a volcano plot showing the resulting p values vs the Log₂-fold change was constructed for the 10 mg/kg M5049 group of the studies. For each study, 10–12 mice were used per group and results are representative of two separate studies for each model. The data presented are the same as those presented in the heatmap in Figure 5.

Fig. 7. Inhibitory effect of M5049 on endogenous natural TLR7/8 stimulatory RNA molecules. PBMCs were stimulated with Let-7c (2 μ M), miR-122 (1.8 μ M), R848 (1 μ M) or Alu RNA (0.4 μ M) complexed with DOTAP. After an overnight incubation IL-6 (A) and IFN- α (B) concentrations in the supernatants were measured by AlphaLISA. Stimulation was also carried out +/- 10 μ M M5049 pretreatment for 30 min (C, D). Values shown are means \pm SD of two donors from two separate experiments. The ability of M5049 to inhibit stimulatory activity of miRNA in vivo was determined by injection of C57BL/6 mice with miR-122 (2 mg/kg) 30 min after pretreatment with M5049. The production of IFN- α (E) and IL-6 (F) was measured in the plasma at 4 hr after miR-122 injection. The results shown are representative of two separate

experiments using 6 mice per group and bars show group medians. Statistical significance was determined by ANOVA (** $P < 0.01$).

TABLE 1

TLR7/8 inhibitor potency and selectivity

TLR	Stimulus	Cells	Endpoint	M5049	CMPD 2		
				IC ₅₀ (nM)	N	IC ₅₀ (nM)	N
TLR7	R848	HEK	NF-κB Luc	11.1 ± 9.94	6	26 ± 10.6	5
TLR8	R848	HEK	NF-κB Luc	24.1 ± 9.16	6	3.6 ± 1.06	5
TLR7	TLR7 ligand	PBMCs	IL-6	68.3 ± 59.8	4	80 ± 28	3
TLR8	TLR8 ligand	PBMCs	IL-6	620 ± 628	4	8.9 ± 6.2	4
TLR3	Poly I:C	PBMCs	MCP-1	No inhibition	2	No inhibition	4
TLR4	LPS	PBMCs	IL-6	No inhibition	2	No inhibition	2
TLR9	CpG	PBMCs	MCP-1	No inhibition	2	No inhibition	4
TLR7	TLR7 ligand	WB	IL-6	2.2 ± 2.6	8	0.58 ± 0.27	6
TLR8	TLR8 ligand	WB	IL-6	120 ± 34	6	0.81 ± 0.31	6

Values are means ± SD

TABLE 2

TLR7/8 inhibitor physicochemical, absorption, distribution, metabolism, and excretion, and safety properties

Parameter	M5049	CPD 2
Molecular weight	356.8	436.6
Therm. Sol. pH 6.5 (mg/mL)	>1	>1
logP	2.2	2.3
PPB (% free) m/r/d/c/h	14/15/33/35/32	56/64/74/74/75
Caco-2 A>B (10^{-6} cm/s)	26	32
Caco-2 efflux ratio	1.5	5.6
Microsomal CLint (μ L/min/mg protein) (m/r/d/c/h)	<10/<10/<10/<10/<10	<10/<10/<10/<10/<10
Hepatocyte CLint (μ L/min/ 10^6 cells) (m,r,d,c,h)	17/11/1.6/1.5/3.1	<10/6.5/<4/5.4/28
AOX Cytosol CLint (μ L/min/mg protein)	2.7	23
AOX cytosol $t_{1/2}$ (h)	4.3	0.51
P450 inhibition IC ₅₀ (μ M)	2D6: 3.6, others >100	All >10
P450 induction (mRNA)	1A2: 2-fold@50 μ M only	
hERG (μ M)	>100	>100
Ames test	Negative	Negative

¹ Kinases inhibited >50% at 1 μ M	CAMKK2	None
² Off-target receptor activities affected	5-HT uptake	ND

¹Tested in Reaction Biology 389 kinase panel; ²CEREP receptor profiling assay.

