Selective Inhibition of the Mitochondrial Permeability Transition Pore Protects against Neurodegeneration in Experimental Multiple Sclerosis

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The mitochondrial permeability transition pore is a recognized drug target for neurodegenerative conditions such as multiple sclerosis and for ischemia-reperfusion injury in the brain and heart. The peptidylprolyl isomerase, cyclophilin D (CypD, PPIF), is a positive regulator of the pore, and genetic downregulation or knock-out improves outcomes in disease models. Current inhibitors of peptidylprolyl isomerases show no selectivity between the tightly conserved cyclophilin paralogs and exhibit significant off-target effects, immunosuppression, and toxicity. We therefore designed and synthesized a new mitochondrially targeted CypD inhibitor, JW47, using a quinolinium cation tethered to cyclosporine. X-ray analysis was used to validate the design concept, and biological evaluation revealed selective cellular inhibition of CypD and the permeability transition pore with reduced cellular toxicity compared with cyclosporine. In an experimental autoimmune encephalomyelitis disease model of neurodegeneration in multiple sclerosis, JW47 demonstrated significant protection of axons and improved motor assessments with minimal immunosuppression. These findings suggest that selective CypD inhibition may represent a viable therapeutic strategy for MS and identify quinolinium as a mitochondrial targeting group for in vivo use.

A considerable body of evidence points to a role for the mitochondrial permeability transition (PT) pore in neurodegenerative and ischemic cell death. The peptidylprolyl cis-trans-isomerase cyclophilin D (CypD, PPIF), which is genomically expressed and imported into mitochondria, is consistently implicated as a key player in the sequence of events leading to PT pore opening and eventual cell death by necrosis. The PT pore forms under conditions of oxidative stress, low adenine nucleotide concentrations, and mitochondrial Ca2+ overload and results in free passage of low molecular mass solutes (<1500 Da) and some proteins across the inner mitochondrial membrane. Under these conditions, mitochondrial proton gradient and membrane potential (ψm) are dissipated, leading to ATP hydrolysis by the reversal of the F1F0-ATP synthase and consequent cellular energy depletion, resulting in cell death. Recent studies point to the F1F0-ATP synthase of mitochondria as being the major component of the PT pore (1), but the subunits involved and the exact pore forming mechanism are controversial (2–4). CypD binds to the lateral stalk of the F1F0-ATPase and positively regulates pore opening (5, 6). In CypD knock-out animals, the pore is desensitized to Ca2+, in an inorganic phosphate (P) dependent manner (7). There is mounting evidence that CypD is key in mediating Ca2+-induced pore opening, and its absence (e.g. in PPIF knock-out animals) desensitizes the pore to Ca2+, in an inorganic phosphate (P) dependent manner (7). Pharmacological inhibition of the pore offers a route to cyto- and neuroprotection.

Multiple sclerosis (MS) is an immunomediated demyelinating and neurodegenerative disease of the central nervous system and the commonest form of non-traumatic disability in young adults (8). Although relapsing autoimmunity in MS can

The abbreviations used are: PT, permeability transition; CRC, calcium reten-

tion capacity; CypD, cyclophilin D; CypA, cyclophilin A; CsA, cyclosporin A; EAE, experimental autoimmune encephalomyelitis; FP, fluorescence polarization; MS, multiple sclerosis; TPP+, triphenylphosphonium; ANOVA, analysis of variance; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MOG, myelin oligodendrocyte glycoprotein; oxazolone, 4-ethoxyacetyl-2-phenyl-2-oxazolin-5-one; SCH, spinal cord homogenate; Sar, sarcosine; Bmt, (4R)-4-[(E)-2-butenyl]-4-methyl-1-threonine.
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be controlled by peripheral immunomodulatory agents, progressive disability that results from neurodegeneration is, so far, untreatable (8, 9). Neurodegeneration in MS is associated with the influence of centrally active inflammatory responses (10, 11). This may relate to metabolic and energy stresses in nerves within the inflammatory penumbra that drive nerve loss during neuroinflammation in MS and other neurodegenerative diseases (12–14). Mitochondrial dysfunction and the irreversible opening of the PT pore are now recognized as a key players in the degeneration of axons (15). In MS lesions (12, 16, 17), the PT pore-induced ATP deficit may result in the inactivation of energy-dependent sodium/potassium pumps, leading to sodium loading and the reversal of the sodium–calcium exchanger that causes toxic accumulation of calcium ions and the induction of cell death effector pathways (16, 18).

CypD is highly expressed in a subset of astrocytes, microglia, and neurons (19), where it may contribute to excitotoxicity and cell death in MS lesions (12, 16, 17). CypD knock-out mice show a less severe phenotype compared with wild type in the experimental autoimmune encephalomyelitis (EAE) model of MS (20, 21). CypD knock-out mouse studies in models of traumatic brain injury (22, 23), Alzheimer disease (24, 25), Parkinson disease (26), amyloid lateral sclerosis (27), and Huntington disease (28, 29), all show a benefit compared with wild type mice. The PT pore is also implicated in ischemia–reperfusion injury in the adult brain (30) and in the heart, where CypD ablation or RNAi knockdown (31, 32) provides cardio-protection (33, 34). A selective inhibitor of PT pore opening could therefore have therapeutic applicability in a range of diseases, particularly MS, where the progressive disability that results from neurodegeneration is so far untreatable (8, 9).

Cyclosporine (cyclosporin A (CsA); Fig. 1A) is a non-selective cyclophilin inhibitor. CsA forms a ternary complex with the cytoplasmic CypA and calcineurin, leading to inhibition of calcineurin signaling. This blocks downstream cytokine production associated with immune cell activation (35, 36) and makes CsA a potent and clinically useful immunosuppressive. Chemical modification to remove calcineurin binding is relatively straightforward, but selectivity for the different cyclophilin proteins is difficult due to their close structural and sequence similarity (37). Neuroprotective actions of CsA via action on mitochondrial CypD are reported; case studies and clinical trials support a neuroprotective effect (38, 39), whereas clinical studies with CsA in traumatic brain injury are in progress (NCT01825044). In vitro CsA shows cytotoxicity and multiple effects on cell health parameters, whereas problems with the clinical use of CsA are nephrotoxicity (35, 39), bilirubinemia, and liver toxicity (40), which can require withdrawal of the drug. These properties combine to make CsA a less than ideal drug candidate for neuroprotection.

A potential solution to the problem of targeting the individual cyclophilins is to enable mitochondrial localization. Triphenylphosphonium (TPP+) is the archetypal mitochondrial targeting, lipophilic cation (41) and has been used in humans for targeting a coenzyme Q analogue to mitochondria (42). TPP+ has non-ideal pharmaceutical properties however, including (a) high molecular weight and lipophilicity, contributing to a lack of “drug likeness”; (b) mitochondrial toxicity (43, 44); (c) effects on respiration (45); and (d) non-ideal biodistribution (46). We have previously shown that TPP+ can be linked to CsA at the [Sar+] position to provide a molecule with immunosuppression blocked and improved cytoprotection in vitro (36, 47).

