

Hyperforin down-regulates effector function of activated T lymphocytes and shows efficacy against Th1-triggered CNS inflammatory-demyelinating disease

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Abstract: Hyperforin (Hyp) is an active compound contained in the extract of *Hypericum perforatum*, well known for its antidepressant activity. However, Hyp has been found to possess several other biological properties, including inhibitory effects on tumor invasion, angiogenesis, and inflammation. In this paper, we show that treatment with Hyp inhibited IFN- γ production, with down-regulation of T-box (T-bet; marker of Th1 gene expression) and up-regulation of GATA-3 (marker gene of Th2) on IL-2/PHA-activated T cells. In parallel, we showed a strong down-regulation of the chemokine receptor CXCR3 expression on activated T cells. The latter effect and the down-modulation of matrix metalloproteinase 9 expression may eventually lead to the inhibition of migratory capability and matrix traversal toward the chemoattractant CXCL10 by activated lymphocytes that we observed in vitro. The effect of Hyp was thus evaluated on an animal model of experimental allergic encephalomyelitis (EAE), a classic, Th1-mediated autoimmune disease of the CNS, and we observed that Hyp attenuates the severity of the disease symptoms significantly. Together, these properties qualify Hyp as a putative, therapeutic molecule for the treatment of autoimmune inflammatory disease sustained by Th1 cells, including EAE. *J. Leukoc. Biol.* 83: 212–219; 2008.

Key Words: inflammation · experimental allergic encephalomyelitis

INTRODUCTION

Hyperforin (Hyp) represents one of the main active constituents of *Hypericum perforatum*, a traditional herb, effective in the treatment of mild to moderate depressive disorders [1–3]. In addition to its antidepressive properties, Hyp shows anti-inflammatory activity by inhibiting the proliferation and induction of apoptosis of PBMC [4, 5]. Moreover, Hyp blocks 5-lipoxygenase (5-LO) and cyclooxygenase 1 (COX-1), i.e., two

crucial enzymes in the biosynthesis of proinflammatory eicosanoids [6]. Finally, in vivo clinical studies reveal significant benefit of Hyp in the topical treatment of mild to moderate atopic dermatitis [7], confirming that Hyp may act as an in vivo anti-inflammatory agent [8].

Mechanisms leading to the anti-inflammatory properties of Hyp are still in debate. It has been demonstrated that Hyp inhibits the generation of reactive oxygen species (ROS) as well as the release of leukocyte elastase (degranulation) and Ca²⁺ mobilization in human isolated polymorphonuclear leukocytes (PMNL) [9]. Less known is the role of Hyp on lymphocytes. Quiney et al. [10] have shown that Hyp promotes apoptosis and capacity to secrete matrix metalloproteinase 9 (MMP-9) on neoplastic B lymphocytes. Concerning T lymphocytes, Shemp et al. [5] have demonstrated that Hyp suppresses the proliferation of PBMC in a dose-dependent manner.

In this study, we evaluate the effect of Hyp on IL-2/PHA-activated T cells. Our results indicate that Hyp down-modulates the T cell population, with particular efficiency on Th1; moreover, we determine the action of Hyp on a classic, Th1-mediated animal inflammatory model of demyelinating autoimmune disease of the CNS, i.e., the experimental allergic encephalomyelitis (EAE). We observed an in vivo activity as an anti-inflammatory agent of Hyp in rats with EAE. The therapeutic potential of Hyp on autoimmune inflammatory disease sustained by Th1 cells is proposed and discussed in comparison with other problematic drugs with similar target.

MATERIALS AND METHODS

Materials

The stable and crystalline dicyclohexylammonium salt of Hyp (Hyp-DCHA) was used in all our experiments. For in vitro experiments, Hyp-DCHA was

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TABLE 1. Primer Sequences Used for RT-PCR

T-bet	5'-TGT GAC CCA GAT GAT CGT CCT-3' 5'-CTG AGT GAT CTC TGC GTT CTG GT-3'
GATA-3	5'-CTG TCG GCA GCA AGG AGA G-3' 5'-CTC TAA CCG ATG GCG GTC AC-3'
CXCR3-A	5'-ACC CAG CAG CCA GAG CAC C-3' 5'-TCA TAG GAA GAG CTG AAG TTC TCC A-3'
CXCR3-B	5'-TGC CAG GCC TTT ACA CAG C-3' 5'-TCG GCG TCA TTT AGC ACT TG-3'
β-ACTIN	5'-TGC CGA CAG GAT GCA GAA G-3' 5'-CTC AGG AGG AGC AAT GAT CTT GA-3'

solubilized 10 mM in DMSO, and the dilutions used never exceeded 1% DMSO final concentration.

Preparation of cell suspensions

T cells were enriched from the entire mononuclear cell suspensions from healthy donor buffy coats, as described previously [11]; >95% cells were viable, as judged by trypan blue exclusion. Staining with CD3 mAb (Caltag Laboratories, Burlingame, CA, USA) showed 98–100% positive T cells in FACS analysis.