Figure 1

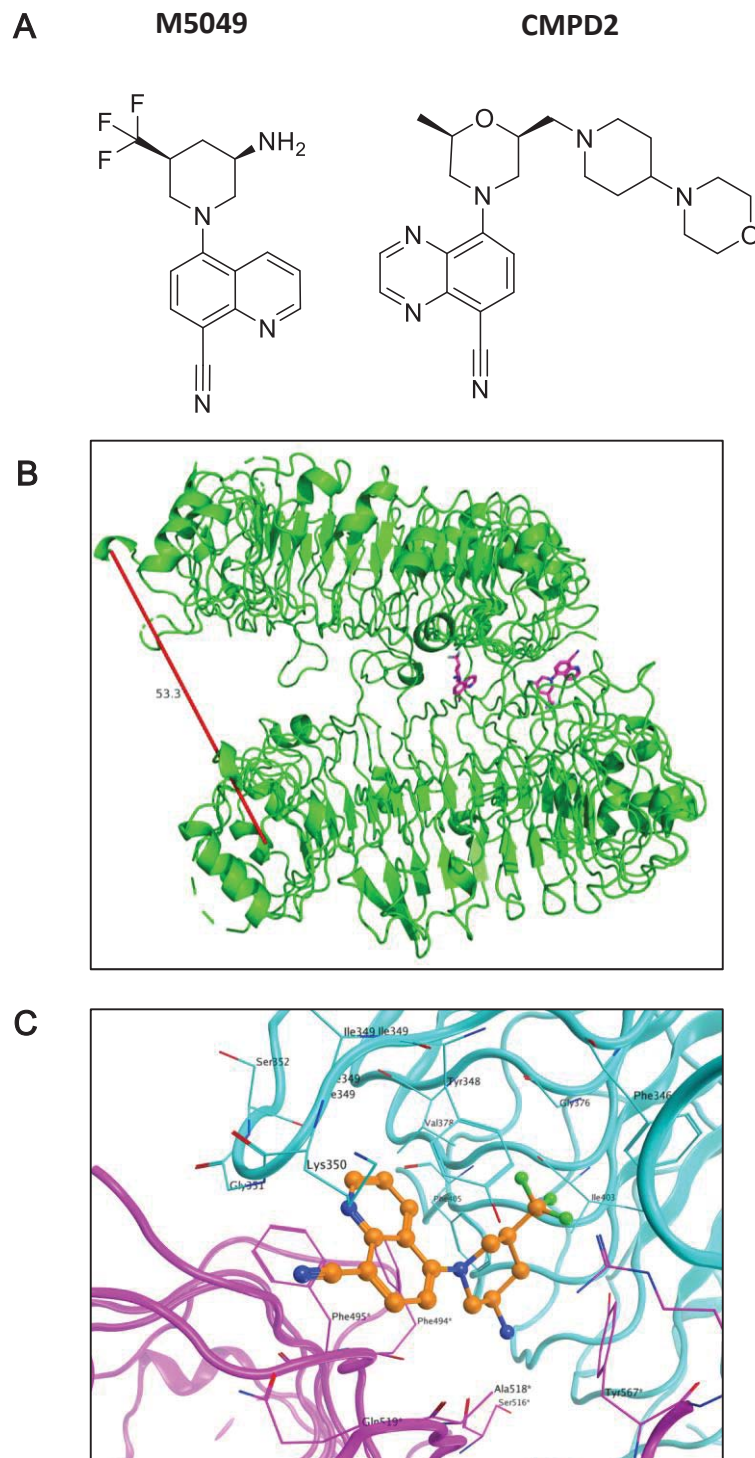
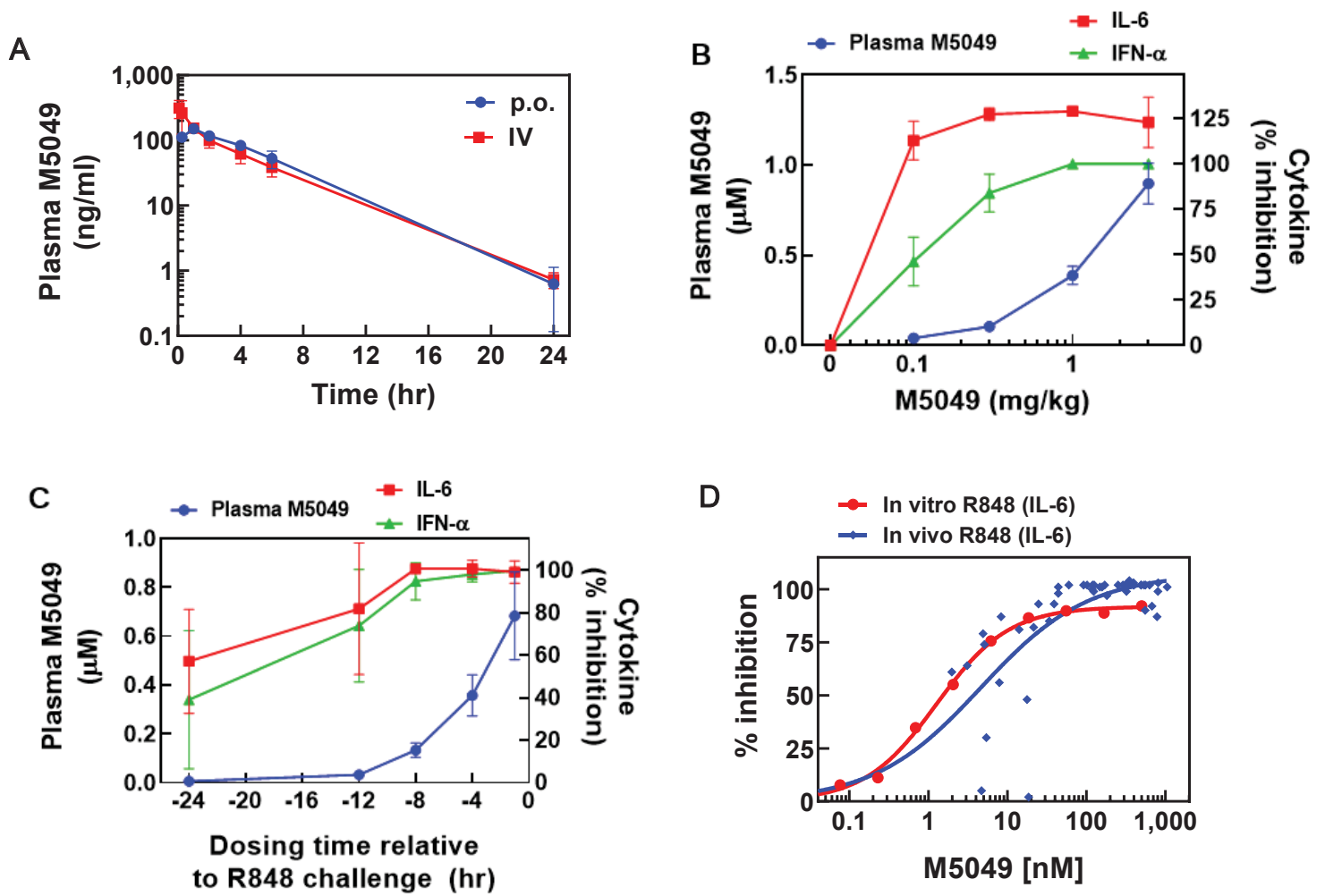