Here we investigated the quinolinium cation as a replacement for triphenylphosphonium. We observed that quinolinium is an effective mitochondrial targeting group: a prototype molecule, JW47, was shown to be more potent at blocking the PT pore and demonstrated less cell toxicity than CsA. In vivo JW47 was less immunosuppressive than CsA and notably achieved significant neuroprotection in an EAE model of MS in mice.

Experimental Procedures

Chemistry—All commercially available solvents and reagents were used without further treatment as received unless otherwise noted. NMR spectra were measured with a Bruker DRX 500- or 600-MHz spectrometer; chemical shifts are expressed in ppm relative to TMS as an internal standard, and coupling constants (J) are reported in Hz. LC-MS spectra were obtained using a Waters ZQ2000 single quadrupole mass spectrometer with electrospray ionization, using an analytical C4 column (Phenomenex Gemini; 50 × 3.6 mm, 5 μm) and an AB gradient of 50–95% B at a flow rate of 1 ml/min, where eluent A was 0.1:5:95 formic acid/methanol/water, and eluent B was 0.1:5:95 formic acid/water/methanol. High resolution mass spectra were acquired on a Waters LCT time-of-flight mass spectrometer with electrospray ionization or chemical ionization.

Preparation of JW47, 1-(Pent-4-en-1-yl)quinolinium—To a solution of quinoline (1 g, 7.74 mmol) in EtOAc was added 5-bromo-pent-1-ene (1.27 g, 8.51 mmol), and this mixture was heated to reflux overnight. The mixture was allowed to cool before concentration under reduced pressure. The product was isolated as a light brown oil (1.54 g, 99%).

LC-MS (m/z): [MH]+ calc. for C14H16N, 174.11; found 174.07. NMR δ1H(acetone- d6, 600 MHz): δ 10.26 (dd, J = 5.8, 1.4 Hz, 1H), 9.41 (d, J = 8.4 Hz, 1H), 8.80 (d, J = 9.0 Hz, 1H), 8.58 (dd, J = 8.2, 1.3 Hz, 1H), 8.36 (dd, J = 8.3, 1.5 Hz, 1H), 8.27 (dd, J = 8.3, 5.8 Hz, 1H), 8.13–8.08 (m, 1H), 5.90 (dd, J = 17.0, 10.3 Hz, 1H), 5.49–5.42 (m, 2H), 5.09 (ddd, J = 17.1, 3.4, 1.6 Hz, 1H), 1H), 5.01–4.96 (m, 1H), 2.41–2.35 (m, 2H), 2.34–2.26 (m, 2H).

Preparation of JW47, 1-(Pent-4-enyl)quinolinium—A solution of cyclosporin A (75 mg, 0.06 mmol) in DCM (2 ml) was added 1-(pent-4-en-1-yl)quinolinium (23 mg, 0.072 mmol) and Hoveyda–Grubbs second generation catalyst (7 mg, 0.01 mmol, 17 mol %). The reaction was stirred in the microwave at 90 °C for 30 min and then allowed to cool. Triethylamine was added to the mixture and then stirred overnight with excess P(CH2OH)3 to coordinate the ruthenium catalyst. This was then washed away with brine and water before the mixture was passed through a Stratospheres PL Thiol MP SPE cartridge (polymer laboratory, Varian Inc.) to remove any remaining catalyst. The crude product was purified by flash reverse-phase chromatography (MeOH/H2O/formic acid) to give JW47 as a brown solid (15 mg, 17%). HRMS (m/z): [MH]+ calc. for C30H115N11O12, 1357.92; found 1357.95. NMR δ1H (CDCl3, 600 MHz): δ 3.49 (s, NMe, 3H), 3.40 (s, NMe, 3H), 3.35 (s, NMe, 3H).
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3H), 3.20 (s, NMe, 3H), 3.12 (s, NMe, 3H), 3.08 (s, NMe, 3H), 2.71 (s, NMe, 3H), 2.68 (s, NMe, 3H).

**NMR δC (CDCl3, 150 MHz):** 39.35, 39.19, 33.67, 31.25, 30.10, 30.01, 29.79 (7 × N-Me).

**Protein Expression**—The CypD expression system was constructed as described by Schlatter et al. (48). Briefly, codon-optimized DNA encoding the CypD-K133I gene was cloned into pET11a (Novogen) digested with NdeI and BamHI. The resulting plasmid was used to transform BL21(DE3)pLysS medium, at A_{600} of 0.6, and the cells were induced with 1 mM isopropyl-1-thiogalactopyranoside. The cultures were then incubated at the same temperature for another 4 h, and the cells were harvested by centrifugation. The cell pellet was resuspended in lysis buffer (100 mM Tris/HCl, pH 7.8, 2 mM EDTA, 0.02% sodium azide) at 4 °C.

**Crystallization**—Crystals were grown using the hanging drop method as described previously (50); drops contained a 50:50 mix of protein solution (30 mg/ml CypD in gel filtration buffer) and reservoir solution. The best crystals were obtained with a reservoir solution consisting of 23% polyethylene glycol (PEG 3350), 50 mM sodium citrate buffer at pH 2.9 as precipitant solution. Prior to data collection, the crystals were cooled to 100 K using 35% PEG 3350 in sodium citrate buffer at pH 2.9 as the cryoprotectant. Data were collected on beamline i04 at Diamond Light Source. Phases were determined by the molecular replacement method using PHASER (51) from the CCP4 suite of programs (52); the CypD-cyclosporin A structure (Protein Data Bank code 2Z6W) was used as the search model but with the cyclosporin A removed from the model. The model was refined using cycles of the program REFMAC (53, 54) (with anisotropic B-factors and Babinet bulk solvent modeling), interspersed with manual checks and model building with COOT (55). The final resolution cut-off was determined during refinement. Ligand topologies were generated with PRODRG2 (56). The final model was validated with PROCHECK (57) and SFCHECK (54) and finally MOLPROBITY (58). Refinement statistics are presented in Table 1. The illustrations were produced with the PyMOL molecular graphics system (Shrodinger LLC, New York).

**Fluorescence Polarization Assay**—Fluorescence polarization (FP) is inversely related to the molecular rotation of the fluorophore. Fluorophores linked to small molecules tumble faster and emit less polarized fluorescence than fluorophore-small molecules bound to proteins. FP is a convenient technique to measure affinity of ligands to cyclophilins (59, 60). We synthesized a new fluorescein-PEG-CsA ligand as the probe (see above), and we determined the binding of our novel ligands to CypD and CypA.

Titration of a single probe concentration against different enzyme concentrations was used to determine the dissociation constant (K_d). From this we also determined the enzyme concentration that would give a high enough polarization signal to measure binding affinities. The inhibitor constants (K_i) were calculated with Equation 1 (61),

$$K_i = \frac{[I]^0}{[L]^0} \frac{[P]^0}{K_d + [P]^0 + 1} \quad \text{(Eq. 1)}$$

where [I]^0 is the concentration of the unlabeled compound at 50% inhibition, [L]^0 is the concentration of the free probe-cyclophilin enzyme at 50% inhibition, [P]^0 is the concentration of the free protein at 0% inhibition, and K_d is the dissociation constant of the probe-protein complex.

