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Preventive and Therapeutic Effects of Adenanthin on Experimental Autoimmune Encephalomyelitis by Inhibiting NF-κB Signaling

Qian-Qian Yin,* Chuan-Xu Liu,† Ying-Li Wu,† Shao-Fang Wu,* Yan Wang,‡ Xia Zhang,‡ Xiao-Juan Hu,‡ Jian-Xin Pu,§ Ying Lu,† Hu-Chen Zhou,† Hong-Lin Wang,‡ Hong Nie,§ Han-Dong Sun,§ and Guo-Qiang Chen*†

Adenanthin, a diterpenoid isolated from the leaves of Isodon adenanthus, has been reported to possess antileukemic activity through targeting peroxiredoxin II/II. However, its other potential activities remain to be explored. Using myelin oligodendrocyte glycoprotein (MOG)35–55-induced experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, we report in this study that adenanthin exerts efficaciously preventive and therapeutic effects on EAE accompanied by significant restriction of infiltration of inflammatory cells and demyelination in CNS. Adenanthin-presented immunomodulatory effects on EAE are correlated with suppressed proliferation of MOG35–55-reactive T cells, decreased Th1 and Th17 cells, increased regulatory T cell populations, decreased production of serum proinflammatory cytokines, and reduced stimulatory capacity of APCs, which might be mediated by its inhibitory action on NF-κB signaling pathway. Our results propose that, as a novel NF-κB inhibitor, adenanthin has potent immunomodulatory activity for the treatment of multiple sclerosis and possibly other autoimmune disorders. *The Journal of Immunology, 2013, 191: 2115–2125.

Multiple sclerosis (MS), a multifocal inflammatory demyelinating disease with progressive damage of CNS caused by an autoimmune response to self-Ags in a genetically susceptible individual (1), is becoming a leading cause for neurologic disability in young adults, especially in females (2). A complex inflammatory cascade involving both adaptive and innate immune system is thought to initiate and control the disease progression (3). Most likely, it involves dysfunction of T cell regulation and multiple signaling events such as NF-κB signaling pathway in inflammatory processes (4–6). Experimental autoimmune encephalomyelitis (EAE), which can be induced in susceptible rodents by immunization with myelin Ags such as myelin oligodendrocyte glycoprotein (MOG)35–55, shares similar characteristics in clinical symptoms and disease course to human MS. It has thus become a widely used animal model of MS (7).

Current disease-modifying drugs for treatment of MS include IFN-β, glatiramer acetate (GA), humanized VLA-4 mAb, and the recently approved oral drugs such as fingolimod (8–10). However, these treatments have the limited efficacy and/or significant safety concerns. Therefore, it is necessary to develop other safe and efficacious drugs for the treatment of MS. In recent decades, natural compounds isolated from higher plants as a source of therapeutic agents have been attracting great interests. Isodon species has long been used in Chinese popular folk medicine for the treatment of bacterial infections, inflammation, and cancers, and its bioactive components are regarded as diterpenoids (11, 12), which are a large and structurally diverse class of secondary metabolites and possess a broad spectrum of important biological activities (13, 14). Therefore, it is of great importance to explore the biological activities and targets of diterpenoids. In this study, we show that adenanthin, a diterpenoid isolated from the leaves of Isodon adenanthus, is effective prophylactically and therapeutically in inhibiting MOG35–55-induced EAE. Furthermore, the natural compound presents modulatory effects on autoreactive T cell responses, inflammatory cytokine pattern, and functions of APCs, and these potent immunomodulatory effects of adenanthin are associated with suppression of NF-κB signaling. Collectively, our data demonstrate that adenanthin presents a promising anti-inflammatory property, and its therapeutic potentials for treatment of MS and possibly other autoimmune disorders warrant further investigation.