Figure 2



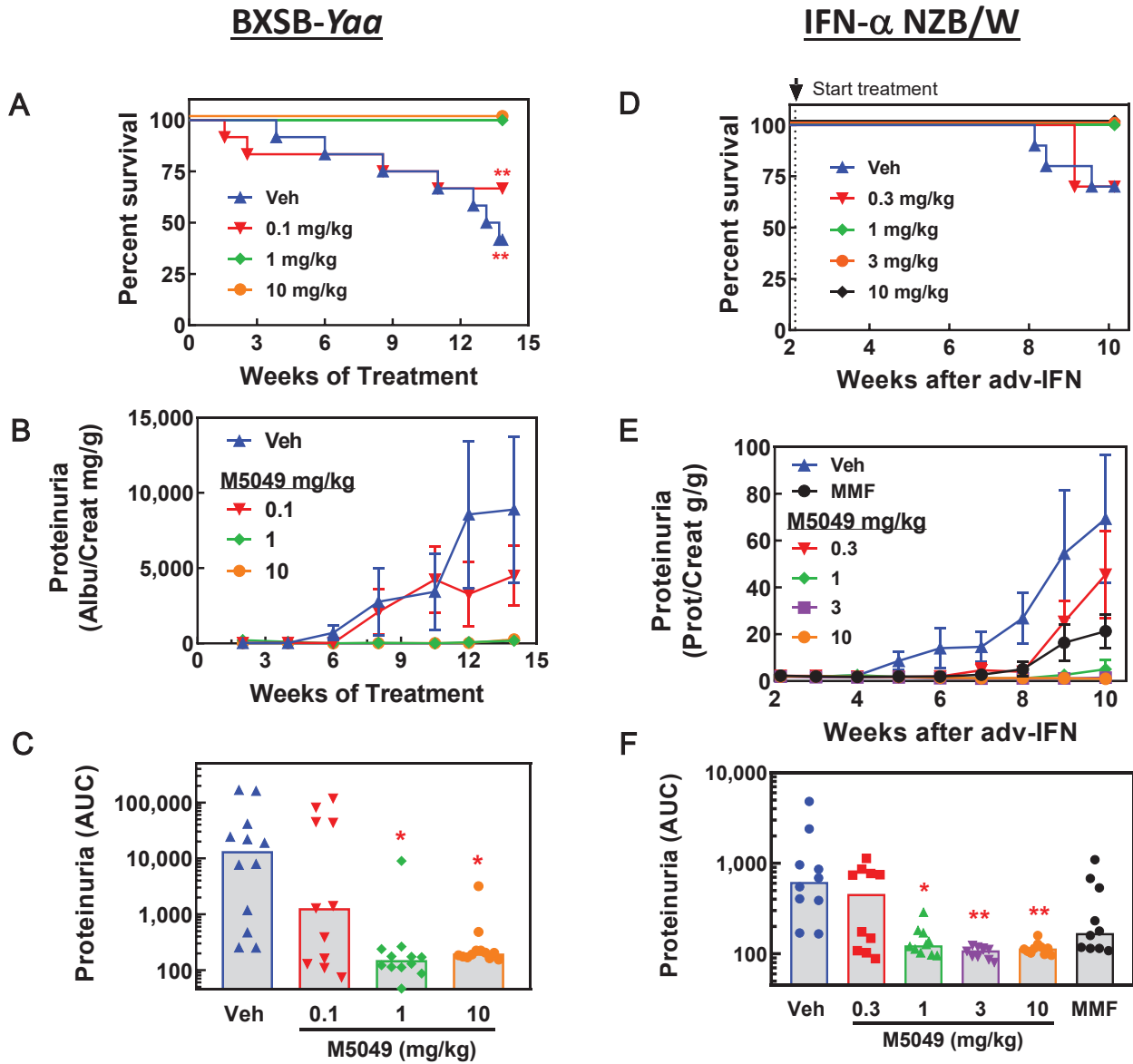


Figure 4

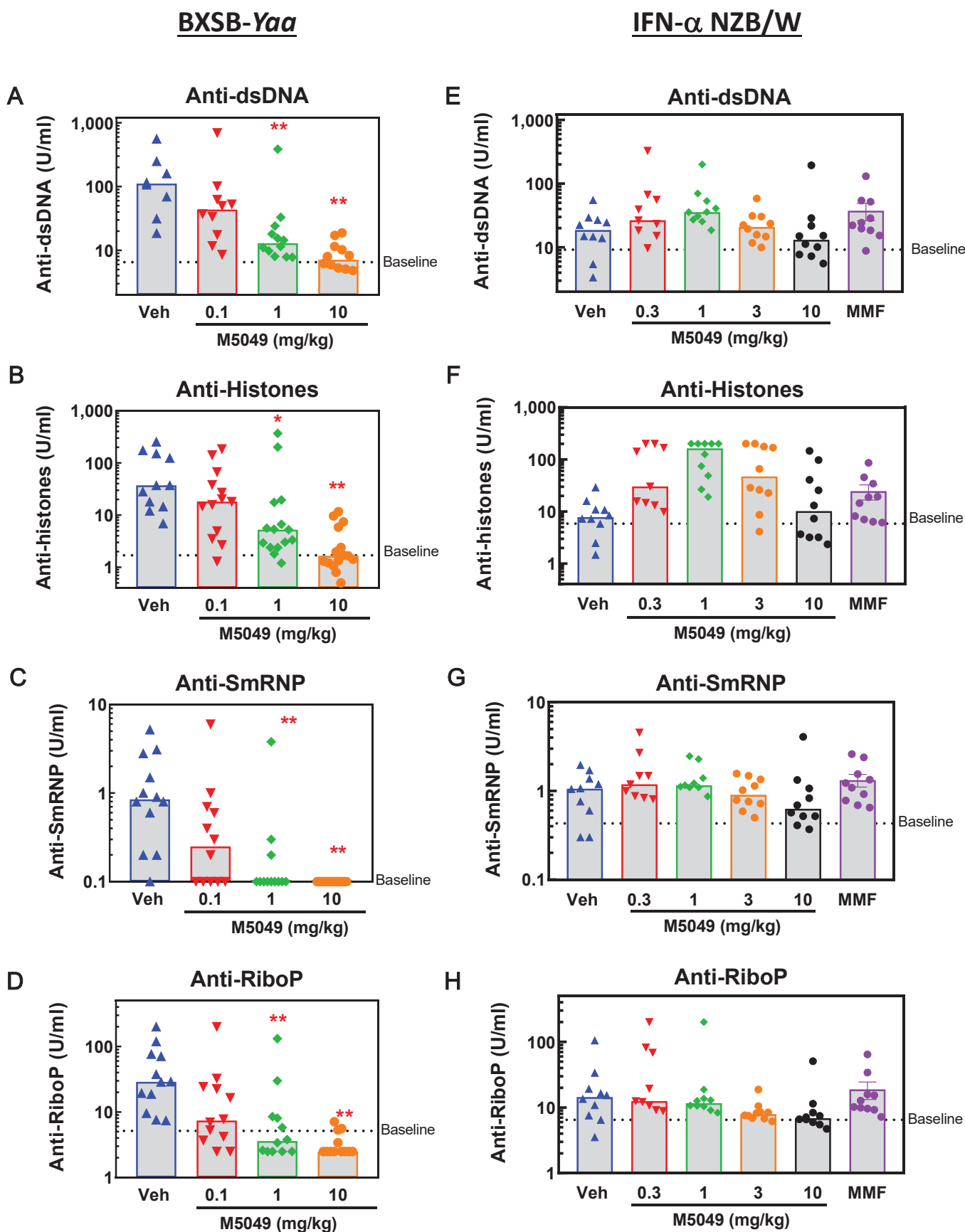


Figure 3