**Mitochondrial Isolation**—Subcellular fractionation was performed as described previously (62). Briefly, C57BL/6J WT or CypD−/− (63) male mice of 3–6 months were sacrificed by cervical dislocation, and their liver was removed and placed immediately into ice-cold isolation buffer (250 mM mannitol, 5 mM HEPES, 0.5 mM EGTA, pH 7.4). At 4 °C, the liver was rinsed in PBS to remove excess blood, and any fat and connective tissue was eliminated. PBS was then replaced with isolation buffer containing 1 mM PMSF, and the liver was chopped into pieces (~2 mm in length). Tissue was then homogenized in this solution until no solid matter remained and then centrifuged at 800 × g for 10 min at 4 °C. The nuclear pellet was then discarded, and the postnuclear supernatant was retained and centrifuged at 10,300 × g for another 10 min at 4 °C. The postmitochondrial supernatant was discarded, and the mitochondrial pellet was resuspended in isolation buffer and PMSF and kept on ice. Protein levels were quantified using a Thermo Scientific BCA protein quantification assay, as per the manufacturer’s instructions.

**Calcium Retention Capacity Assay**—Isolated mitochondria were resuspended (500 μg of protein/ml) in MSK buffer (75 mM mannitol, 25 mM sucrose, 5 mM potassium phosphate monobasic, 20 mM Tris-HCl, 100 mM KCl, and 0.1% bovine serum albumin, pH 7.4) supplemented with 10 mM succinate, 1 mM rotenone, and 1 μM Fluoro5N. 200 μl of mitochondrial suspension per well was used in 96-well microplates. Compounds were incubated for 10 min before the plate was assayed in a Fluostar Optima plate reader, using excitation/emission filters at 480/520 nm; CaCl_2 was injected approximately every 6.5 min for 80 min (12 total injections, final concentration of 75 μM). To cal-

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calculate percentage inhibition of Ca\(^{2+}\)-induced pore opening, first areas under each curve were calculated, and controls without CaCl\(_2\) addition were subtracted as background. The background-corrected values were then expressed as the fraction of controls without mitochondria, representing the total amount of Ca\(^{2+}\) added, unbuffered by mitochondria. Percentage inhibition for each [compound] was then calculated as the percentage of the corresponding value for the untreated condition. Significance was assessed by one-way ANOVA, in comparison with CsA control. For experiments with CypD\(^{-/-}\) mice, 100 \(\mu\)l of mitochondrial suspension per well was used. CaCl\(_2\) was injected approximately every 6.5 min for 135 min (20 total injections, final concentration of 266 \(\mu\)M). Data were background-corrected and expressed as the fraction of controls without mitochondria and then normalized to the wild type no drug condition. Significance was assessed by one-way ANOVA.

Respirometry—Oxygen consumption was measured using Oroboros Oxygraph-2K as described previously (62). Prior to the assay, the Oxygraph chambers were calibrated with Miro5 (at 100–200 nM mitochondrial suspension per well was used. CaCl\(_2\) was injected approximately every 6.5 min for 135 min (20 total injections, final concentration of 266 \(\mu\)M). Data were background-corrected and expressed as the fraction of controls without mitochondria and then normalized to the wild type no drug condition. Significance was assessed by one-way ANOVA.

Measurement of Mitochondrial Membrane Potential—Rat cortical neurons, cultured for 8–9 days, were incubated for 40 min at 37 °C with the cell-permeant cationic dye tetramethylrhodamine methyl ester (25 \(\mu\)mol), and fluorescence was measured using the ImageXpress Micro XL system (Molecular Devices). Fluorescence was measured for 7 min prior to the addition of DMSO, CsA, or JW47 (both at 40 \(\mu\)mol and 1 \(\mu\)mol) and then for a further 50 min before the addition of the mitochondrial uncoupler FCCP (2.5 \(\mu\)mol) as a positive control. The minimum value after the addition of compound (prior to the addition of FCCP) was taken, and this was expressed as a percentage (using the baseline as 100% and FCCP as 0%) and then normalized to DMSO (100%). Significance was assessed by one-way ANOVA, in comparison with DMSO control.

Measurement of Mitochondrial Membrane Potential (ex Vivo)—Freshly isolated mouse liver mitochondria were suspended in MSK buffer containing 10 \(\mu\)g/ml rhodamine 123 (dequench mode), at a concentration of 500 \(\mu\)g/ml, and plated in an opaque black 96-well plate. Baseline fluorescence was then measured every 60 s for 5 min in a Fluostar Optima (excitation 480 nm/emission 520 nm) before the manual addition of compounds (concentrations as specified). Fluorescence measurements were continued for 45 min until the addition of 2 \(\mu\)M FCCP, followed by a further 10 min of fluorescence readings.

ATP Production—Freshly isolated mitochondria were resuspended in MSK buffer (containing 10 mM glutamate and 2 mM malate) at 1 mg/ml and plated in opaque white 96-well plates, or for neuronal assays, neurons were used 9 days after plating at 15,000 cells/well. Drugs were added at the concentrations specified and, for mitochondrial assays, were incubated for 10 min before the addition of ADP (5 mM), followed by another 45 min. For neuronal assays, drugs were added in neurobasal medium and incubated for 60 min. Cell Titer Glo reagent was then added, and the plate was shaken for 2 min in the dark to lyse cells/mitochondria and release ATP. The plates were incubated a further 10 min, and then luminescence values were read using an Optima Fluostar. ATP production data were normalized to DMSO control, and significance was assessed by one-way ANOVA.

Cytotoxicity in HepG2 Cells—HepG2 cells were seeded in black, clear-bottom 96-well tissue culture plates at a density of 3000 cells/well. The cells were incubated for 24 h in culture medium and then exposed (in three replicates) to increasing doses of test compound or to vehicle control (0.5% DMSO). The cells were exposed for 72 h before running the high content screening assays. The high content screening assay was multiplexed to determine mitochondrial membrane potential and mitochondrial mass using MitoTracker® (Life Technologies), cytochrome c release (antibody, Abcam), and membrane permeability (YO-PRO-3M,1, Life Technologies). Cell count, nuclear size, and DNA structure were also measured (Hoechst 33342, Life Technologies). Following staining of the HepG2 cells, fluorescence was analyzed by image acquisition with a Thermo Fisher Cellomics® ArrayScan VTI High Content Screening Reader (Thermo Fisher Scientific) and vHCS™View software (Thermo Fisher Scientific). 20 fields were imaged per well using a \(\times 10\) wide field objective. The image acquisition data were normalized to vehicle control values. Dose-response curves were defined and evaluated with the following equations,

\[ \xi(C; c; \omega) = \left( \ln(C) - c \right) / \omega; \]  

\[ t(\xi) = (1 + \tanh(\xi)/2; \]  

\[ R(t; R_0; R_\infty) = R_0(1 - t) + R_\infty t; \]

in which \( C \) represents the test compound concentration, and \( R_0, R_\infty, c, \) and \( \omega \) are fitting parameters. The final response at a given concentration \( C \) is expressed as \( R(t; \xi(C; c; \omega));R_0;R_\infty \). It was restricted such that \( \omega > 0 \), which implies \( R \to R_0 \) as \( C \to 0 \) and \( R \to R_\infty \) as \( C \to \infty \). The coefficient of determination \( R^2 \) was calculated for each compound and dose-response curve. An \( R^2 \) value of \( >0.65 \) was used as quality control criteria and was required in all response curves.

