Multiple functional therapeutic effects of the estrogen receptor β agonist indazole-Cl in a mouse model of multiple sclerosis

Spencer M. Moore*a, Anna J. Khalaj*a,b, Shalini Kumar*c, Zachary Winchester*a,b, JaeHee Yoonb, Timothy Yoob, Leonardo Martinez-Torres*a,b, Norio Yasuido, John A. Katzenellenbogenb, and Seema Kaushalya Tiwari-Woodruffb,c,e,1

*Division of Biomedical Sciences at the School of Medicine, University of California, Riverside, CA 92521; †Department of Neurology, ‡Brain Research Institute, and §Intellectual and Developmental Disabilities Research Center, Semel Institute for Neuroscience at the School of Medicine, University of California, Los Angeles, CA 90095; and ‡Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

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Currently available immunomodulatory therapies do not stop the pathogenesis underlying multiple sclerosis (MS) and are only partially effective in preventing the onset of permanent disability in patients with MS. Identifying a drug that stimulates endogenous remyelination and/or minimizes axonal degeneration would reduce the rate and degree of disease progression. Here, the effects of the highly selective estrogen receptor (ER) β agonist indazole chloride (Ind-Cl) on functional remyelination in chronic experimental autoimmune encephalomyelitis (EAE) mice were investigated by assessing pathologic, functional, and behavioral consequences of both prophylactic and therapeutic (peak EAE) treatment with Ind-Cl. Peripheral cytokines from autoantigen-stimulated splenocytes were measured, and central nervous system infiltration by immune cells, axon health, and myelination were assessed by immunohistochemistry and electron microscopy. Therapeutic Ind-Cl improved clinical disease and motor performance and also decreased peripheral Th1 cytokines and reactive astrocytes, activated microglia, and T cells in brains of EAE mice. Increased callosal myelination and mature oligodendrocytes correlated with improved callosal conduction and refractoriness. Therapeutic Ind-Cl-induced remyelination was independent of its effects on the immune system, as Ind-Cl increased remyelination within the cuprizone diet-induced demyelinating model. We conclude that Ind-Cl is a refined pharmacologic agent capable of stimulating functionally relevant endogenous myelination, with important implications for progressive MS treatment.

Significance

In the search for effective multiple sclerosis treatment, much effort has been invested in estrogens and estrogen receptor (ER) agonists because of their neuroprotective benefits. However, because estrogens can produce estrus-based feminizing effects and cancer, ERβ agonists represent more desirable therapeutic candidates. The structurally unique ERβ ligand indazole chloride (Ind-Cl), a halogen-substituted phenyl-2H-indazole core, is a preclinical development candidate that is administered s.c. and can be developed for oral administration.

Here, we explored pathologic, functional, and behavioral consequences of prophylactic and therapeutic (after onset of peak EAE) Ind-Cl in chronic EAE mice. Importantly, our recent finding of Ind-Cl-induced RM was confirmed, using the chronic cuprizone (CPZ)-induced demyelinating model (9), supporting Ind-Cl’s remyelinating capabilities independent of its effects on primary inflammation. Our results demonstrate that prophylactic and therapeutic Ind-Cl have significant beneficial effects in a murine model of progressive MS. Specifically, Ind-Cl attenuates remyelination | experimental autoimmune encephalomyelitis | cuprizone diet | motor deficit | PI3/Akt/mTOR

Multiple sclerosis (MS) is an autoimmune, demyelinating, and neurodegenerative disease of the central nervous system (CNS) that affects 2–2.5 million people worldwide. Currently approved MS drugs reduce relapse rates but fail to reverse or prevent neurodegeneration and disability progression. Disease-modifying drugs capable of restoring neuronal function via axon remyelination (RM) represent a major unmet goal for MS therapeutics. Oligodendrocyte (OL) progenitor cells (OPCs) are responsible for remyelinating axons, make up at least 3% of all white matter cells, and are present in and around MS lesions; however, they remain largely quiescent in the adult CNS (1). Although endogenous OL may be present in patients with MS, as evidenced by shadow plaques, it is short-lived, incomplete, and relatively ineffective (2). Transition to progressive MS is characterized by increased axon loss, which correlates with RM failure (3). Hence, a treatment that stimulates endogenous OPCs to differentiate and remyelinate axons would reduce axon degeneration and restore neuronal function.