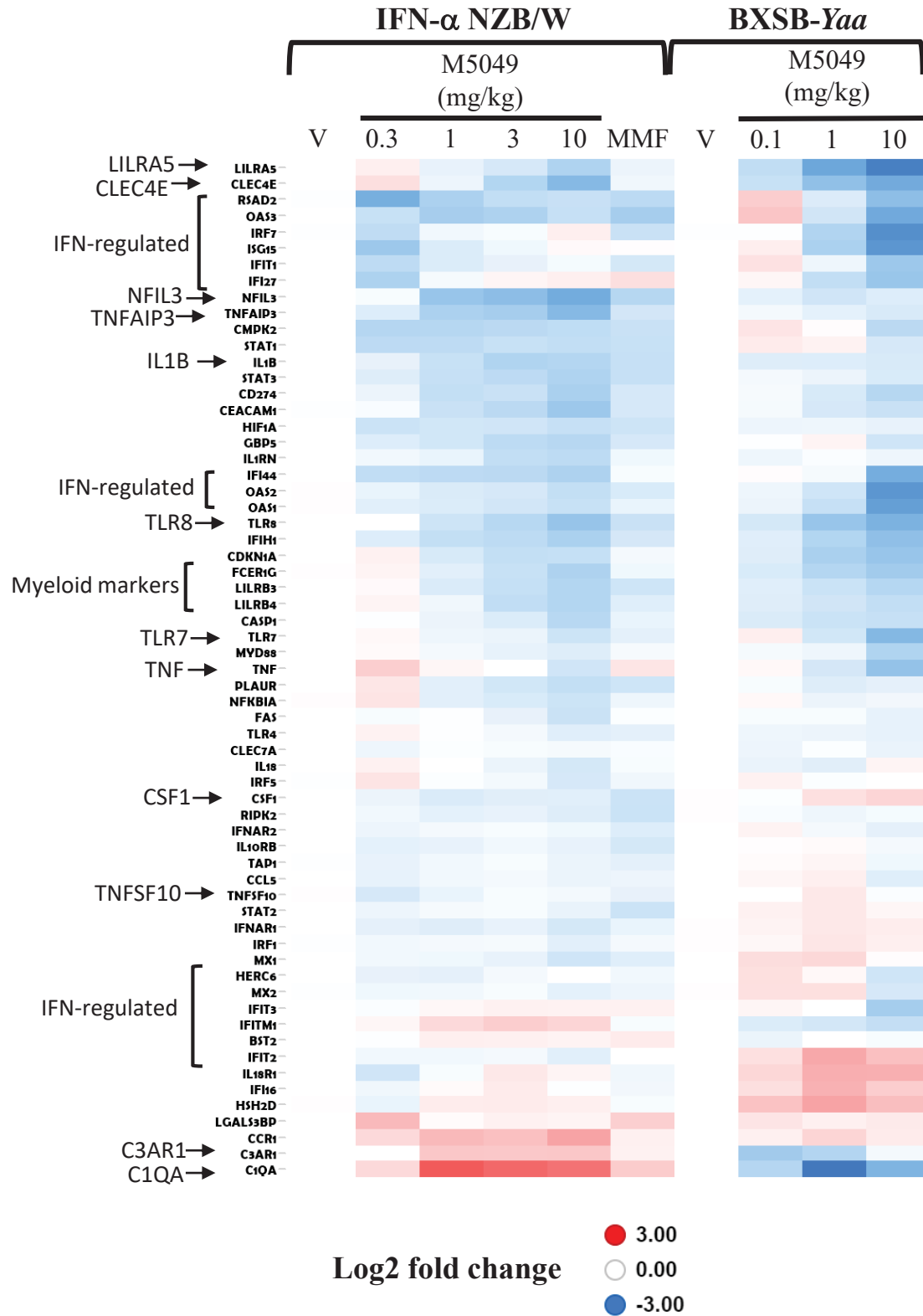


Figure 6

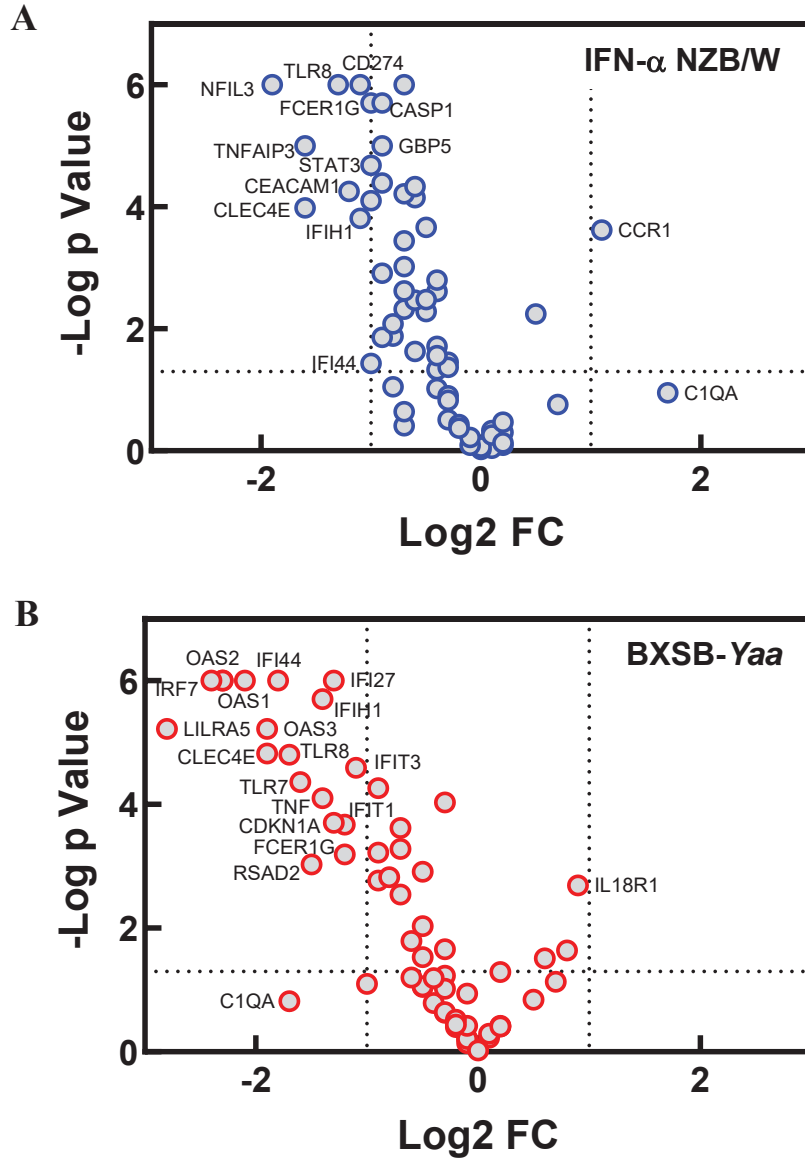
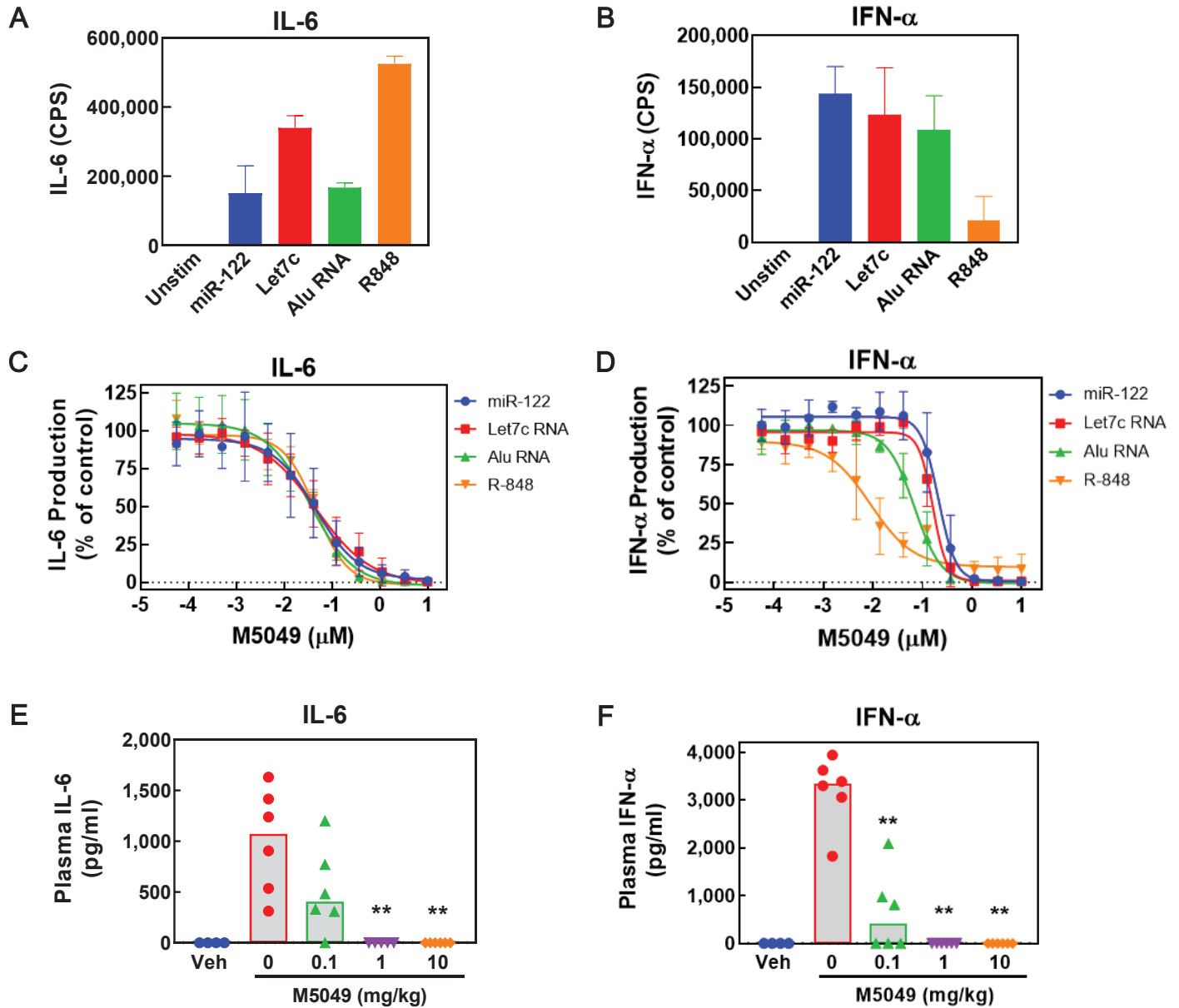