Cell-based Assay for CypA Activity—VSV-G pseudotyped GFP-encoding HIV-1 vector was prepared by triple plasmid transfection of 293T cells with Fugene 6 (Roche Applied Science) as follows. Confluent 293T cells in a 10-cm dish were transfected with a mixture of 10 \(\mu\)l of Fugene-6 in 200 \(\mu\)l of
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Opti-MEM (Gibco) with 1 µg of pMDG VSV-G expression vector (64), 1 µg of p8.91 HIV-1 gag-pol expression vector (65), and 1.5 µg of lentiviral expression vector encoding enhanced GFP protein, CSGW (66). Viral supernatant was collected 48 h post-transfection and stored at −80°C.

To generate CRFK cells stably expressing N-terminally HA-tagged TRIM-CypA from an EXN-based vector, murine leukemia virus (MLV) vector was prepared as above, using pMDG, CMVi MLV gag-pol expression vector, and γ-retroviral expression vector encoding a fusion protein comprising human CypA downstream of owl monkey TRIM5 RBC (EXN-TRIM-CypA) (67). CRFK cells, which are null for TRIM5α activity (68), were then transduced with vector, followed by selection of cells in 1 mg/ml G418 (Invitrogen).

To test for the ability of drug to rescue HIV-1 infectivity in the presence of TRIM-CypA, CRFK cells were infected with a single dose of virus that infected around 20% of the cells, in the presence of DMSO, CsA (0.3–10 µM), or JW47 (0.6–20 µM). Infectivity was measured by flow cytometry 48 h postinfection.

P-glycoprotein Activity—Assessment of drug transporter activity was conducted using the Pgp-Glo™ assay (Promega, Madison, WI) containing recombinant human ABCB1 in membranes and according to the manufacturer's instructions. Briefly, samples were preincubated with ATP before incubation with 100 mM compound or positive control. The residual ATP was assayed by luciferin bioluminescence.

In Vitro Mitogenic T Cell Stimulation—Spleens were isolated from ABH mice, and tissue was homogenized through a cell strainer (BD Biosciences) into Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS; Gibco, Invitrogen), 2 mM l-glutamine (Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 50 µM 2-mercaptoethanol (Invitrogen). Cells were centrifuged at 500 × g for 5 min, and erythrocytes were lysed using 0.87% ammonium chloride following incubation for 5 min at 37°C. Cells were washed, and viable cells were counted using 0.87% ammonium chloride following incubation for 5 min at 37°C. Cells were washed, and viable cells were counted using trypsin blue (Sigma-Aldrich) exclusion. 4 × 10^5 cells/well were incubated in 96-well microtest U-bottom plates (Falcon BD, Oxford, UK) in a final volume of 200 µl of DMEM. Cells were incubated with either 10-fold dilutions (range 10 nM to 10 µM) of CsA (Sandoz, Basel, CH) or JW47 diluted in DMEM from a 50 mM stock in DMSO. Cells were incubated with either 5 µg/ml concanavalin A (Sigma-Aldrich) mitogen or 0.5 µg/ml mitogenotic mouse CD3 and mouse CD28-specific antibodies (Pharmingen, Oxford, UK). The cells were incubated at 37°C for 18–22 h before the addition of 1 µCi of [3H]thymidine (PerkinElmer Life Sciences)/well. After additional incubation for 16–20 h, the 96-well plates (Microtest U-bottom, Falcon BD) were harvested (Harvester 96, Mach III M, TOMTEC) onto glass fiber filters (PerkinElmer Life Sciences). After drying, a scintillation sheet (MeltiLexA, PerkinElmer Life Sciences) was melted onto the filter using a hot plate (RET Basic, IKA, Staufen, Germany). Samples were analyzed using scintillation counting (MicroBeta Plus liquid scintillation counter, PerkinElmer Life Sciences), and [3H]thymidine incorporation was assessed in at least triplicate samples.

Myelin Antigen-induced T Cell Proliferation—ABH mice were injected subcutaneously in the flank with 100 µg of myelin oligodendrocyte glycoprotein (MOG) peptide residues 35–55 (Cambridge Research Biochemicals Ltd., Billingham, UK) emulsified in Freund's adjuvant containing 200 µg of Mycobacterium tuberculosis H37Ra (Difco, Becton Dickinson, Oxford, UK) on days 0 and 7 (69). Spleens were collected and prepared and analyzed as above except that mitogens were replaced with 5 µg/ml MOG 35–55 peptide, and cells were incubated for 72 h before the addition of tritiated thymidine.

Pharmacokinetic Analysis—ABH mice (n = 4) were injected intraperitoneally with 0.1 ml of 10 mg/kg JW47. Animals were killed 2 and 4 h later with a CO₂ overdose, and blood was immediately collected from the heart following death. Blood was then added to Microtainer (BD Biosciences) tubes and centrifuged using an Eppendorf microcentrifuge, and plasma was collected. Following the removal of blood, the brain was rapidly (<30 s) dissected from the skull and stored at −80°C prior to analysis by a contract research organization using liquid crystal mass spectroscopy.

In Vivo T Cell Proliferation—The contact sensitizer 4-ethoxyxylene-2-phenyl-2-oxazolin-5-one (oxazolone; Sigma) was dissolved (25 mg/ml) in 4:1 acetone/olive oil. Mice (n = 3/group) received epicutaneous application of either 25 µl of 2.5% oxazolone or acetone/olive oil on the dorsum of the ear on day 0 (70). The draining auricular lymph nodes were removed 3 days later, and the induced proliferative response was assessed as described previously. Briefly, 5 × 10^5 cells/well were cultured in RPMI 1640 medium with glutamate (Gibco®, Invitrogen Ltd., Paisley UK), supplemented with 0.5 mM sodium, in round-bottomed 96-well plates overnight at 37°C in a humidified atmosphere of 5% CO₂ in the presence of 1 µCi of [3H]thymidine (PerkinElmer Life Sciences) per well. DNA synthesis was estimated using β-scintillation counting as above. Animals received daily intraperitoneal injections of either vehicle or JW47 from day 0 to day 3 (14, 70). Results are expressed as mean ± S.E. thymidine incorporation counts/min.

Induction of Relapsing-Progressive EAE—Mice were injected subcutaneously with 1 mg of freeze-dried mouse spinal cord homogenate (SCH) in Freund's adjuvant on days 0 and 7 as described previously (71). After the initial paralytic disease and subsequent remission, a relapse was induced by a further injection of SCH in Freund's incomplete adjuvant on day 28 to induce a relapse 7 days later (71). Studies were randomized, blinded, and powered as described previously (71). Neurological scores were graded as 0 = normal, 1 = limp tail, 2 = impaired righting reflex, 3 = hind limb paresis, 4 = complete hind limb paralysis, and 5 = moribund/death (71). Results are expressed as mean ± S.E. maximum or minimum neurological score and mean day of onset ± S.D. The clinical scores are presented as the mean daily neurological score ± S.E. Differences in clinical scores were assessed using non-parametric, Mann-Whitney U statistics (71). Motor control and coordination were assessed on an accelerating (4–40 rpm, accelerating at 6 rpm/25 s) RotaRod (ENV-575M, Med Associates Inc., St. Albans, VT) as described previously (71). This was performed 1 day before induction of relapse and at the termination of the experiment on day 45. RotaRod assessment was performed blinded to treatment. Animals were randomized to vehicle or treatment based on their RotaRod scores. Results are expressed

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as mean ± S.E. time that animals maintained rotarod activity. Differences in rota activity and quantitative neurofilament ELISA were assessed using Student’s t test incorporating tests for equality of variance using Sigmamap (Systat Software, Inc., San Jose, CA) (71).