Cell viability

Cell viability under Hyp-DCHA (0.3–2.5 μM) was evaluated after 4 and 24 h on T cells, preactivated 72 h with 2.5 μg/ml PHA and 100 U/ml IL-2 with 0.4% trypan blue or 10 μg/ml propidium iodide (PI) by microscope or by flow cytometry, respectively.

Flow cytometry

Purified T cells from four donors were cultured in the presence of 2.5 μg/ml PHA and 100 U/ml IL-2 for 72 h. After stimulation, nontoxic concentrations of Hyp-DCHA (0.6–2.5 μM) were added to the cultures for 4 and 24 h, and the expression of cytokine and chemokine receptors was evaluated by FACS [11]. The cells were treated with commercially available FITC- or PE-conjugated mAb: anti-CD3, -CD4, and -CD8, isotype-matched controls (BD PharMingen, San Diego, CA, USA), anti-IL-4 and -IFN-γ (BD PharMingen), FITC-conjugated anti-human CXCR3 (clone 49801.111), and anti-human CCR3 (R&D Systems Inc., Minneapolis, MN, USA). FITC- or PE-conjugated, control isotype-matched mouse mAb were used to set the fluorescence background (IgG1 and IgG2a, BD PharMingen).

T lymphocytes were gated with two different approaches: physical characteristic of cells and expression of CD4 in the area of lymphocytes to identify the T helpers. Expression of cytoplasmic cytokines was evaluated after fixing and permeabilization of the cell membranes with Fix & Perm reagent (Caltag Laboratories, Burlingame, CA, USA), followed by PE-conjugated anti-IFN-γ and anti-IL-4.

RNA purification and analysis by real-time PCR

T cells were stimulated with PHA plus IL-2 for 72 h in the absence or presence of Hyp-DCHA (0.6, 1.2, 2.5 μM). After 4 and 24 h, the levels of mRNA for T-bet (T-bet), GATA-3, CXCR3-A, and CXCR3-B were measured. Total cellular RNA was extracted from 4×10^6 cells, using the RNA mini kit isolation and purification system (Qiagen, Valencia, CA, USA). On-column Dnase, digestion was performed according to the manufacturer's instructions, and RNA was quantified by measuring the absorbance at 260 nm. cDNA was synthesized from 1 μg total RNA at 42°C for 15 min in the presence of avian myeloblastosis virus RT (2.5 U), 2.5 μM oligo(dT) primer (Promega, Madison, WI, USA), using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, P/N 4309155), premixed with a fraction of 5 μM primers and 2.5 μl cDNA to a final 25-μl reaction volume. The primers used for T-bet, GATA3, CXCR3-A, CXCR3-B, and β-actin amplifications are detailed in **Table 1**. The primers used for β-actin, T-bet, and GATA-3 were obtained using the Primer Express (Applied Biosystems) computer software and for CXCR3-A and CXCR3-B, the primers found in Lasagni et al. [12]. Fifty cycles of 95°C/10

min, 95°C/15 s, and 60°C/1 s were repeated in duplicate. A no-template control for each master mix, and three standard curves were generated for β-actin, T-bet, GATA-3, CXCR3-A, and CXCR3-B using total T cDNA in 1, 1:5, 1:25, and 1:125 serial dilutions. The relative amounts of mRNA were determined by comparison with standard curves. For each sample, the results were normalized for β-actin expression. To distinguish specific from non-specific amplicons, a dissociation curve was generated.

Chemotaxis of T cells

T cell migration was measured in a 48-well-modified Boyden chamber (AC48, Neuro Probe Inc., Gaithersburg, MD, USA), using polyvinylpyrrolidone-free polycarbonate membranes (5 μm pores, Osmonics, Livermore, CA, USA). The bottom face of the filters was pretreated with fibronectin to maximize attachment of migrating cells: In preliminary experiments, only a trivial number of cells were recovered in the bottoms of the wells, and fibronectin failed to induce chemotaxis.

T cells were stimulated 2.5 days with IL-2 (100 U/ml) plus PHA (2.5 μg/ml) and the following 2 days with IL-2 alone; then, different doses of Hyp-DCHA (0.0, 0.3, 0.6, 1.2 μM) were used to treat the cells. A total of 28 μl 200 ng/ml recombinant human IFN-inducible protein 10 (rhIP-10)/CXCL10 (R&D Systems) or medium was added to the bottom well; 50 μl T cells (5.0×10^6 cells/ml) in RPMI 1640, preincubated or not 30 min at 37°C with 5% CO₂ with Hyp-DCHA, were added to the top wells. After 2 h at 37°C with 5% CO₂, the membranes were removed, washed with PBS on the upper side, fixed, and stained with DiffQuik (Dade AG, Düringen, Switzerland), and the cells were counted in three fields/well at $\times 800$. All assays were in triplicate.