Materials and Methods
Reagents and cell lines

Adenanthin was isolated from the dried aerial parts of I. adenanthus and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) at 40 nM (20 mg/ml).
Biotin-tagged adenanthin was synthesized as described previously (15). TNF-α and LPS were purchased from Sigma-Aldrich. Abs against NF-κB/p65 (C-20), p50, and OCT-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IκBα, anti–phospho-IκBα (Ser32), anti–IκB kinase (IKK)α/β, and anti-IKKβ were acquired from Cell Signaling Technology (Beverly, MA). Human acute monocytic leukemic cell line THP-1, mouse macrophage-like cell line RAW264.7, human acute pro-myelocytic leukemic cell line NB4, and HEK 293T cells were maintained using electroporation (designated as THP-1NF-, RAW264.7NF-, CD4+ T cells, CD11b+ cells, or CD11c+ cells purchased from BD Biosciences). Th2 systemic inflammation was induced by s.c. immunization on the upper dorsal flanks with 500 ng/ml MOG peptide for 24 h, followed by stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of GolgiPlug for 5 h. Cells were surface stained with mAbs against CD4 and then stained for intracellular cytokines with Abs for 30 min on ice. To analyze MOG-specific Th1 and Th17 cells, splenocyte mononuclear cells (MNCs) were stimulated with 20 μg/ml MOG peptide for 24 h, followed by stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of GolgiPlug for 5 h. Cells were surface stained with Abs against CD4. The cells were then washed, fixed, and permeabilized with Cytofix/Cytoperm buffer, and intracellular cytokines were stained with Abs against IL-17 and IFN-γ (BD Biosciences). Foxp3 staining was carried out using a commercial kit according to the manufacturer’s instructions (eBioscience). Flow cytometric analysis was performed on a BD FACSCalibur (BD Biosciences) and results were analyzed using FlowJo 7.0 software (Tree Star, Ashland, OR).

Nuclear extracts of THP-1 cells or HEK 293T cells, which were transiently transfected with pCMV4-FLAG-p65 expression vector by PolyFect (Qiagen), were prepared with the NE-PER nuclear extraction reagent (Pierce Biotech) and biotin-labeled using a biotin 3'-end DNA labeling kit (9-end DNA labeling kit, Ambion, Austin, TX). The wild-type oligonucleotides (5’-AGTT-GAGGGACTTTCCCAGGC-3’ and 5’-GGCTTGAAGAATCCCTT-CACACT-3’) were biotin-labeled using a biotin 3’-end DNA labeling kit (Pierce Biotech) and annealed with their complementary strands. Reaction mixtures were then separated on a 5% native polyacrylamide gel, and shifted bands that corresponded to protein/DNA complexes were captured by a HRP-based detection system.

Pulldown of adenanthin-bound proteins
THP-1 cell lysates were incubated with biotin or biotin-adenanthin beads in the absence and presence of unlabeled adenanthin overnight at 4˚C as
described previously (15), and the bead-bound proteins were detected by Western blot for proteins as indicated.

**IKKβ kinase activity assay**

The IKKβ kinase activity was performed by DELFIA assay using recombinant human active IKKβ kinase protein (Cell Signaling Technology) in the presence or absence of adenanthin at different concentrations with biotinylated IκBα peptide as the substrate according to the manufacturer’s instructions of an HTScan IKK kinase assay kit (Cell Signaling Technology).

**Western blot**

Cell lysates were equally loaded onto SDS-PAGE, electrophoresed, and transferred to ECL nitrocellulose membranes (Amersham Biosciences). After blocking with 5% nonfat milk in TBS, the membranes were incubated with indicated Abs overnight at 4˚C, followed by HRP-linked secondary Ab (Cell Signaling Technology) for 1 h at room temperature. Detection was performed by a SuperSignal West Pico chemiluminescent substrate kit (Pierce).

**Real-time quantitative RT-PCR**

Total RNA was isolated by a TRIzol kit (Invitrogen, Carlsbad, CA). RNA was treated with DNase (Promega). cDNA was synthesized according to the manufacturer’s instructions. Real-time quantitative PCRs were performed with SYBR Green PCR Master Mixture reagents (Applied Biosystems, Foster City, CA) on the ABI Prism 7900 system (Applied Biosystems). Primers are summarized in Supplemental Table I.

**Statistical analysis**

An unpaired Student t test was used to evaluate the difference between two different treatments. A p value <0.05 was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Irvine, CA).

**Results**

**Adenanthin effectively delays the onset and suppresses the severity of EAE**

Adenanthin is a single diterpenoid molecule with 99.9% purity (Supplemental Fig. 1). To investigate its potential effects on EAE, we i.p. administered adenanthin at 10 and 20 mg/kg daily from day 3 before immunization with MOG35–55 peptide for the prevention protocol. Notably, mice could tolerate these doses of adenanthin without the remarkable macroscopic damages of important organs such as kidney, lung, heart, stomach, and spleen, suggesting that adenanthin had no general toxicity at least at the doses used. However, adenanthin presented a dose-dependent preventive effect on EAE and 20 mg/kg adenanthin had the greatest effect. Notably, vehicle treatment had similar effects on EAE mice compared with untreated controls (Supplemental Fig. 2). Therefore, we focused on the potential preventive and therapeutic effects of adenanthin at 20 mg/kg on EAE mice only with the vehicle treatment as control. This dose of adenanthin was administered daily for the prevention protocol as described above or on day 8 after immunization with MOG35–55 peptide for the treatment protocol, and the occurrence of clinical signs of EAE and body weight of mice were continuously monitored. As depicted in Fig. 1, mice with vehicle treatment developed clinical symptoms from day 7 to 8 and reached a maximum clinical score at day 18 after immunization, whereas mice with adenanthin application in the prevention protocol showed no symptoms of EAE until day 22 and the symptoms were also significantly inhibited compared with those of vehicle EAE mice (Fig. 1A). GA, a synthetic random basic copolymer, has been approved for treatment of MS for >2 decades (19, 20). In this study, we used GA at 400 μg/mouse, a similar dose that was used in human patients (17, 18), as a positive control in the treatment protocol. The results showed that GA treatment was effective in reducing the clinical scores of EAE. Likewise, adenanthin treatment showed significant inhibitory effect on the severity of EAE compared with vehicle control (Fig. 1B). In line with these observations, mice in prevention and treatment protocols with adenanthin administration also presented significantly less weight loss than did vehicle EAE mice along the progression of disease (Fig. 1C, 1D). All of these data support that adenanthin is an effective natural compound for the prevention and treatment of EAE.