At the end of the experiment, the spinal cord was removed, and an ELISA for heavy chain neurofilament on spinal cord was performed. Total nerve content of each spinal cord was estimated following calibration against neurofilament protein standards as described previously (71, 72).

**Neurofilament ELISA**—Neurofilament level as a validated correlate of spinal cord axonal content as determined by histology was determined as follows. Spinal cords were collected from the spinal columns of untreated (n = 11) and JW47 (1 mg/kg)-treated (n = 13) animals at the second remission phase of disease postrelapse at day 45 after disease induction. Tissues were snap-frozen and stored at −80 °C before homogenization. Tissues were homogenized in a glass homogenizer in 1 ml/100 mg of spinal cord tissue wet weight homogenization buffer (0.2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 4 mM urea, 10 mM Tris-HCl (Sigma), pH 7.2) plus 1:100 HALT protease inhibitor mixture (Thermo Fisher Scientific) and further homogenized by sonication twice for 10 s (Cole-Parmer Instruments, Vernon Hills, IL). Samples were spun down at 13,000 rpm in a bench top centrifuge (Eppendorf), and the supernatant was collected and stored at −80 °C before neurofilament determination. Samples were thawed on ice, and an ELISA for heavy chain neurofilament was performed. Briefly, a 96-well plate was coated overnight at 4 °C with capture antibody (1:5000; SMI35 anti-neurofilament H, Cambridge Bioscience, Cambridge, UK) in coating buffer (0.15 M Na2CO3, 0.35 M NaHCO3 (Sigma), pH 9.6). Following one wash in wash buffer (150 mM NaCl, 10 mM Tris-HCl, 0.1% Tween 20 (Sigma), pH 7.5), nonspecific binding was blocked by incubation with 5% bovine serum albumin (Sigma) in wash buffer for 1 h at room temperature. Following a wash step, samples and standards (porcine neurofilament heavy chain, Chemicon International) were diluted in wash buffer with 1% bovine serum albumin and incubated on the plate for 1 h at room temperature. Following five wash steps, the detector antibody was applied (1:1000 rabbit anti-NF200, Sigma) and incubated for a further 1 h at room temperature. The plate was washed five times, and the reporter antibody was applied (1:1000 swine anti-rabbit HRP conjugate, DAKO). Following a final five washes, tetramethylbenzidine substrate (Sigma) was applied, and color production was measured on a BioTek Synergy HT plate reader at 450 nm.

The protein content of the samples was determined by a micro-BCA assay (Pierce, Thermo Fisher), and axonal neurofilament levels in each were calculated as μg of neurofilament/mg of total protein in each sample.

**SMI32/SMI35 Ratio**—A 96-well plate was coated with either SMI35 anti-phosphorylated NF-H or SMi32 anti-non-phosphorylated NF-H, which is a marker of axonal damage/dystrophy (Cambridge Bioscience) antibodies at a 1:5000 dilution as above. Due to the nature of the epitope, an absolute standard for SMi32-reactive neurofilaments was unavailable. NF-HSMI32 was therefore presented as a proportion of total neurofilament as measured by absorbance level and corrected for total protein levels in each sample.

**Study Approval**—All animal procedures were approved by the local ethical review processes and government inspectors in accordance with the UK Animals (Experimental Procedures) Act 1986, which incorporates directive 2010/63/EU. Experimental details, including randomization, powering, and blinding, to conform with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines have been reported previously (71).

**Results**

**Design and Synthesis of a Selective CypD Inhibitor**—We required a molecule with the following profile: (a) good CypD potency, (b) selectivity for CypD over other cellular cyclophilins by mitochondrial targeting, (c) significant brain levels indicating an ability to target MS lesions, (d) low immunosuppressive activity, and (e) a better cytotoxic profile than CsA. CsA is famously non-druglike in terms of small molecule parameters, such as Lipinski’s rules of five; however, the macrocyclic structure has been proposed to cancel out some of the non-druglike features, such as excessive hydrogen bonding (73). In chemical terms, the compound is an uncharged, 11-amino acid, macrocyclic peptide. It is lipophilic with a measured logP of 2.7 (74).

A ternary complex of CsA, CypA, and calcineurin is required to trigger the immunosuppressive response via inhibition of nuclear factor of activated T-cells dephosphorylation (75). Residues 3–7 are available from the Cyp-cyclosporin complex to bind calcineurin. The ternary complex is closely packed and less tolerant of changes than for Cyp binding alone. The crystal structure of SmBzCsA (Fig. 1C) reveals that substitution at the [Sar3] position does not affect cyclophilin binding but blocks calcineurin binding (76). We chose to modify CsA at the [Bmt1] side chain, where we predicted that substitution would have a similar negative effect on immunosuppression. Because the activation of calcineurin is also proposed to mediate some of the cytotoxic effects of CsA, we anticipated that selective molecules would improve the toxicity profile, whereas additional selectivity for CypD over the cytoplasmic cyclophilins would provide further gains.

The CsA binding cleft in cyclophilins is highly conserved, and mitochondrial targeting provides a convenient way to obtain selectivity for the mitochondrial CypD. A variety of compounds have been suggested to localize to mitochondria. Mostly, these are lipophilic cations, such as TPP+ but other cations are known (77). We were attracted by the possibility of utilizing the quinolinium cation as a targeting group for several reasons, including its small size and chemical simplicity. The cation is also present in dequalinium, a topical antibiotic that can also be given in vivo (78, 99). We utilized SmBzCsA as a non-immunosuppressive, non-targeted control (Fig. 1B) (36, 76). We also designed the chemical linker to be small and to be a hydrocarbon chain to minimize any effects on membrane permeability.

**Synthesis**—We employed olefin metathesis as a direct and relatively mild way to alter the natural structure of CsA. The synthesis of the [Bmt1]−linked CsA derivatives is outlined in Fig. 1A. Olefin cross-metathesis using the Grubbs-Hoyeda second
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FIGURE 1. A, synthesis of [Bmt]<sup>1</sup>- and [Sar]<sup>3</sup>-substituted analogues by olefin cross-metathesis and alkylation. Reagents were DCM, Hoveyda-Grubbs catalyst, second generation, reflux, 30 h (i) and ethyl acetate reflux (ii). B, structure of SmBzCsA. C, the crystal structure of SmBzCsA (Protein Data Bank code 4IPZ) is shown bound to CypA (solid magenta surface). The aligned structure of CypD (Protein Data Bank code 5A0E) is shown as a wire mesh. Amino acids 9 to 2 are involved in cyclophilin binding. The [Bmt]<sup>1</sup> residue (pink) side chain and the [Sar]<sup>3</sup> substituent do not affect cyclophilin binding.