Migration of T cells through Matrigel

The ability of T cells to pass through a tissue barrier mimicking that on which endothelial cells reside [basement membrane (BM)] was tested using a modified Boyden chamber assay with filters coated with 12 μg/filter Matrigel (reconstituted BM) [13]. Cells were stimulated as above (2.5+2 days), and their response to rhIP-10/CXCL10 (200 ng/ml) was measured by seeding 1.5×10^6 cells in serum-free RPMI 1640 with 0.0, 0.3, 0.6, and 1.2 μM Hyp-DCHA onto 5 μm pore-size-coated filters. After 2 h incubation at 37°C with 5% CO₂, migrated cells were collected from the lower chamber and counted under a microscope, and the results of sextuplicate experiments were averaged. Non-migrated cells were immunostained for IL-4 and IFN-γ and analyzed by FACS to assess differential inhibition exerted on Th1 or Th2.

Gelatin zymography

T cells were purified and stimulated as above (2.5+1 days), seeded onto 12-well plates at 4×10^6 /well, and incubated for an additional 24 h in serum-free RPMI 1640 in the presence of IL-2 (100 U/ml) and Hyp-DCHA (0.0, 0.3, 0.6, 1.2 μM). The conditioned medium was analyzed by 0.1% gelatin zymography as described already [13]. The bands of gelatinolysis were quantitated using Gel-Doc 2000 and QuantityOne software (Bio-Rad, Hercules, CA, USA).

Animals and treatments

EAE was induced in 10 female pathogen-free Lewis rats (Charles River, Italy) of 125–150 g by direct immunization with guinea pig spinal cord tissue using a standard protocol [14, 15]; 10 uninjected rats were used as control. Five animals of each group were treated with the Hyp-DCHA added to the food pellets (150 mg/kg) to achieve a (assumed) daily dose of 3 mg/animal [16]. The rats were weighted regularly and examined for clinical signs of EAE by two independent observers according to a semiquantitative score for walking attitude [13]: 1 = loss of tail tone; 2 = weakness in one or both hind legs or middle ataxia; 3 = ataxia or paralysis; 4 = severe hind leg paralysis; 5 = severe hind leg paralysis accompanied by urinary incontinence.

Histopathology

Twenty-seven days after immunization, the rats were perfused under anesthesia with Tyrode Ca⁺⁺-free buffer followed by paraformaldehyde 4% + saturated picric acid, 14% solution in PBS 0.2 M, pH 6.9. Cryostat sections (14 μm) were collected from lumbar spinal cord and stained with H&E, and the inflammatory infiltration was evaluated by two independent operators on two

replicate sections using the following score [17]: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. Sections were also stained for the detection of the CD4 Th/inducer lymphocytes (mouse mAb-7 clone PE2, dilution 1:200, Lab Vision, Fremont, CA, USA) by indirect immunofluorescence using fluorescein-conjugated sheep anti-mouse F(ab')₂ fragment (Boehringer Mannheim, Germany).

Statistical analysis

Data were analyzed with the assistance of the statistical analysis system. Data are expressed as mean ± SE. Mean values were compared using the ANOVA test. A *P* < 0.05 was considered as significant. Two-way ANOVA was used for analysis of the clinical score and body weight. Student's *t*-test was used to compare histological measurements.

RESULTS

Cytotoxic effect of Hyp-DCHA on PHA and IL-2-activated T lymphocytes

We have established the nontoxic range of Hyp-DCHA to use to investigate the effect on activated T lymphocytes. A significant decrease of cell viability was measured by a PI exclusion test only after 24 h incubation with the higher dose of Hyp-DCHA (2.5 μM) with respect to the untreated control: The percentage of viable cells fell from 89.3% ± 4.0 (untreated cells) to 71.3 ± 3.2 (*P* < 0.05). Analogous results have been obtained with the trypan blue assay (data not shown).

Hyp-DCHA exhibits an inhibitory activity on Th1 cytokine IFN-γ production from activated T lymphocytes

After stimulation with PHA and IL-2 and treatment with Hyp-DCHA, T cells were double-immunostained for intracellular cytokines and CD4 or CD8; the cells were then analyzed by flow cytometry to discriminate whether the action of Hyp-DCHA was preferentially addressed to a type 1 or type 2 T cell subpopulation. A dose-dependent decrease of IFN-γ⁺/CD4⁺ T cells was observed after 4 h of treatment with Hyp-DCHA, in terms of percentage and mean fluorescence intensity (MFI) compared with untreated T cells—these data resulting statistically significant (*P* < 0.05, **Fig. 1a**)—and there was a mild increase, in particular, in terms of MFI, of IL-4⁺/CD4⁺ T cells after treatment with Hyp-DCHA (not significant, **Fig. 1b**). Therefore, our results suggested a preferential inhibitory activity of Hyp-DCHA on Th1 cells.

The effect on the cytokine production after 24 h of treatment with the drug was prevented because of the undetectable levels of two cytokines at this time-point.