Figure 7



Article Title: Discovery of M5049: A Novel Selective TLR7/8 Inhibitor for Treatment of Autoimmunity

Authors: Jaromir Vlach, Andrew T. Bender (co-first authors), Melinda Przetak, Albertina Pereira, Aditee Deshpande, Theresa Johnson, Sonja Reissig, Evgeni Tzvetkov, Djordje Musil, Nouné Tahmassian Morse, Philipp Haselmayer, Simone Favre-Zimmerli, Shinji L. Okitsu, Robert Walsky, Brian Sherer

Journal: Journal of Pharmacology and Experimental Therapeutics

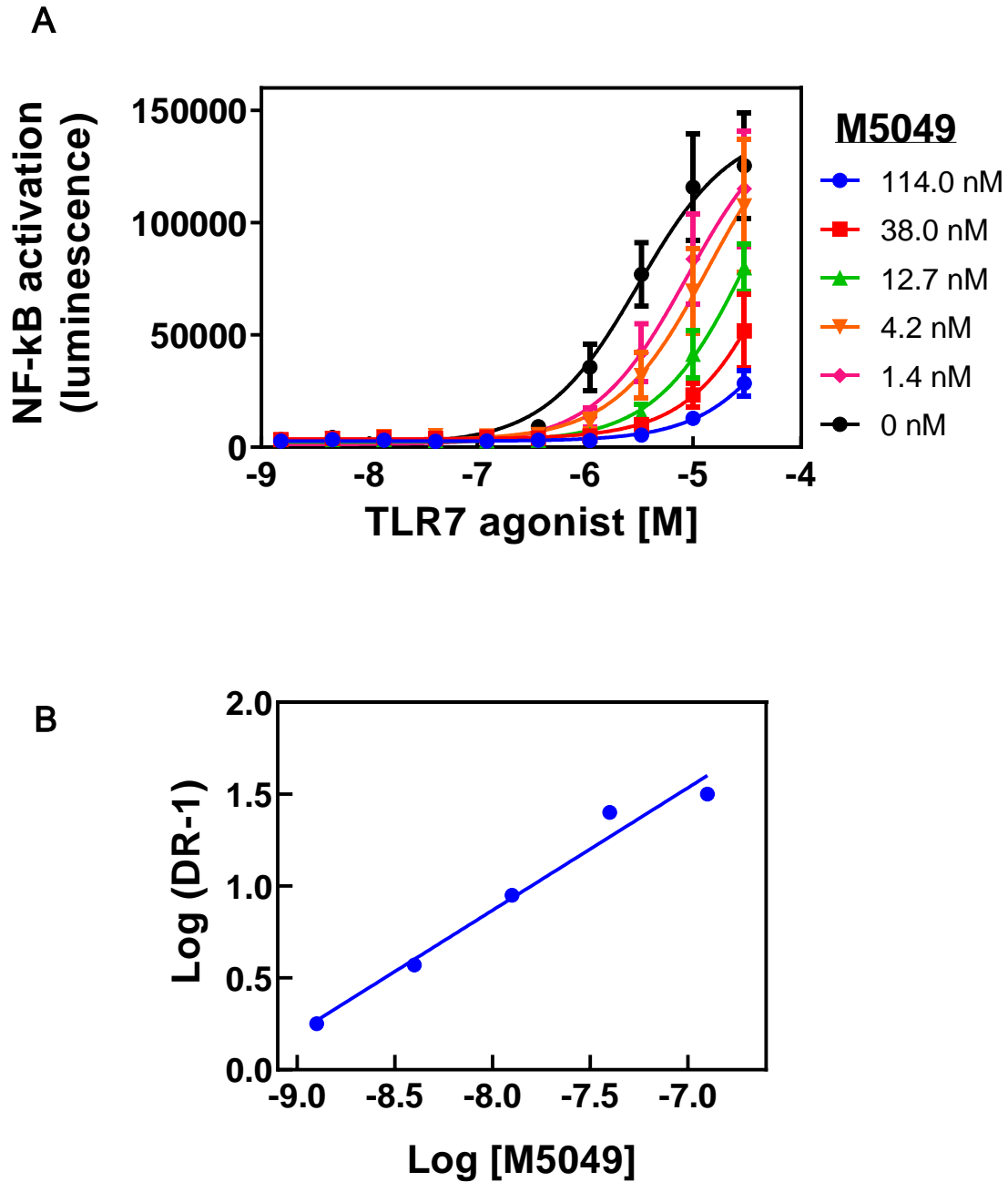
Article number : Journal JPET-AR-2020-000275R2