FIGURE 2. A, crystal structure of JW47 in complex with CypD and its effect on Ca<sup>2+<sup>1</sup>-induced PT. Electron density of JW47 in the CypD catalytic site is shown as a wire mesh with JW47 modeled into the density as a ball and stick model (green). B, JW47 adopts two poses in the crystal structure shown in orange and magenta. The orange pose illustrates a possible stabilizing interaction with Ala-103, Pro-105, and Lys-125. The surface in green is generated from the chain that co-crystallized with this pose. Note that Lys-125 also adopts two conformations. C, fluorescence polarization assay for cyclophilin binding obtained using a fluorescein-labeled CsA probe. Typical data for CypD are shown. The concentration of the probe is 45 nM, and the enzyme concentration is 40 nM. Millipolarization values are fitted to a dose-response curve using Origin. D, fluorescence polarization data for CypA. Error bars, S.D.
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TABLE 1
Crystallographic statistics of JW47

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2_1,2_1</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>38.12</td>
</tr>
<tr>
<td>b (Å)</td>
<td>69.51</td>
</tr>
<tr>
<td>c (Å)</td>
<td>109.17</td>
</tr>
<tr>
<td>Matthews coefficient</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Data statistics

| Resolution (Å)       | 35.99-1.08 (1.11-1.08)*       |
| No. of measurements  | 1,255,737 (13,170)            |
| No. of unique reflections | 101,679 (2352)            |
| Completeness (%)     | 81.5 (24.7)                  |
| R_{work} (%)         | 0.061 (1.279)                |
| R_{free} (%)         | 0.017 (0.518)                |
| I/standard deviation | 22.0 (1.4)                   |

Refinement statistics

| R_{work} (%)         | 0.0981                       |
| R_{free} (%)         | 0.1312                       |
| No. of protein atoms | 2600                         |
| No. of water atoms   | 304                          |
| No. of ligand atoms  | 194                          |
| Mean overall B value (Å²) | 15.02                       |

Crystallographic parameters are shown in Table 1. The core of the macrocycle is almost identical to that of CsA itself, whereas the pendant quinolinium group can adopt two poses (Fig. 2B). In both poses, the position of the quinolinium group is partially stabilized by crystal contacts with a symmetry-related molecule. In one of the poses, the 1-(pنت-4-etyl)quinolinium (salmon color) extends back over the macrocycle and makes an intramolecular hydrophobic contact with [MeLeu]₈ of JW47. In the other pose (magenta) the 1-(pنت-4-etyl)quinolinium moiety extends in the opposite direction to lie along the surface of the CypD, making hydrophobic contacts with Ala-103, Gly-104, and Pro-105.

**Cyclophilin Enzyme Binding**—A fluorescence polarization assay was used to determine cyclophilin binding. CsA gives binding constants of \( K_i = 1.4 \text{ nM} \) for CypD and 22.5 nM for CypA. These affinities are broadly in agreement with data from other laboratories (59, 60). The CsA analogue, SmBzCsA, also demonstrated potency in this assay with \( K_i \) of CypD = 236 nM and \( K_i \) of CypA = 202 nM (Table 2), consistent with our data utilizing the classical chymotrypsin-based system (36). JW47 showed similar binding to SmBzCsA at \( K_i \) of CypD = 202 nM and \( K_i \) of CypA = 236 nM. Representative dose-response curves for CypD and CypA are shown (Fig. 2, C and D).

**The Quinolinium Group Localizes Fluorescein to Mitochondria**—To confirm that quinolinium cation can localize cargo to mitochondria, we took a membrane-impermeant dye, carboxyfluorescein, and conjugated it to quinolinium using an alkyl linker (see supplemental Methods). The linked dye diffused into cells (although some remained extracellularly) and co-localized with tetramethylrhodamine methyl ester, demonstrating the mitochondriotropic properties of quinolinium (data not shown).

**JW47 Is a Highly Potent Inhibitor of Ca²⁺-mediated PT Pore Formation**—In order to assess the efficiency of compounds on Ca²⁺-mediated PT pore formation, we measured the calcium retention capacity (CRC) of isolated mouse liver mitochondria. The Ca²⁺ concentration in the extramitochondrial solution was measured using the membrane-impermeable low affinity fluorescent Ca²⁺ sensitive dye Fluo-5N following repeated addition of Ca²⁺ boluses (10 μM). Energized mitochondria take up Ca²⁺, resulting in a declining fluorescent signal following the Ca²⁺ bolus-induced peak. Mitochondria take up and buffer Ca²⁺ up to a threshold when intramitochondrial [Ca²⁺] reaches the threshold to induce PT. This results in loss of mitochondrial membrane potential, preventing further Ca²⁺ uptake, resulting in lack of Ca²⁺ buffering, represented by a stepwise increase in extramitochondrial [Ca²⁺] at each Ca²⁺ addition (Fig. 3A). The amount of Ca²⁺ required to induce PT characterizes its Ca²⁺ sensitivity and defines mitochondrial CRC. Inhibition of CypD, the Ca²⁺ sensor of PT, thus leads to increased CRC. JW47 inhibited Ca²⁺-induced PT (i.e. increased CRC) with significantly higher potency as compared with CsA and the non-immunosuppressive inhibitor SmBzCsA. JW47 showed half-maximal inhibition at ~10 nM as compared with ~40 nM for CsA in the CRC assay (Fig. 3B). These results show that JW47 is an approximately 4-fold
more potent inhibitor of Ca\(^{2+}\)-mediated PT pore opening than CsA. In order to confirm that JW47 selectively targets CypD to reduce Ca\(^{2+}\) sensitivity of PT pore formation, the efficiency of the compound was tested on mitochondria isolated from CypD knock-out mice. Neither CsA nor JW47 had any effect on CRC from CypD KO mice (Fig. 3C), whereas CRC in the mitochondria isolated from WT and CypD KO animals. Percentage inhibition denotes increase in CRC compared with DMSO treatment, normalized to WT. * p < 0.05 (t test). Error bars, S.E.

**JW47 Has a Wide Safety Window over Effects on Mitochondrial Membrane Potential or Oxidative Phosphorylation**—To assess the potential adverse effects of JW47, we measured fundamental mitochondrial functional parameters both in cultured rat neurons and in isolated mitochondria and compared the effects of CsA and JW47 above concentrations causing maximal inhibition of the PT pore (>200 and 40 nM, respectively). Neither mitochondrial membrane potential (Fig. 4, A and B), oxygen consumption (Fig. 4, C and D), nor ATP production (Fig. 4, E and F) were affected by supramaximal JW47 (up to 200 nM) or CsA (up to 1 mM) in either models. JW47 inhibited neuronal mitochondrial membrane potential only at ~25 times higher concentrations (1 \(\mu\)M), than the concentration which produced maximal inhibitory effect (40 nM) on the PT pore.

**Cytotoxicity in HepG2 Cells**—HepG2 cells represent a convenient model for exploring hepatotoxicity and can be used to estimate multiple cell health parameters simultaneously. JW47 showed less toxicity in all measures examined, most notably cytochrome c release (Fig. 4G).