T-bet but not GATA-3 gene expression is down-regulated by Hyp-DCHA on activated T lymphocytes

As shown in **Figure 1c**, T-bet mRNA expression was down-modulated under 1.2 and 2.5 μM Hyp-DCHA by 24.6% and 38.9%, respectively, after 4 h; a stronger down-regulation was measured at 24 h (46.8%, 61.6%, and 71.1%, with doses 0.6, 1.2, and 2.5 μM, respectively). In contrast, for the same time intervals, GATA-3 mRNA expression proved to be up-regulated with the two higher doses of Hyp-DCHA by 88.7% and

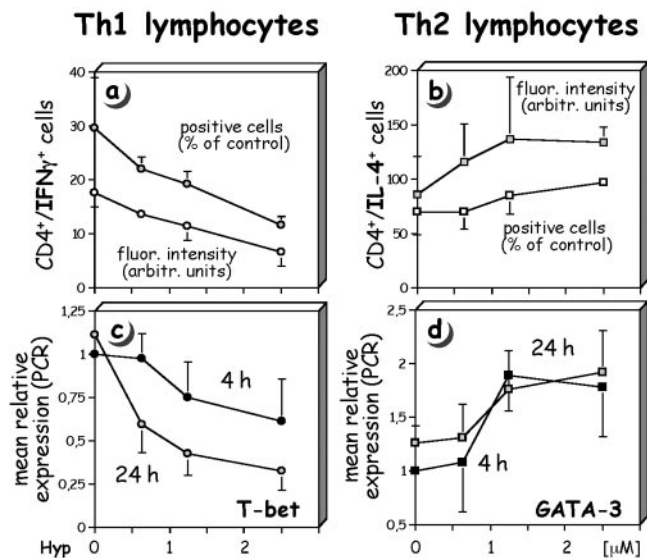


Fig. 1. Hyp-DCHA down-modulates the Th1 but not the Th2 component of activated lymphocytes. T cells were cultured with 2.5 μg/ml PHA and 100 U/ml IL-2 for 72 h and treated with Hyp-DCHA; positive cells and MFI for CD4⁺/IFN-γ⁺ (a) and CD4⁺/IL-4⁺ (b) were evaluated by flow cytometry after 4 h (*P* < 0.05). Each point represents the mean value from four donors. Relative expression of mRNA for T-bet (c) and GATA-3 (d) was evaluated by RT-PCR on T cells treated as above after 4 and 24 h (*P* < 0.05). Statistical evaluation was performed using ANOVA.

78.3% at 4 h and 50.7% and 66.0% at 24 h (**Fig. 1d**). This finding provides evidence that the immunomodulatory action of Hyp-DCHA was likely exerted at a level of regulation of cytokine production with more efficacy on Th1 cytokine production.

Hyp-DCHA induces down-regulation of CXCR3 expression on activated T lymphocytes

The expression of mRNA for CXCR3-A and CXCR3-B, alternatively spliced variants of the CXCR3 receptor, associated to the Th1 and Th2 population, respectively [18], was examined in activated T cells in the absence or presence of various doses of Hyp-DCHA. CXCR3-A mRNA levels were consistently higher than CXCR3-B, and the expression of both variants was dose-response decreased after 4 h under 0.6–2.5 μM Hyp-DCHA (data not shown).

A down-regulation of CXCR3 expression by flow cytometry was observed under Hyp-DCHA treatment in a dose- and time-dependent manner (data not shown). After 4 and 24 h (**Fig. 2**) in the presence of 2.5 μM Hyp-DCHA, a 20% and 38% reduction of the CXCR3 expression was observed. Hyp-DCHA induced a more pronounced reduction (34% and 72% after 4 and 24 h) in terms of CXCR3 MFI. This effect was less marked on the resting T cells and only mild on CCR3 expression, with 65% reduction of percentage expression but only 8% reduction in terms of CCR3 MFI after 24 h under 2.5 μM Hyp-DCHA (**Fig. 2**).

Hyp-DCHA inhibits CXCL10-induced migration of activated T lymphocytes

On uncoated filters, the migration of activated T cells in response to CXCL10 chemoattractant was increased by 133%