Supplemental Table 1
Pharmacokinetic properties of TLR7/8 inhibitors

Compound	Species	Gender	Dose (mg)	CL (IV) (L/h/kg)	t_{1/2} (h)	C_{max} (ng/mL)	V_{ss} (L/kg)	AUC_{0-24h} (ng•h/mL)	F (%)
M5049	Mouse CD1	Female	1	1.4	1.4	180	2.7	830	100
CMPD2			1	6.6	3.2	35	24	140	91
M5049	Rat Wistar	Female	1	1.2	5.0	85	8.7	670	87
CMPD2			1	1.4	14	50	23	633	86
M5049	Dog Beagle	Female	1	0.59	13	90	5.7	1400	84
CMPD2	Monkey Cynomolgus	Male	1	0.91	10	340	9.2	1100	97

Supplemental Figure 1

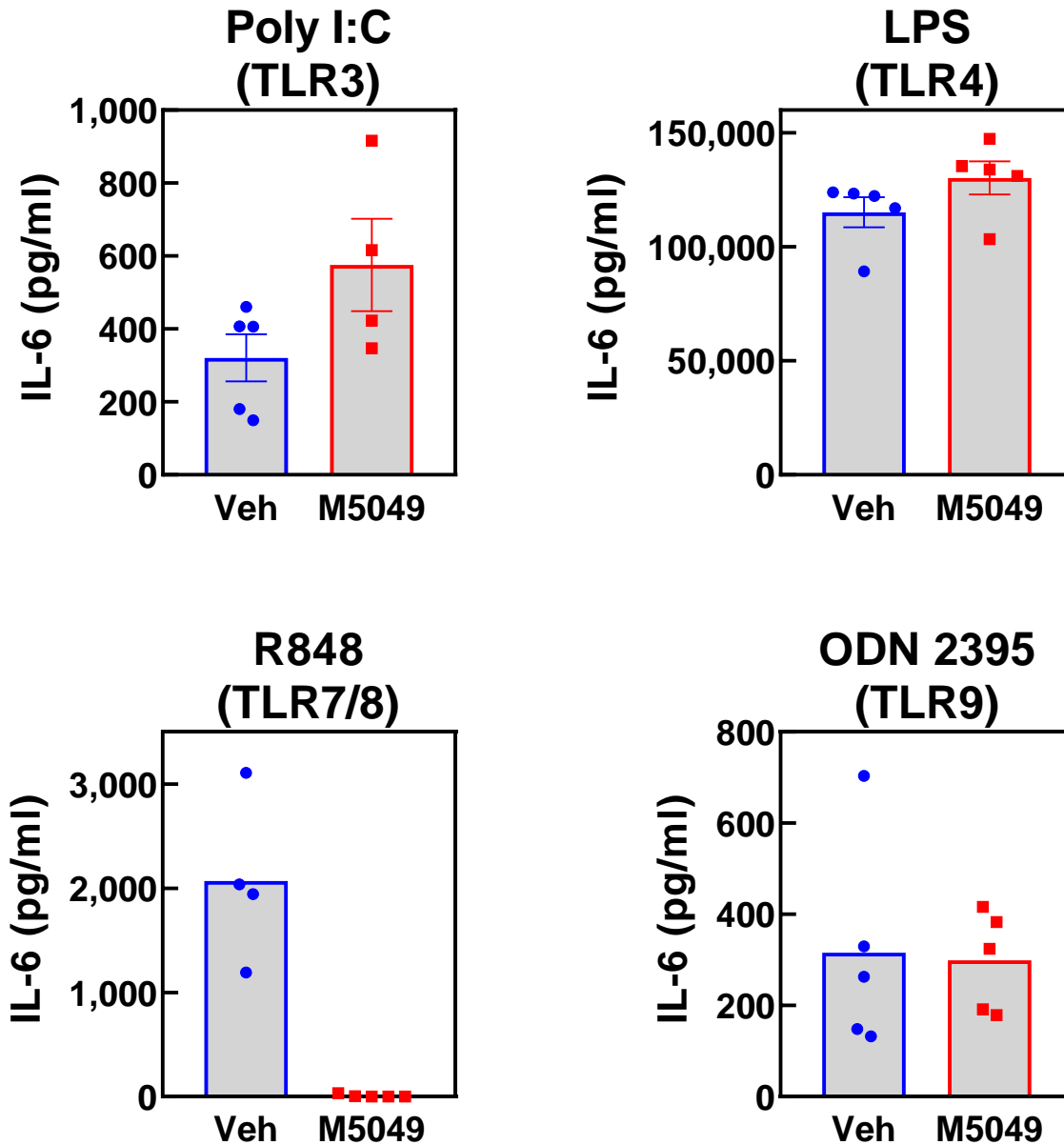
M5049 is a competitive inhibitor of TLR7 and TLR8



M5049 is a competitive TLR7 inhibitor. HEK-NF-kB-luciferase reporter cells transfected with human TLR7 were stimulated with a TLR7 agonist in a dose response in the presence of increasing concentrations of M5049. The EC_{50} was determined for the TLR7 agonist in the presence of the different concentrations of M5049 and this data was used to construct a Schild plot. The straight line fit in the Schild plot suggests that M5049 is a reversible competitive inhibitor.

Supplemental Figure 2

In vivo M5049 selectivity



Selectivity of M5049 in vivo. To test the selectivity of M5049 in vivo the inhibitory activity of the compound against different TLRs was compared. Mice were dosed via oral gavage with M5049 (10 mg/kg) and 0.5 hours later were dosed i.p. with different TLR agonists: 200 µg/mouse poly I:C (TLR3), 20 µg/mouse LPS (TLR4), 25 µg/mouse R848 (TLR7/8), or 25 µg/mouse ODN 2395 (TLR9). After 2-hour, blood samples were collected and plasma concentrations of IL-6 were measured.