Experimental autoimmune encephalomyelitis (EAE) affords researchers an in-depth, mechanistic understanding of immune-mediated, demyelinating neurodegeneration and anti-inflammatory effects of currently approved MS drugs. Our recent work has demonstrated promising neuroprotective effects of the estrogen receptor (ER) β agonist 2,5-bis(4-hydroxyphenyl)propionitrile (DPN) (4, 5). Although DPN, acting through ERβ, has a desirable palliative effect in EAE, it possesses only 70-fold binding selectivity for ERβ and lacks anti-inflammatory effects (6, 7). A more selective ERβ agonist capable of immunomodulation would be more efficacious in treating inflammatory demyelinating neurodegeneration.

The structurally unique ERβ ligand indazole chloride (Ind-Cl), based on a halogen-substituted phenyl-2H-indazole core, is a preclinical development candidate with a strong dossier, including in vitro pharmacology using rodent and human cells, selectivity and potency data, promising absorption-distribution-metabolism-excretion findings, and pharmacokinetic profiling that includes confirmation of brain penetrability (mouse brain/plasma: ~1:0) (7, 8). It is a highly ERβ-selective (> 100-fold) small molecule agonist that is administered s.c. and can be developed for oral administration (7).

In the search for effective multiple sclerosis treatment, much effort has been invested in estrogens and estrogen receptor (ER) agonists because of their neuroprotective benefits. However, because estrogens can produce estrus-based feminizing effects and cancer, ERβ agonists represent more desirable therapeutic candidates. The structurally unique ERβ ligand indazole chloride (Ind-Cl), a halogen-substituted phenyl-2H-indazole core, is a preclinical development candidate with a strong dossier. Our results indicate that Ind-Cl is effective in functionally ameliorating disease even when treatment is initiated at peak experimental autoimmune encephalomyelitis clinical disease. Ind-Cl’s immunomodulatory and direct remyelinating effects result in motor dysfunction amelioration. These findings support Ind-Cl’s potential to provide unique therapeutic benefits to patients with multiple sclerosis, as well as patients affected by other demyelinating disorders.

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1To whom correspondence should be addressed. Email: seema.tiwari-woodruff@ucr.edu.

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clinical disease, and its functional immunomodulatory, remyelinating, and neuroprotective effects manifest in axon conduction and myelination improvements. Importantly, these effects correlate with improved motor function. Thus, Ind-Cl could impart much-needed, unique therapeutic benefits in progressive MS and other demyelinating disorders.

Results

Prophylactic Ind-Cl Decreases EAE Clinical Disease Severity Equally in Female and Male Mice. Vehicle-treated EAE mice developed a persistent, chronic disease course starting at ~day 12–15. Female and male prophylactically Ind-Cl-treated (5 mg/kg) EAE mice showed attenuated clinical disease severity, with no sex difference in treatment response (Fig. 1A). Ind-Cl's effect was more pronounced than that of DPN, as DPN-treated female mice trended toward the same degree of clinical disease onset as vehicle-treated mice, but attenuated clinical disease after day 21–25 (4, 10). Ind-Cl reduced severity of peak clinical disease.

Ind-Cl During EAE Does not Increase Uterine Weight. Estrogen augments uterine proliferative processes (11). Before detailed analyses, we assessed the effects of various ER ligands on body and uterine weight. Similar to observations with DPN (10), WAY 200070, WAY 200241, and Ind-Cl did not increase uterine/body weight, whereas E2 induced a fourfold increase (Fig. 1B).

Therapeutic Ind-Cl Attenuates EAE Clinical Disease Severity. A therapeutic regimen (i.e., initiated after peak EAE disease) is most translationally relevant to MS treatment. Female mice were administered Ind-Cl beginning on day 18–21 (postEAE), when the mean clinical score was ~2.5. Control groups consisted of prophylactic E2-treated and vehicle-treated EAE mice (Fig. 1C). A significant decrease in body weight of EAE mice (12) treated with vehicle and Ind-Cl was observed, whereas prophylactic E2 treatment induced an increase compared with untreated normal control groups (Fig. S1). Therapeutic Ind-Cl attenuated a further increase in clinical scores. Importantly, therapeutic Ind-Cl-treated mice exhibited a steady decline in clinical scores, reaching ~1.0 after 19–22 d of treatment.