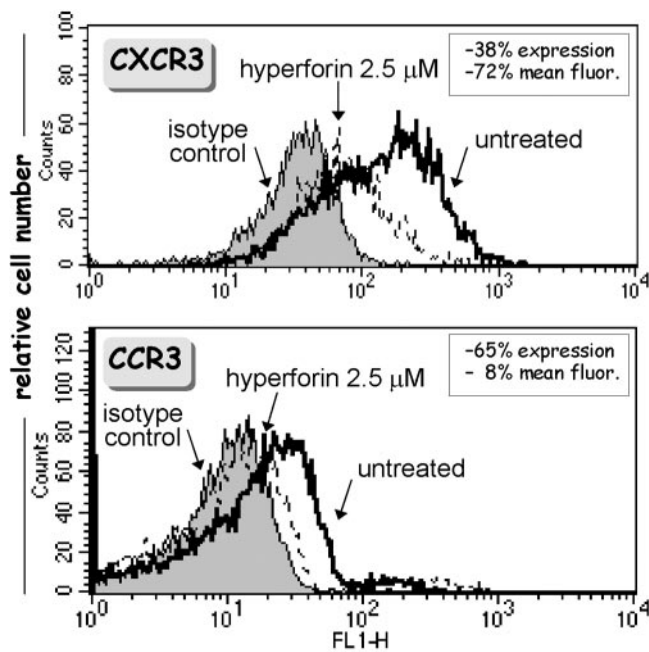


Fig. 2. Hyp-DCHA down-regulates CXCR3 expression on activated T cells. Surface expression of CXCR3 on the membrane of 72 h PHA and IL-2-activated T cells as detected by FACS is shown. CXCR3 expression was evaluated on cells treated 24 h with 2.5 μ M Hyp-DCHA (upper panel); the gray peak shows the fluorescence of cells incubated with FITC-conjugated isotype control mAb (anti-IgG1). Unimodal distribution with a statistically significant shift (down-regulation) between a Hyp-DCHA-treated and untreated sample is observed. The down-regulation of CCR3 after 24 h of treatment is not significant (lower panel). Representative results of four independent experiments are shown.

with respect to the nonstimulated control; in these conditions, migration toward CXCL10 was decreased significantly under all tested doses of Hyp-DCHA, already by 63.7% at 0.3 μ M (Fig. 3a).

On Matrigel-coated filters, the migration of activated T cells toward CXCL10 was increased by 72% with respect to the nonstimulated control; under Hyp-DCHA, migration toward this chemoattractant was progressively inhibited, with almost complete suppression under 0.3 μ M (Fig. 3b).

The cytofluorimetric analysis (antibody against CD4, IFN- γ , IL-4) of the cells recovered from the upper chambers showed a progressive relative increase of Th1 over the Th2 population, with an IFN- γ /IL-4 ratio of 0.18, 0.20, 0.23, and 0.57, relative to untreated or 0.625, 1.25, and 2.5 μ M Hyp-DCHA-treated cells, respectively, reinforcing the conclusion that Hyp-DCHA inhibits the migration of Th1 cells (CD4⁺/IFN- γ ⁺).

Hyp-DCHA effects on secretion of gelatinases

Gelatin zymography of activated T cell-conditioned medium revealed a major gelatinolytic band identified as pro-MMP-9 and a minor one identified as pro-MMP-2 (Fig. 3c). Under Hyp-DCHA (0.3, 0.6, 1.2 μ M), the zymography shows a clear, dose-dependent reduction of the MMP-9 gelatinolytic band (>50% at 0.6 μ M), and that of MMP-2 (barely visible) seems unaffected (Fig. 3c).

Hyp-DCHA effects on rats with EAE

Active immunization of Lewis female rats using CNS tissue suspended in CFA induces a T cell-mediated inflammation and antibody response and little demyelination. This is considered a reliable model, commonly used for preclinical testing of anti-inflammatory therapeutic strategies [19]. Immunized animals developed a severe EAE disease, which peaked at 12–14 days after immunization and was followed by a substantial recovery (Fig. 4a). At approximately Day 20, a less-severe relapse was observed. As expected, body weight followed the same course, showing a substantial decrease at the peak of the disease and a further recovery (Fig. 4b). Disease severity was significantly lower in animals treated with Hyp-DCHA [two-way ANOVA, group effect $F(1,176)=25.66$, $P<0.001$]. This is confirmed by the body weight of treated animals, which is higher than untreated animals [two-way ANOVA, group effect $F(1,72)=5.813$, $P<0.5$].