Supplemental Figure 3

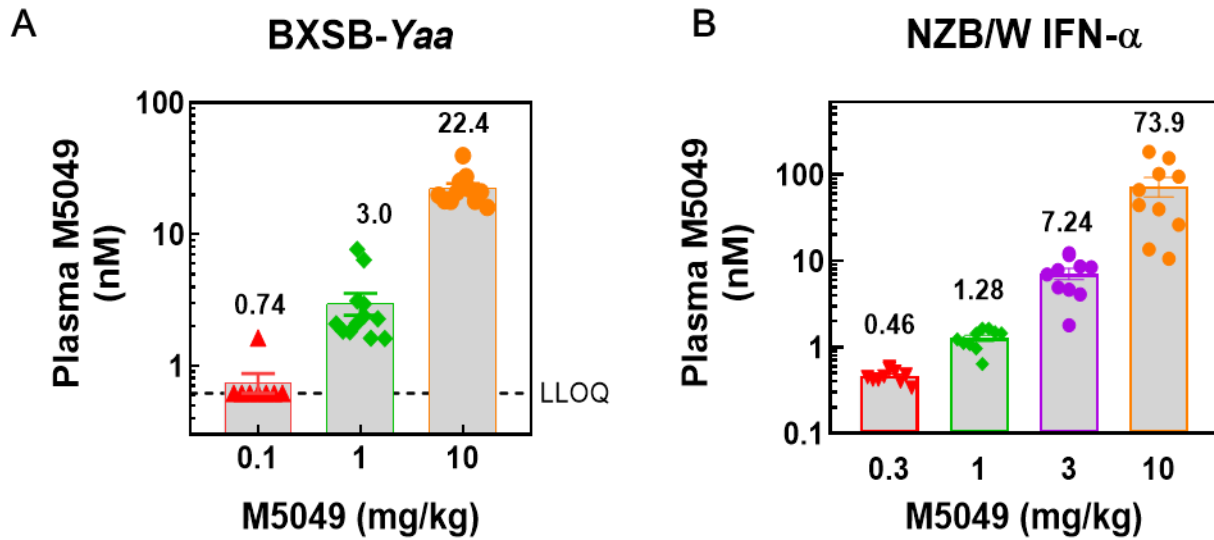
BioMap profiling of M5049



BioMap Profiling of M5049. M5049 was profiled in the BioMap (Discover X – Eurofins) Diversity PLUS Panel to assess its activity in a variety of human primary cell co-culture systems. M5049 was tested at concentrations ranging from 10 nM to 10 μ M and showed little activity.

Supplemental Figure 4

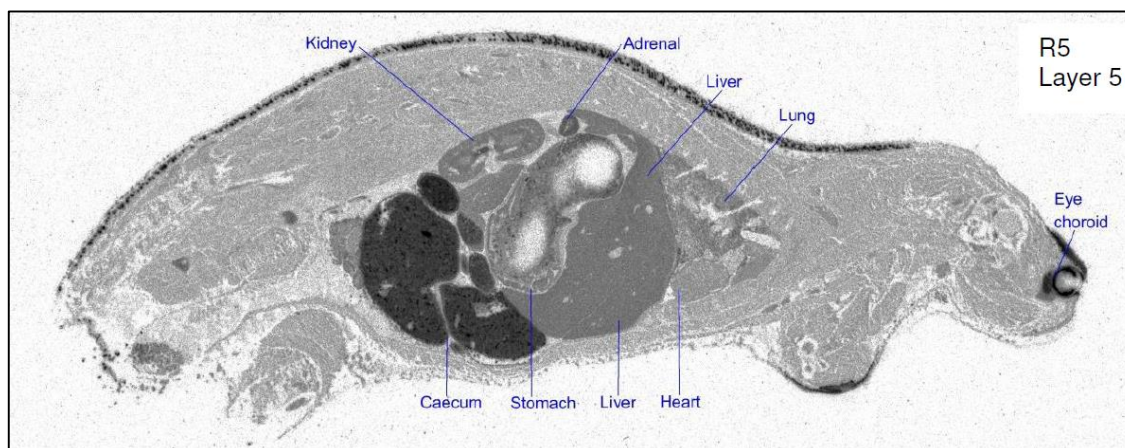
24 hr plasma concentrations of M5049 in NZB/W and BXSB-*Yaa* mice



24 hr plasma concentrations of M5049 in NZB/W IFN- α and BXSB-*Yaa* mice. Concentrations of M5049 in the plasma of lupus mice at 24 hr after the last dose were measured by LC-MS. Numbers above the bars represent the group means.