Prophylactic and Therapeutic Ind-Cl Reduces Th1 Cytokine Production by Peripheral Immune Cells in EAE Mice. To examine the mechanism of Ind-Cl effects on EAE, we evaluated peripheral immune response by measuring cytokine production from splenocytes. T-cell-secreted IFN-γ, IL-6, and proinflammatory IL-17 levels were comparable in vehicle-treated and prophylactic DPN-treated EAE animals but decreased with prophylactic Ind-Cl. Similarly, vehicle-treated and DPN-treated groups showed similar levels of macrophage-secreted TNF-α, which was reduced with Ind-Cl. Interestingly, IL-10, IL-13, and IL-5 levels were reduced only with prophylactic Ind-Cl. Overall, these results point to a profound immunomodulatory effect of Ind-Cl (*P < 0.05; Fig. 2A).

Prophylactic and Therapeutic Ind-Cl Reduces Immune Cells in EAE CNS. Thoracic spinal cords of vehicle-treated EAE mice (day 36–40) displayed numerous multinucleof to coalescing cell infiltrates (represented by DAPI + nuclei stain; Fig. 2B i). Prophylactic (day 0; preEAE) or therapeutic (day 21; postEAE/peakEAE) Ind-Cl reduced infiltrates. The majority of infiltrating cells were positive for pan-leukocyte marker CD45 + microglia/macrophages and T cells (CD3 +). Glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS)-positive astrocytes were also increased. Ind-Cl regimens reduced infiltrating CD3 + and GS + cells and CD45 + and GFAP + intensity in the dorsal column (DC) of EAE mice (Fig. 2B).

Fig. 1. Prophylactic and therapeutic treatment with Ind-Cl decreases clinical scores, with no effect on uterine weight. (A) Mice were immunized with MOG35-55. Normal mice did not receive MOG35-55 or treatment. Treatments began on day 0 (prophylactic) until day 32. Vehicle-treated severe disease course beginning at ~day 15. E2 (0.04 mg/kg/d, orange) prevented the onset of clinical disease. In addition, 8 mg/kg/48 h DPN (blue) displayed decreased clinical scores over time, as observed previously (5). Ind-Cl (5 mg/kg/d) treatment in males (dark purple) and females (light purple) reduced clinical scores (A). One of three representative EAE experiments is shown. n = 8–10 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA Friedman test. (B) Assessment of postperfusion uterus to body weight ratios from intact normal, EAE-vehicle, EAE-DPN (10 mg/kg/d), EAE-Ind-Cl (5 mg/kg/d), EAE+WAY 200241 (10 mg/kg/d), and EAE+WAY 200070 (10 mg/kg/d) and EAE+E2 (0.04 mg/kg/d) injected female mice revealed a fourfold increase in the E2-treated group and no differences between all other treatment groups (n = 4–5 mice/group; ***P < 0.001, ANOVA). (C) Mice were administered 5 mg/kg/d Ind-Cl therapeutically, beginning on day 21 (purple; postEAE), or 0.04 mg/kg/d E2 at day 0 (gold; preEAE) of active EAE or vehicle (red). EAE mice treated with Ind-Cl showed significant improvement. One of three representative EAE experiments is shown (n = 10 mice/group; **P < 0.01, ANOVA Friedman test). Reprinted from ref. 5 with permission from Elsevier (www.sciencedirect.com/science/journal/09699961).

Fig. 2. Treatment with Ind-Cl suppresses cytokine production by peripheral immune cells and reduces CNS inflammation and infiltration. (A) Cytokine production by MOG35-55-stimulated splenocytes was assessed from EAE mice killed on postinduction day 34. Pretreatment with DPN or Ind-Cl was initiated at day 0, and posttreatment with Ind-Cl began on day 21. DPN-treated (blue) and vehicle-treated (red) animals displayed similar cytokine levels. Pretreatment (light purple) with 5 mg/kg/d Ind-Cl resulted in significant reduction of all measured cytokines. Posttreatment with Ind-Cl (dark purple) significantly decreased TNF-α levels. Data are representative of experiments repeated twice. n = 4–6 mice/group; *P < 0.05, t test. (B) CNS inflammation was assessed using immunohistochemistry. Asterisks within the representative 4x image of vehicle-treated EAE spinal cord indicate lesions and areas of infiltration and demyelination, unlike Ind-Cl-treated mice (i and ii). Ten times and 40x images of the DC (area delineated by the white dashed box) in i shows decreased infiltration by peripheral CD45 + immune cells with Ind-Cl treatment (i and iii) and decreased CD3 + T-cell (red) numbers (iv). Pretreatment with Ind-Cl resulted in decreased GFAP + (red) intensity and GS + (red) numbers, and a trend toward this effect was observed with posttreatment (v and vi). n = 10 mice/group; *P < 0.05, **P < 0.01, ANOVA.