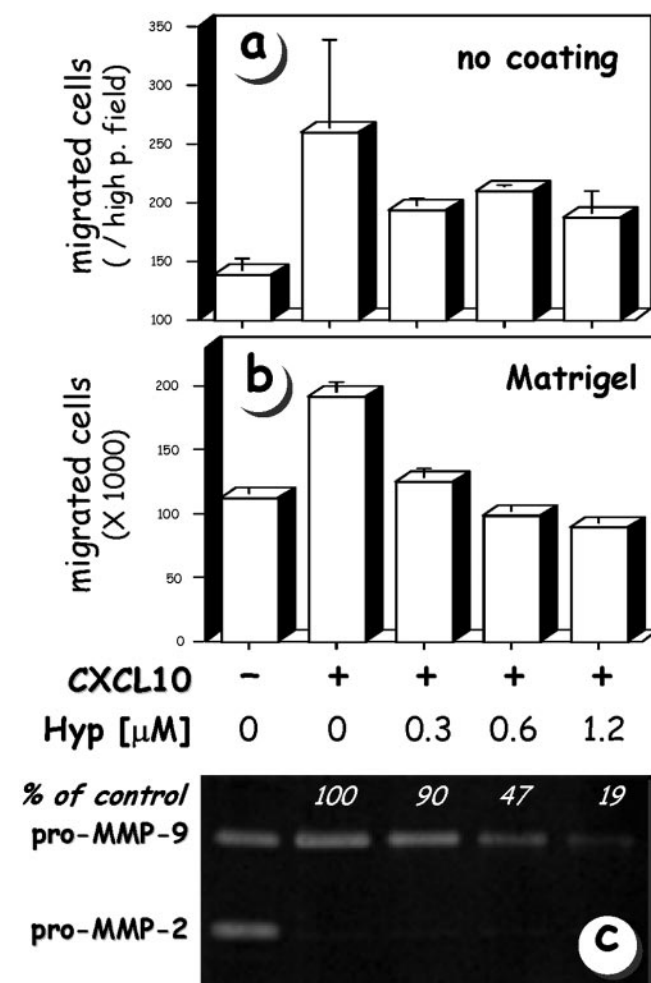


Fig. 3. Hyp-DCHA inhibits chemotaxis through gelatin and Matrigel and MMP-9 secretion by activated T cells. The modified Boyden chamber assay shows that lymphocyte chemotaxis toward CXCL10, through a filter without (a) or with (b) Matrigel coating, is restrained by below- μ M Hyp-DCHA in the upper chamber. Examples are of triplicate experiments in the upper histogram and the most effective one of sixuplicate in the lower histogram; average of quadruplicate \pm SD. (c) Gelatin zymography of equal volumes of medium conditioned by activated T cells shows that under Hyp-DCHA, the secretion of pro-MMP-9 is restrained in a dose-dependent manner down to 19% at 1.2 μ M; the first lane shows a pro-MMP-9 and pro-MMP-2 standard.

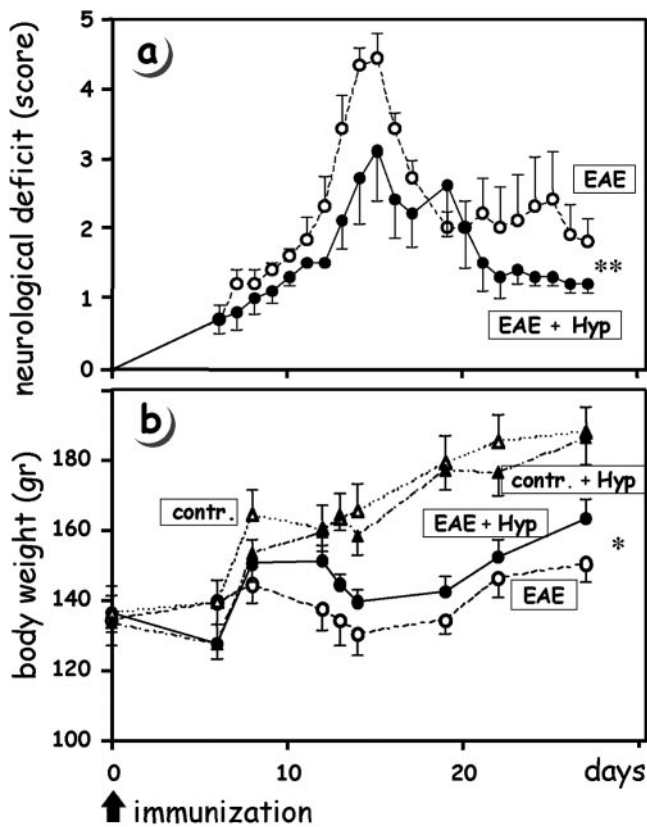


Fig. 4. Hyp-DCHA ameliorates the clinical EAE of rats. (a) Hyp-DCHA (Hyp) decreases disease severity in all phases of the disease, also preventing relapse; mean values (\pm SEM). Efficacy is confirmed by the higher body weight of EAE-treated animals (b). Statistical analysis: one-way ANOVA; *, $P < 0.05$; **, $P < 0.001$.

Histopathological examination of EAE animals revealed multiple, confluent foci of inflammation in white and gray matter of the spinal cord (Fig. 5, b and c). Mononuclear cells, which were immunoreactive for CD4 (Fig. 5g), were mostly observed, together with neutrophilic infiltration. Representative micrographs for quantitative evaluation of scores 1–3 are presented in Figure 5, d–f, respectively. Hyp-DCHA treatment effectively lowered central inflammation 27 days after immunization (Fig. 5c). Infiltrates, where present, were less severe, as confirmed by quantitative analysis (Fig. 5h).

DISCUSSION

The data reported here show that Hyp-DCHA inhibits IFN- γ production, with a down-regulation of T-bet gene expression and CXCR3 expression on IL-2/PHA-activated T cells, and attenuates the severity of the symptoms in rats with EAE. This molecule represents a novel approach for treatment of autoimmune inflammatory disease sustained by Th1 cells.

The role of Hyp-DCHA as an anti-inflammatory agent has been widely investigated. Hyp acts as a dual inhibitor of 5-LO and COX-1, key enzymes in the formation of proinflammatory eicosanoids from arachidonic acid, suggesting a therapeutic potential in inflammatory and allergic diseases connected to eicosanoids [6]. Furthermore, Hyp inhibits the generation of

ROS as well as the release of leukocyte elastase in human-isolated PMNL; blocks receptor-mediated Ca^{2+} mobilization in PMNL and monocytic cells; and targeting component(s) within G protein signaling cascades that regulate Ca^{2+} homeostasis [9], causes a rapid decline of the intracellular Ca^{2+} concentration in resting cells.