Supplemental Figure 5

Quantitative Whole-Body Autoradiography of rats dosed with ^{14}C -M5049

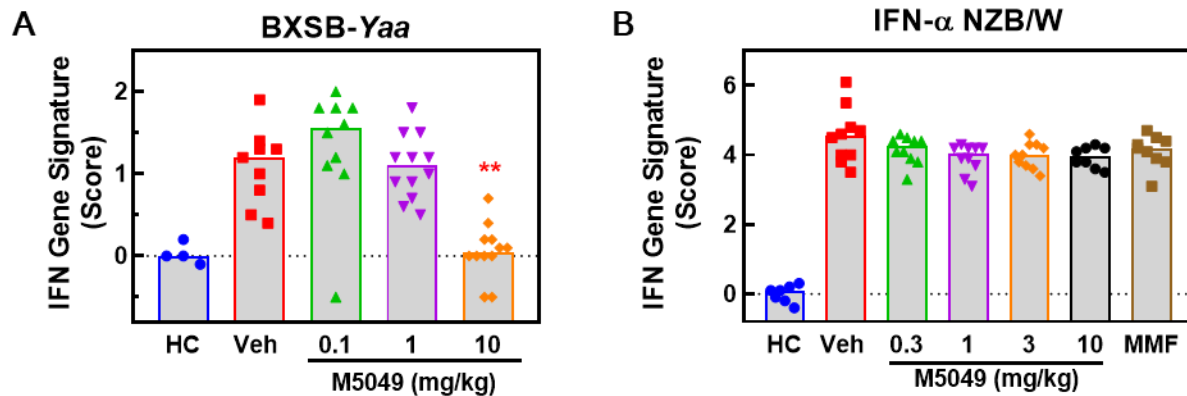


Tissue	t_{\max} (hr)	C_{\max} (nmol-eq/g)	AUC (hr-nmol-eq/g)	C_{\max} ratio	AUC ratio	$t^{1/2}$ (hr)
Blood (QWBA)	2	29.1	475	1.0	1.0	41.5
Skin high pigmented	8	118	8520	4.0	17.9	23.4
Pituitary gland	2	198	5490	6.8	11.6	20.7
Spleen	0.5	250	3040	8.6	6.4	27.4
Intestinal wall (small intestine)	8	152	2990	5.2	6.3	11.4
Lung	4	252	2930	8.6	6.2	26.0
Liver	0.5	268	2920	9.2	6.1	27.3
Thyroid gland	8	157	2820	5.4	5.9	34.6
Stomach glandular	2	293	2610	10.1	5.5	39.8
Kidney (total)	2	142	2420	4.9	5.1	29.9
Intestinal wall (colon)	4	122	2230	4.2	4.7	34.0
Pancreas	2	140	2050	4.8	4.3	28.1
Bone marrow	4	127	1970	4.4	4.1	24.1
Lymph nodes submandibular	2	102	1620	3.5	3.4	26.4
Thymus	2	107	1610	3.7	3.4	14.4
Heart	2	82.3	1200	2.8	2.5	24.5
Stomach non glandular	2	135	989	4.6	2.1	30.2
Skin low pigmented	4	42.1	949	1.4	2.0	37.7
Fat brown	2	56.6	911	1.9	1.9	30.7
Muscle	2	66	912	2.3	1.9	13.6
Skin subcutis	4	44.3	731	1.5	1.5	40.0
Brain	2	35.6	529	1.2	1.1	8.5
Fat white	2	7.88	144	0.3	0.3	12.6

Quantitative Whole-Body Autoradiography of rats dosed with ^{14}C -M5049. Rats were given a single 30 mg/kg dose of ^{14}C -M5049 and at 0.5, 2, 4, 8, 24, 48, 72, 120, and 168 hours after dosing concentrations of the compound were measured in the plasma or different tissues. One animal per timepoint was used and PK properties were extrapolated from the time course curves.

Supplemental Figure 6

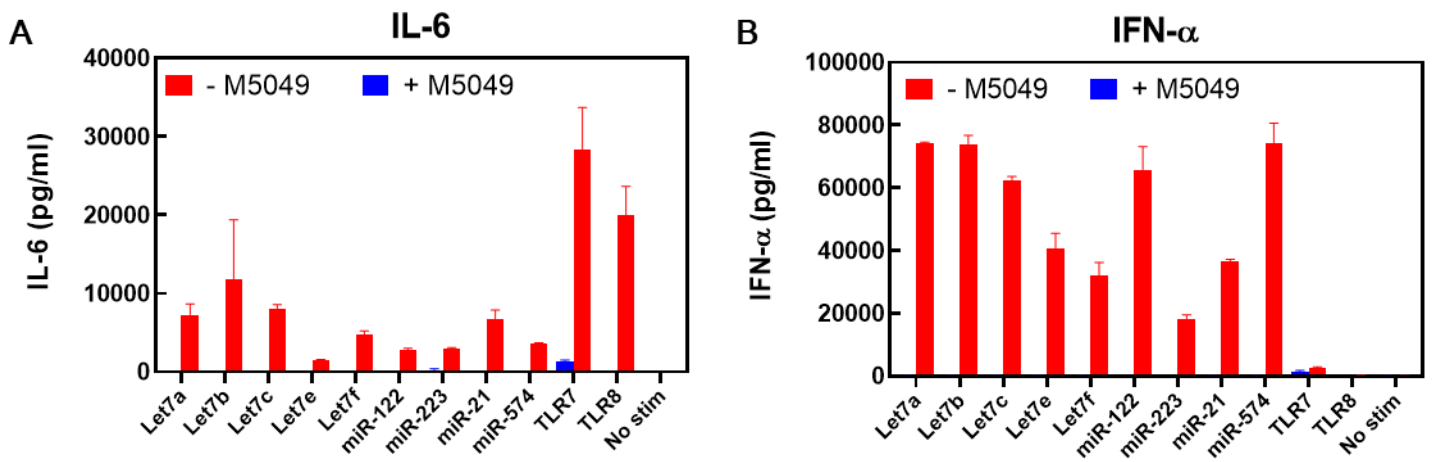
Effect of TLR7/8 inhibitors on the IFN gene signature in lupus mouse models



Comparison of M5049 effects on the IFN gene signature in mouse lupus models. At the end of each study blood samples were collected and RNA was extracted. Gene expression analysis was performed using NanoString and a panel of 17 IFN-regulated genes was used to calculate an IFN gene signature score for each mouse. The gene signature score calculation was performed relative to a healthy control group of DBA/1 mice for all studies. Results are plotted as group medians and statistical significance was assessed by ANOVA (** $p < 0.01$) and the results are plotted with bars showing the group medians.

Supplemental Figure 7

Various miRNA stimulate PBMCs and are inhibited by M5049



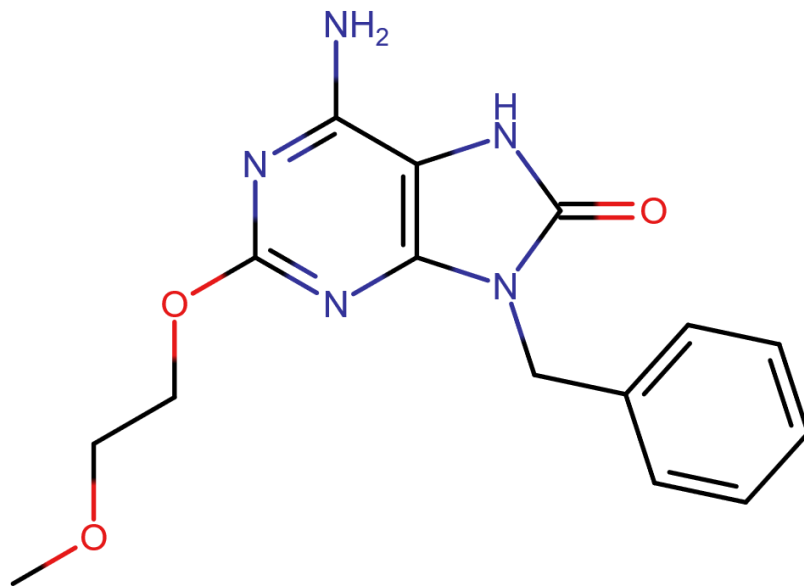
Various miRNA stimulate PBMCs and are inhibited by M5049. PBMCs were treated +/- 10 μ M M5049 for 30 min and were then stimulated with various miRNA at 10 μ g/ml. After an overnight incubation IL-6 (A) and IFN- α (B) release in the supernatants was measured by AlphaLISA. Results are presented as means \pm SD for 2 donors tested in 2 separate experiments.

Supplemental Figure 8

Structure of TLR7 and TLR8 specific agonists

TLR7 agonist CL-087

A



TLR8 agonist (similar to motolimod)

B