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Ind-Cl Increases OL Survival and Activates the PI3K/Akt/mTOR Pathway. During EAE, many cell types (e.g., OLs) undergo apoptosis (5, 13, 18). Increased colloidal OLs in Ind-Cl-treated EAE mice could be a result of decreased OL apoptosis and/or increased OL survival. Similar to previous observations, significant increases in caspase-3 activity were observed in vehicle-treated EAE groups (5), but not preEAE+ or postEAE+Ind-Cl– groups, via immunohistochemistry (Fig. S4). Overexpression of B-cell lymphoma 2 (BCI-2) is known to inhibit cell death. 2′,3′-Cyclic nucleotide-3′-phosphodiesterase (CNPase) is a myelin-associated enzyme expressed exclusively by differentiating OLs and makes up 4% of total CNS myelin protein. CNPase and BCI-2 levels were assessed in CC homogenates. During EAE, continued demyelination and cell death results in decreased CNPase and BCI-2 (Fig. 4 II, A and B). Therapeutic Ind-Cl increased CNPase and BCI-2 compared with vehicle-treated EAE and normal mice (*P < 0.05, **P < 0.01; Fig. S5 A and B).

To assess Ind-Cl effects on the potentially BDNF-mediated PI3K/Akt/mTOR pathway [also modulated by DPN (6, 19–21)] in EAE mice, CC homogenates were probed for relevant proteins.
TO CHARACTERIZE THE FUNCTIONAL CONSEQUENCE OF IND-CL IN VIVO, EAE 
MICE WERE TESTED FOR THEIR ABILITY TO REMAIN WALKING ON A ROTATING 
CYLINDER. THE ROTOROD TEST HAS STRONG TRANSLATIONAL CORRELATES TO 
MOTOR ASSESSMENTS, USING THE KURZKE EXPANDED DISABILITY STATUS 
SCALE, IN PATIENTS WITH MS. EAE MICE DISPLAY AN INCREASED TENDENCY TO 
FALL FROM THE CYLINDER COMPARED WITH NORMAL MICE, WHICH ARE 
CAPABLE OF REMAINING ON THE CYLINDER FOR THE FULL TRIAL PERIOD. AT 
DAY 21, MICE WERE RANDOMLY ASSIGNED TO RECEIVE THERAPEUTIC 
VEHICLE OR IND-CL (FIG. 5 II, A). IND-CL ATTENUATED EAE CLINICAL DISEASE, 
WHEREAS VEHICLE-TREATED MICE DISPLAYED SEVERE AND CHRONIC 
DISABILITY BEGINNING AT −DAY 15 (FIG. 5 II, A). ROTOROD PERFORMANCE 
DECLINED SHARPLY AS CLINICAL DISABILITY PROGRESS, BUT IND-CL RESCUES 
MOTOR FUNCTION; IND-CL-TREATED MICE APPROACHED NORMAL PERFORMANCE 
LEVELS WITHIN 10–12 D OF TREATMENT INITIATION (FIG. 5 II, A AND B). BY DAY 30–40, IND-CL-TREATED EAE MICE EXHIBITED RECOVERY OF 
MOTOR FUNCTION (***P < 0.01, ANOVA; FIG. 5 II, A AND B).

Ind-Cl Improves Callosal Axon Conduction. To characterize the functional consequence of 
neuropathology during EAE and with Ind-Cl, callosal compound action potentials (CAPs) 
were recorded (FIG. 5 I, A). Typical voltage traces showing two downward 
phases of the N1 and N2 CAP amplitudes, likely representing fast 
depolarizing large, myelinated axons and slower depolarizing non-
myelinated axons, respectively, are shown (13) (FIG. 5 I, A AND B). 
During EAE (red), both N1 and N2 CAP amplitudes were 
decreased to nearly 50% of normal (black; FIG. 5 I, A AND B). 
Prophylactic Ind-Cl (light purple) and DPN (navy) increased N1 and 
N2 CAP amplitudes compared with vehicle treatment. Therapeutic 
Ind-Cl (dark purple) induced an increase in N1, but not 
N2, CAP amplitude (FIG. 5 I, C AND D; **P < 0.001, *P < 0.05).