The balance of type 1 and type 2 T cells plays a major role in the regulation of cellular and humoral immune responses to give rise to an appropriate immune response against foreign antigens. Type 1 T cells are characterized by expression of the cytokine receptors IL-12R β 2-chain and IL-18R [20] and the chemokine receptors CXCR3, CCR5, CCR2 [21], and CXCR6 [22]. The transcription factor T-bet is associated with a type 1 response, such as IFN- γ activation [20]. The chemokine receptors CCR3 and CCR4 [21] and the transcription factor GATA-3 are associated with type 2 T cells [20]. We show here that micromolar doses of Hyp-DCHA down-regulate CXCR3 expression on IL-2/PHA-activated T lymphocytes, and the compound has a mild effect on CCR3 expression. We thus hypothesized a selective action of Hyp-DCHA on Th1 population, and indeed, a decrease of the percentage of CXCR3-positive cells has now been measured, which was paralleled by a significant decrease of CD4⁺/IFN- γ ⁺ (Th1) cells. The CD4⁺/IL-4⁺ (Th2) cells were not influenced significantly by the drug, and this result reinforces the initial hypothesis.

Furthermore, under 0.3–1.2 μM Hyp-DCHA (0.4 μM is the registered hematic value in patients under a usual antidepressant regimen; 900 mg/day *Hypericum* extract), the migratory capacity of activated T cells toward CXCL10, a specific ligand for CXCR3, was remarkably inhibited in the absence of an extracellular matrix barrier (uncoated filters) and abolished completely when the filters were coated with Matrigel (reconstituted BM). MMPs have been recognized as instrumental to the migration of cells across tissue barriers such as BMs, and T lymphocytes are widely dependent on secretion of MMP-2 and MMP-9 [23]. Hyp-DCHA has already been shown to inhibit secretion and activation of these gelatinases in various types of normal and transformed cell lines, remarkably blunting the raf/MEK/ERK signaling pathway [16, 17], and the present results show that it restrains MMP-9 secretion with a more pronounced effect on activated T lymphocytes with respect to resting T cells. More recently, we have shown that Hyp-DCHA restrains PMN chemokine-triggered migration and protects against inflammatory events taking place in animal models of angiogenesis and bleomycin-induced lung fibrosis [17]. We have herein observed a strong inhibition of chemoinvasion toward CXCL10, and as shown by cytofluorimetric analysis of nonmigrated cells, the inhibitory effect on chemoinvasion by Hyp-DCHA involved mainly Th1 cells (CD4⁺ IFN- γ ⁺).

Multiple sclerosis (MS) is an inflammatory/autoimmune disease with severe demyelination of the CNS, which is mediated by proinflammatory Th1 lymphocytes. Similar to MS, the pathology of EAE consists of an inflammatory infiltrate, primarily T cells and macrophages in the spinal cord and in the brain parenchyma [13, 24]. In EAE, leukocytes access the white matter of the CNS parenchyma by trafficking across the blood-brain barrier (BBB) vascular endothelium into the perivascular space. There is evidence that all activated T cells are capable