**Estimation of Cellular CypA Activity Using an HIV-based Cellular Assay**—To test cellular cyclophilin selectivity of JW47, we conducted an HIV-1-based cellular assay responsive to CypA inhibition. HIV-1 infection of cell lines can be inhibited by the expression of an artificial antiviral protein, comprising the RBCC domains of owl monkey tripartite motif-containing protein 5 (TRIM5) fused to human CypA (TRIM-CypA). TRIM-CypA inhibited viral infection by 32-fold in the absence of drug (Fig. 5A). CsA rescued infectivity through CypA inhibition, whereas JW47 rescued infectivity poorly and only at concentrations of >10 \(\mu\)M (Fig. 5B). A drop to infectivity in non-restricting cells was due to drug toxicity at 5 \(\mu\)M CsA and above. JW47 showed no evidence for toxicity at any of the concentrations tested.

**Activity against Drug and Anion Transporters**—The ability of the compounds to affect human P-glycoprotein (ABCB1) drug transporter activity was assessed using a bioluminescence assay. The standard inhibitor verapamil at 100 \(\mu\)M showed a 40% reduction in luminescence (Fig. 5C). Despite being noted as a P-glycoprotein inhibitor (79), CsA showed only a 16% inhibition at 100 \(\mu\)M. JW47 showed less inhibition than CsA, 8% at 100 \(\mu\)M (Fig. 5C). Activity against organic anion transporters OATP1B1 and OATP1B3 has been linked to drug-induced unconjugated hyperbilirubinemia in patients receiving alisporivir (80). JW47 demonstrated 7–8-fold less inhibition of \(\beta\)-estradiol-17-\(\beta\)-d-glucuronide uptake for the OATP1B1 transporter than CsA (2.56 ± 0.47 \(\mu\)M versus 0.44 ± 0.084 \(\mu\)M for CsA). Similarly, for the OATP1B3 transporter, JW47
showed 4-fold less activity than CsA for inhibition of CCK-8 transport (IC$_{50}$ = 0.20 ± 0.11 μM versus 0.82 ± 0.07 μM).

Pharmacokinetics—JW47 pharmacokinetics was determined in normal ABH mice at 10 mg/kg intraperitoneally at 2 and 4 h. JW47 showed high plasma levels of 10.1 μM at 2 h (Table 3) and appreciable although much lower brain levels (13.2 nM). A 10 mg/kg dose was used to enable detection in the brain. This is broadly comparable with CsA in rodents (79).
The inhibitory effect of JW47 on T cell responses was examined in vitro. Concanavalin A and mitogenic CD3/CD28 monoclonal antibodies induce mitogenic T cell proliferative responses that were inhibited by CsA typically in the 1–10 nM range (Fig. 6, A–C). JW47 only exhibited marked immunomodulation in the 1–10 μM range and was cytopathic at 100 μM. Similarly, JW47 exhibited markedly less immunosuppressive activity compared with CsA in an antigen-driven proliferation assay using myelin peptide (myelin oligoglycoprotein residues 35–55) antigen-induced T cell proliferation.

The dominant pathogenic antigen in spinal cord homogenate-induced disease in ABH mice is a hydrophobic residue in proteolipid protein that does not produce robust T cell proliferative responses in vitro. Therefore, to identify non-immunosuppressive doses of potential neuroprotective compounds for use in models of MS (14, 70), we employed a model using epidermal application of the ear skin sensitizer, oxazolone, to induce a T cell proliferative response in the draining auricular lymph node peaking 3 days later (Fig. 7) (70). Dose response of JW47 in this contact hypersensitivity model showed that daily injection of 1 and 0.1 mg/kg intraperitoneally had no effect, whereas 10 mg/kg intraperitoneally inhibited the T cell response. CsA was immunosuppressive (Fig. 6D) at doses known to inhibit T cell proliferation and EAE (70). Daily dosing of 1 mg/kg intraperitoneal JW47 was therefore chosen as a non-immunosuppressive dose for in vivo studies.

JW47 Induces Neuroprotection in Vivo and Slows the Accumulation of Disability in an Experimental Model of Multiple Sclerosis—The EAE model of multiple sclerosis can be used to assess both inflammatory and neurodegenerative aspects of the disease (81). It has been shown previously that the severity of neurological disease during disease worsening, associated with weight loss, is directly correlated to the degree of immune infiltration into the spinal cord (81–83). Immunosuppression that inhibits the incidence or severity of disease is usually associated with a reduction in the histological detection of infiltrates, demyelination, and axonal loss (81–83). Clinical disease was assessed by scoring neurological signs. In addition to this subjective read-out, objective motor outcomes were detected by assessing loss of motor coordination on an accelerating rotorod, which has been shown previously to exhibit a strong positive correlation with spinal nerve content (71). JW47 was administered daily at 1 mg/kg intraperitoneally shortly before the anticipated onset of relapse. This failed to inhibit the development or the severity of relapse, which would occur with a T cell immunosuppressive agent (84), and limited the accumulation of disability due to the inflammatory penumbra (Fig. 8A). Thus, there were no differences in the incidence of disease (14 of 14 for JW47 versus 12 of 12 for vehicle), the onset of induced relapse following injection of SCH in Freund’s adjuvant on day 28 (34.5 ± 0.72 days for JW47 versus 34.1 ± 0.8 days for vehicle), or the maximal severity disease score (3.9 ± 0.1 for JW47 versus...
Animals relapsing during treatment with daily 50 mg/kg CsA developed clinical disease with a score of 3.0 ± 0.4 (n = 7), which is significantly \( p < 0.05 \) lower than found with either vehicle or JW47 treatment and is indicative of immunosuppression. Treatment with an immunosuppressive agent, such as 250 μg of CD4-depleting (YTS191) monoclonal antibody or other immunosuppressive agents, which are optimized for treatment at this late stage, will completely eliminate relapse (70). Therefore, there was no clinical evidence that JW47 was inducing overt immunosuppression in this paradigm. However, although the minimal clinical scores during the first remission before treatment were not different (0.6 ± 0.1 JW47 versus 0.6 ± 0.1 vehicle), following induced relapse, JW47-treated animals accumulated less deficit as a consequence of the attack and demonstrated a significantly \( p < 0.001 \) better recovery during the second remission (minimal neurological score 1.4 ± 0.3 for JW47 versus 3.1 ± 0.1 for vehicle \( p < 0.001 \)). This subjective outcome was supported by objective rotarod activity outcomes (Fig. 8B). Animals exhibited comparable rotarod activity on day 27 during the first remission (168.8 ± 21.8 s for JW47 versus 161.1 ± 16.0 s for vehicle), but there was significantly \( p < 0.001 \) less loss of motor coordination following treatment with JW47 (Fig. 8B). During the second remission after relapse, JW47-treated animals maintained activity on an accelerating rotarod for 135.0 ± 42.9 s compared with only 46.3 ± 10.1 s in vehicle-treated animals. This activity strongly correlates with spinal nerve content in this assay (71), and it was found that JW47 lost significantly \( p < 0.01 \) fewer nerves (Fig. 8C) and axons (Fig. 8D) within the spinal cord than vehicle-treated animals, which was measured using a quantitative neurofilament ELISA. The SMI35 antibody stains neurofilaments with high and low degrees of phosphorylation and reveals thick and thin axons. SMI32 does not stain thin axons. The degree of neurofilament phosphorylation is modulated by myelination (85), so our readout directly quantifies loss of small axons and, by inference, demyelination. This assay system has been validated against the histological detection of nerve loss during EAE (72, 83) and is a biomarker of

**FIGURE 6. The oxazolone contact hypersensitivity test.** A low severity in vivo measure of T cell proliferation is used as a rapid screen for immunosuppressive doses of agents. Oxazolone administered to the ear induces an increase in cell number and proliferation in the draining auricular lymph node. Error bars, S.E.
neurodegeneration in MS. Thus, JW47 exhibits neuroprotective potential and can inhibit loss of nerves due to the inflammatory penumbra during EAE.