To further investigate Ind-Cl effects on EAE-induced CC axon deficits, axon refractoriness was examined (9). In the normal group, 
the N1 component evoked by the second pair of pulses was 50% of 
the amplitude of a single pulse presentation at an interpulse interval (IPI) of 2.2 ± 0.1 ms (FIG. 5 I, E). The IPI for the vehicle-treated 
EAE group had a slower response of 5.1 ± 0.2 ms. Prophylactic Ind-
Cl and DPN, and therapeutic Ind-Cl, callosal axons had faster IPIs 
of 3.8 ± 0.1, 3.7 ± 0.2, and 3.7 ± 0.1 ms, respectively. The N2 component 
IPIs from vehicle-treated mice (8.8 ± 2.2 ms) were slower 
than those of normal mice (3.2 ± 0.2 ms). Ind-Cl induced a small 
but significant recovery: preEAE+Ind-Cl = 4 ± 0.2 ms, preEAE+ 
DPN = 4.4 ± 0.2 ms, and postEAE+Ind-Cl = 4.7 ± 0.2 ms.

**Therapeutic Ind-Cl Decreases EAE-Induced Rotorod Motor Performance Deficit.** To assess the functional significance of Ind-Cl in vivo, EAE 
mice were tested for their ability to remain walking on a rotating 
cylinder. During EAE (red), both N1 and N2 CAP amplitudes were 
decreased to nearly 50% of normal (black; FIG. 5 I, A AND B). 
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Ind-Cl-Induced Axon RM Improvement Occurs Independent of Primary Inflammation Effects. In contrast to EAE, CPZ diet-induced demyelination does not compromise the blood-brain barrier and elicits no primary infiltrating autoimmune response. To examine the direct effects of Ind-Cl on RM or protection in the absence of its inflammatory effects on primary inflammation, the CPZ diet model was used. Nine weeks of CPZ diet (9 wk CPZ) resulted in extensive demyelination and loss of PLP_EGFP+ cells throughout the cortical layers, CC, and dorsal hippocampus (Fig. 6A). Assessment of CC myelin and mature OLs revealed decreases in the 9-wk-DM group (9wk-DM). A subset of 9wk-DM mice were switched to 3 wk of normal diet (ND) and underwent RM (9wkCPZ+3wkND), during which half were administered vehicle (+V) or Ind-Cl (+Ind-Cl). Both RM groups showed myelin density and mature OL recovery (Fig. B). Ind-Cl induced a 50% increase in CC myelination and OLs compared with the vehicle-treated RM group. Direct positive effects of Ind-Cl on axon RM were confirmed by EM analysis, as Ind-Cl during RM resulted in a lower g-ratio compared with 9wk-DM alone and vehicle-treated 9wkCPZ+3wkND-RM groups (normal = 0.86 ± 0.01; 9wkCPZ = 0.96 ± 0.01; 9wkCPZ+3wkND+V = 0.94 ± 0.01; and 9wkCPZ+3wkND+Ind-Cl = 0.89 ± 0.003; *P < 0.5, **P < 0.01, ***P < 0.001, ANOVA; Fig. 6C).

Discussion
In MS, demyelinated areas containing damaged axons are associated with inflammatory reactions orchestrated by activated T cells, macrophages, and endogenous glia, which produce proinflammatory and neurotoxic factors and attenuate repair/RM of damaged/demyelinated axons (22). This manifests as clinical deficits (23, 24). Thus, these cell types are immunomodulation targets in MS. Currently approved immunomodulators are only modestly effective in reducing relapses by slowing disability accumulation; these treatments fail to stop axon loss and/or stimulate RM. OL and myelin rescue and sustenance, with immunomodulation, are of high priority for effective MS therapy development.