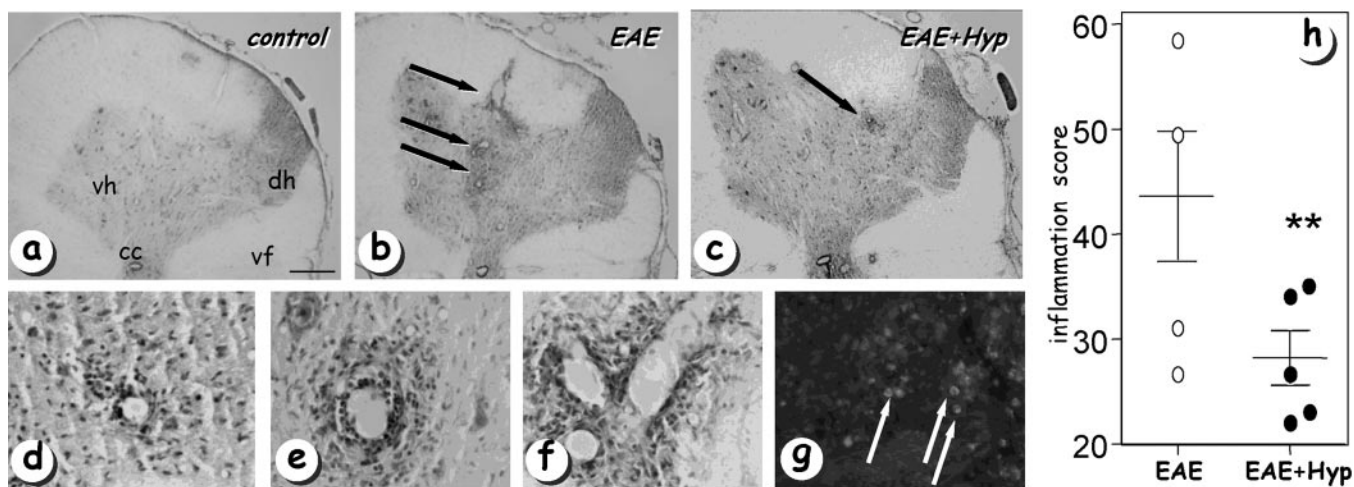


Fig. 5. Hyp-DCHA (Hyp) restrains inflammation in EAE rat spinal cord. Histopathological examination of rat lumbar spinal cord, 27 days after immunization. Control (a), EAE animals (b), EAE animals under Hyp-DCHA (c). cc, Central canal; dh, dorsal horn; vf, ventral funiculus; vh, ventral horn. Original bar, 100 μ m. Confluent inflammatory infiltrates and necrosis can be observed in the white and gray matter in EAE animals, mostly located in perivascular areas (arrows in b and c). Higher magnification micrographs (d–f) illustrate perivascular inflammatory cuff and immunofluorescence staining (g) in the presence of CD4 Th/inducer lymphocytes. (h) Quantitative evaluation indicates a positive effect of Hyp in reducing inflammatory cellular infiltrate; measurements in single animals are reported as untreated (○) and treated (●), and the horizontal bars indicate mean value. Statistical analysis: Student's *t*-test; **, *P* < 0.01. Animals treated with Hyp show less abundant and smaller cellular infiltrates (arrow in c) and almost absent necrotic areas.

of entering the CNS parenchyma [25]. Owing to the primary role of inflammation in the pathogenesis of MS and EAE, anti-inflammatory steroids are the first-line therapy for acute attack. Several anti-inflammatory agents have been used in EAE with controversial results, being protective or detrimental under different circumstances [26]. However, molecules acting on redox balance—as scavengers of ROS, such as α -lipoic acid [27], or as inhibitors of ROS generation, such as aminoguanidine [15]—ameliorate the EAE clinical course and histopathological index. It has also been demonstrated that the major green tea polyphenol, epigallocatechin-3-gallate, suppresses EAE in a murine model by limiting brain inflammation and reducing neuron damage [28]. Moreover, a substantial reduction of EAE severity has also been reported by preventive and therapeutic administration of methylthioadenosine, which probably acts through the suppression of TNF production and inducible NO synthase gene expression [29] or by aminoimidazole-4-carboxamide ribonucleoside, which inhibits the infiltration of inflammatory cells across the BBB.

More recently, promising results were obtained by drugs, specifically designed to target Th1 lymphocytes. Transmigration of T cells from the circulation to the brain parenchyma has been recognized as a primary pathogenic event in MS and thus, as a primary therapeutic target [30]. The humanized mAb to α -4 integrin, Natalizumab (Tybrasi, Biogen Idec, Cambridge, MA, USA/Elan, Dublin, Ireland), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of relapsing MS. This antibody prevented the interaction of lymphocytes with endothelial cells, thus reducing the transmigration of lymphocytes to areas of infiltration [31]. Published, randomized control of trials have indicated that Natalizumab, alone [32] or in combination with IFN- β 1a [33], reduced the risk of the sustained progression of disability and the rate of clinical relapse, thus, a highly promising drug. However, development of cases of progressive multifocal leukoencephalop-

athy in patients treated with Natalizumab led to withdrawal of this drug from the market [34] and subsequent resumption by FDA under a special restricted distribution program [35].

Here, we report a substantial reduction of EAE severity by Hyp-DCHA in a rat EAE model characterized by severe inflammation and scarce demyelination, which is coupled to a 40% reduction of inflammatory cellular infiltrates in the spinal cord. This model has been used to test antibody to α 4 β 1 integrin in rat preclinical studies [36], which has been developed afterwards in the drug Natalizumab. The strong down-regulation of the CXCR3 expression by Hyp-DCHA is a likely mechanism for this effect. In fact, inhibition of T cell activation by periplocoside E, which inhibits IFN- γ -dependent CXCR3 expression in T cells, is also effective in reducing EAE severity and mononuclear cell infiltration in the CNS [37]. Moreover, CXCR3 $-/-$ mice showed reduced EAE severity, and a mean EAE score was approximately 2.0 compared with 2.5–3.5 of wild-type animals [38]. Conversely, a neutralization therapy with decoy chemokine receptor DNAs encoding the binding site of CXCR3, aimed at neutralizing macrophage chemoattractive effects, suppressed relapse of chronic-relapsing EAE [39]. However, restraint in MMP-9 secretion might also have a part in the clinical and histopathological effects of Hyp-DCHA, as the antioxidant α -lipoic acid seems to reduce MMP-9 synthesis and secretion, thus ameliorating the EAE course [40]; targeting MMPs and leukocyte transmigration is indeed considered a potential therapy for MS [41].

Our findings thus suggest a potential, therapeutic action of Hyp-DCHA on autoimmune diseases sustained by Th1 cells. Further studies by our group are in progress to determine the activity of this drug in other autoimmune disease T cell-mediated animal models, including OVA-specific TCR transgenic mice, experimental autoimmune neuritis, and experimental autoimmune uveoretinitis.

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