Discussion

The permeability transition pore is linked to necrotic cell death but has been hitherto underexploited as a therapeutic target. The most well known modulator of the pore, CypD, is located on the inner mitochondrial membrane and is resistant to blockade by small molecules. CsA is the standard pan-cyclophilin inhibitor, but it shows no selectivity for CypD and exhibits significant cytotoxicity. We have shown previously that CsA can be targeted to mitochondria using the well known TPP+ cation. Unfortunately, triphenylphosphonium attached by long alkyl or PEG linkers increases the overall lipophilicity and molecular weight of the conjugate substantially and adversely affects pharmaceutical properties. In this study, we utilized quinolinium attached by a short alkyl chain as a mitochondrial targeting cation with a minimal effect on molecular weight and lipophilicity. Olefin cross-metathesis enabled functionalization of CsA using mild conditions that did not affect the sensitive functionality of the cyclic peptide (86). Using this methodology, we identified JW47 as a prototype inhibitor. In isolated mitochondria, JW47 delayed opening of the PT pore and was more active than CsA. Detailed measures of oxidative phosphorylation indicated that JW47 only affected cellular ATP production at supramaximal concentrations but not at the effective concentration (40 nM) or the lowest maximally effective concentration (200 nM). In cortical neurons, mitochondria were not affected by 40 nM JW47 but were significantly depolarized by 1 μM JW47. The greater effect of JW47 in mitochondrial Ca2+ buffering compared with CsA indicates that quinolinium is effective in directing the drug to mitochondria in accordance with its more negative electropotential and in agreement with the Nernst equation (87). JW47 has less intrinsic CypD binding than CsA but is a more potent PT pore inhibitor. In addition, in isolated mitochondria from CypD knock-out mice, JW47 had no effect on Ca2+-mediated PT, proving that its principal mechanism of action is mediated by CypD binding. In a system designed to detect restriction of HIV replication via CypA, JW47 showed no activity, in contrast to both CsA and SmBzCsA, indicating that JW47 has minimal ability to inhibit
CypA in cells. Drug transporter studies on JW47 indicate less propensity to cause bilirubinemia via inhibition of the bilirubin transporter OATP1B1 in comparison with CsA itself and an analogue, alisporivir (88). A multiparameter study of JW47 in hepatocellular carcinoma-derived HepG2 cells showed less toxicity than CsA (89) with perhaps a surprising drop in mitochondrial toxicity, despite the expected sequestration of the ligand to mitochondria (88).

EAE is a T cell-mediated, autoimmune model of MS, which can develop relapsing autoimmunity and progressive neurodegeneration (20, 81, 84). Studies using transgenic mice in the EAE model have shown that the PT pore pathway can determine neurodegeneration independently of the peripheral immune response (20, 21, 90). Although CsA can prophylactically inhibit the generation of EAE, it is therapeutically less effective during spontaneous relapsing EAE and did not inhibit relapsing MS also (70, 91). Furthermore, the nephrotoxicity of CsA also limited its clinical utility in MS (39). Progressive MS is associated with the accumulation of nerve loss and disability, shows minimal response to peripheral immunosuppression, and is currently untreatable (92). It was found that doses of JW47 could be selected to induce neuroprotection without causing overt T cell immunosuppression. We chose ELISA detection of total spinal cord nerve content as a robust non-biased way to fully quantify nerve loss (and, by implication, myelin content). This protection may be via direct effects on
nerves, although it is possible that JW47 also exhibited some influence on innate inflammatory cells, which are believed to drive progressive neurodegeneration. The level of efficacy of JW47 is similar to that found previously with other neuroprotective agents in this experimental paradigm (14), which appear to have some predictive value for identifying neuroprotective agents in multiple sclerosis (14, 93). Relapsing disease was not inhibited with JW47, in contrast to the inhibition that would occur following T cell immunosuppression with drugs used to treat relapsing neuroimmunological disease (94). These current agents, however, do not stop progressive neurodegeneration (92); therefore, it is believed that drugs like JW47 should be used in combination with current immunosuppressive disease-modifying drugs to target the neurodegenerative effects that are currently not treated.

It is therefore of interest that CsA treatment showed some modest benefit in progressive MS (39) and in neuromyelitis optica, another immunemediated demyelinating disease (95). This suggests that inhibition of CypD activity in humans may offer some clinical benefit and would benefit from delivery of a CypD-selective inhibitor. We anticipate that JW47, which is more selective and less toxic in cells, would be better tolerated than CsA in vivo.

CsA is thought to be excluded from the CNS by adenosine-binding cassette drug transporters, such as an ABCB1, ABC1, ABCC4, and ABCG2, which are present in the brain (14, 96, 97). JW47 exhibited limited activity as ABCB1 substrate compared with CsA and could be detected in the brain of normal animals. A pharmacokinetic experiment with JW47 showed potential for in vivo evaluation with high plasma levels of JW47 that are within the active in vitro dose range and significant although lower brain levels. Whereas brain levels may be higher at Cmax, it is also probable that brain levels and importantly spinal cord levels may be significantly higher in animals with active and chronic EAE, which exhibit mainly spinal cord disease, due to alterations in metabolism and blood-brain barrier breakdown that occurs in EAE and MS (11, 71, 98). Furthermore, CNS adenosine-binding cassette drug transporters, notably P-glycoprotein (ABCB1), are down-regulated from vasculature such that compounds, even ABCB1 substrates, can be selectively targeted to lesions, despite poor apparent global CNS penetration (14). Further studies to determine the oral bioavailability, pharmacokinetic/pharmacodynamics, and toxicology of JW47 and related molecules are warranted. The action of JW47 demonstrates that pharmacological inhibitors of the PT pore are neuroprotective in EAE, as predicted using genetic ablation studies of CypD (20) and P66ShcA in the PT pore pathway (21, 99), and may provide a treatment for neurodegeneration and progression of disability during relapsing-remitting and progressive MS and other neurological diseases.

In summary, we have shown that quinolinium is an effective mitochondrial targeting group, and the CsA-tagged molecule is less cytotoxic than CsA and shows little ability to interact with cytoplasmic proteins. In mitochondria, JW47 is a more potent inhibitor of pore opening than CsA and does not affect mitochondrial function at a concentration of maximal inhibition. Furthermore, JW47 demonstrates a pronounced neuroprotective effect in vivo and should be a useful tool for investigation of the role of the PT pore in other neurodegenerative conditions and in models of ischemia-reperfusion injury. The quinolinium cation is smaller and is more amenable to pharmaceutical optimization than TTP+®, and we anticipate that it will become a useful addition to the toolbox of mitochondrial targeting groups.

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Selective Inhibition of the Mitochondrial Permeability Transition Pore Protects against Neurodegeneration in Experimental Multiple Sclerosis


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