For effective RM treatments, much has been invested in estrogens and ER agonists because of their neuroprotective benefits (5, 10, 25, 26). Different ERβ ligands analogs have distinct effects on gene transcription in signaling pathways for chromosome replication, cell death, and OPC differentiation (27). The therapeutic potential of ERβ-selective compounds is particularly favorable because beneficial effects of ERβ activation are independent of undesired proliferative effects on breast and uterine tissue, which are principally ERα-mediated (28). Certain halomindazoles, synthetic ERβ-specific ligands based on a halogen-substituted indole core (8), potently inhibit their functional activation of inflammatory response genes in microglia and astrocytes (7). Our study demonstrates that the halomindazole Ind-Cl ameliorates chronic EAE even when treatment is initiated at peak clinical disease. We analyzed callosal white matter integrity in addition to spinal cord, as MS CC reflects demyelinating lesions, diffuse tissue damage, and neural connectivity abnormalities (29, 30). Specifically, Ind-Cl inhibits ongoing demyelination and axon damage in EAE, leading to substantial recovery of axon conduction, a functional indicator of axon myelination and neuroprotection. Ind-Cl increased BDNF, decreased cell death markers, and activated the PI3K/Akt/mTOR signaling pathway required for OPC proliferation and OL differentiation. Furthermore, therapeutic Ind-Cl reversed ongoing motor deficit. In contrast to DPN’s effects, the present study confirms a reduction of reactive astrocytes by Ind-Cl. Reactive astrocytes respond to and magnify ongoing inflammatory response. We report that both EAE-induced peripheral immune response and CNS immune cell increases are reduced with prophylactic Ind-Cl, which is further evidence of this drug’s promising immunomodulatory properties. ERβ is present in various cell types within the peripheral immune system and CNS, including neurons, astrocytes, microglia, OLs, and immune cells (31). Using conditional gene knockout mice, we have shown that the functional beneficial effects of the less-selective ERβ agonist DPN in EAE mice are largely attributable to its action on ERβ in OL lineage cells (6). Further, increased BDNF expression in DPN-administered mice lacking ERβ in OLs is not sufficient to reduce clinical disease or demyelination or to increase the PI3K/AKT/mTOR pathway activation, although it may explain partial improvement of axonal loss and conduction (5, 6). It would follow that the functional benefits of therapeutic Ind-Cl, a more selective ERβ agonist, are at least partly attributable to the drug’s actions on ERβ in OL lineage cells. However, unlike DPN, Ind-Cl exhibits immunomodulatory capabilities in both the peripheral immune system and CNS (7). Ind-Cl may concurrently act on ERβ in multiple cell types. An effect of Ind-Cl on peripheral cells does not exclude a direct effect on the CNS. Time of treatment initiation may contribute to predominant Ind-Cl mechanism of action in EAE. For example, immunomodulatory capabilities may yield indirect neuroprotection (i.e., prevention of neurodegeneration caused by immunomodulatory response) if treatment is initiated early in disease, whereas treatment initiation late in disease may rely on the direct neuroprotective (i.e., restoration of neuronal components, including myelinating OLs, and function) capabilities of Ind-Cl, which we have demonstrated here in the CPZ diet-induced demyelination, a model with an intact blood-brain barrier and no primary inflammatory response.

The difference in immunomodulatory plus remyelinating properties of Ind-Cl and the solely remyelinating properties of DPN raises questions about ERβ binding affinity, selectivity, gene regulation, and mechanisms of action of various ERβ ligands. Thus, it is not surprising that structurally related ERβ ligands, even ones having similar ERβ versus ERα binding affinity, selectivity, and efficacy in standard reporter gene assays, have distinct patterns of endogenous gene regulation. In light of such findings, one may understand why the actions of Ind-Cl through ERβ are different.
Materials and Methods

Treatment. Ind-Cl [synthesized by J.A.K.’s laboratories (8)] was dissolved in 10% ethanol + 90% (vol/vol) Miglyol 812N (vehicle; Sasol) and administered s.c. daily at 0.04 mg/kg body weight. Control groups received (s.c.) either 0.04 mg/kg/d 17-estradiol (E2) or 8 mg/kg/d 4-HDP (4, 10). Treatment was initiated at EAE postinduction day 0 (preEAE) or day 21 (postpeakEAE) and continued until day 40. For CPZ experiments, animals received Ind-Cl or vehicle during RM only (n = 10–15 per group; two to three experiments).

EAE. Active EAE was induced in 8-wk-old male and female PLP_EGFP C57BL/6 mice (4, 10, 13). All procedures were conducted in accordance with the NIH and approved by the Animal Care and Use Committee at the University of California, Los Angeles.

CPZ. Male PLP_EGFP mice were randomly assigned to one of two groups. The normal myelination group received normal chow. The demyelination group (n = 32) received 0.2% CPZ-milled chow for 9 wk (33). DM animals (48(4):1132 90% (vol/vol) Miglyol 812N (vehicle; Sasol) and administered 10% ethanol s.c. daily at 5 mg/kg body weight. Control groups received (s.c.) either 0.04 mg/kg/d 17-estradiol (E2) or 8 mg/kg/d 4-HDP (4, 10). Treatment was initiated at EAE postinduction day 0 (preEAE) or day 21 (postpeakEAE) and continued until day 40. For CPZ experiments, animals received Ind-Cl or vehicle during RM only (n = 10–15 per group; two to three experiments).

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