**Adenanthin inhibits infiltration of inflammatory cells and demyelination in CNS**

Pathologically, EAE is characterized by infiltration of inflammatory cells and severe demyelination in CNS (21). Thus, we performed histological analysis of inflammatory infiltration and demyelination of spinal cord at day 18 after immunization, the peak phase of EAE disease in vehicle control mice. As demonstrated in Fig. 2A, the vehicle EAE mice presented profound infiltration of MNCs and demyelinating lesions in spinal cord compared with naive mice, as assessed by H&E staining (Fig. 2A) and Luxol fast blue staining (Fig. 2B), respectively. In the prevention protocol, as expected, adenanthin could effectively restrict the development of these EAE-related histological signs, which were also remarkably ameliorated in the treatment protocol with adenanthin and GA as well (Fig. 2A, 2B). Consistently, the percentages and numbers of infiltrating CD4+ T cells and F4/80+ macrophages in CNS, the two prominent inflammatory cell subtypes in active lesions of CNS of EAE mice (22), were markedly decreased in adenanthin prevention and adenanthin/GA treatment groups compared with those of the vehicle EAE mice (Fig. 2C).

**Adenanthin modulates MOG35–55-reactive CD4+ T cell responses**

Adenanthin treatment did not reduce the viability of MNCs in spleen and lymph nodes (LNs) of EAE mice at day 18 after immunization (Fig. 3A), suggesting that adenanthin-mediated amelioration of EAE was not due to the nonspecific toxic effects on MNCs. It is thought that the activation of autoreactive CD4+ T cells and their differentiation into different Th effector subsets are crucial events in the pathogenesis of MS (23), where Th1 and Th17 effector subtypes are critically involved in the development of autoimmune diseases, whereas regulatory T cells (Tregs) act on maintaining self-tolerance (24). To examine whether adenanthin has impacts on MOG35–55-reactive T cell responses, the proliferation of splenocytes from adenanthin- or vehicle-treated EAE mice at day 18 after immunization was examined upon the in vitro restimulation by MOG35–55 peptide. As illustrated in Fig. 3B, MOG35–55-stimulated proliferation of splenocytes derived from vehicle EAE mice in a dose-dependent manner whereas the stimulatory effect was far less on splenocytes from adenanthin-treated EAE mice. Of note, splenocytes from naive mice had no response in proliferation to MOG35–55 peptide. As depicted in Fig. 3C, in vitro proliferation of MOG35–55-specific CD4+ T cells derived from splenocytes of vehicle EAE mice at day 18 after immunization were pretreated with vehicle or adenanthin, followed by coculture with splenic APCs loaded with or without 20 μg/ml MOG35–55. The results showed that adenanthin significantly inhibited the proliferation of MOG35–55-specific CD4+ T cells (Fig. 3E). As depicted in Fig. 3F, adenanthin-treated EAE mice presented the reduced splenic IFN-γ-producing Th1 and IL-17–producing Th17 cells (3.67 ± 0.42 versus 12.43 ± 0.91%; p < 0.05; 0.77 ± ...
in both splenic CD11b decreased PD-L1 but without significant changes of MHC class II expressions of costimulatory molecules CD80 and CD86 and vehicle-treated EAE mice presented substantially increased CD80, CD86, and PD-L1 (25). Compared with naive mice, the together with the expression of costimulatory molecules, such as presentation through the tricomplex of the MHC, Ag, and TCR to-

together with the expression of costimulatory molecules, such as CD80, CD86, and PD-L1 (25). Compared with naive mice, the vehicle-treated EAE mice presented substantially increased CD80, CD86, and PD-L1 as compared with those of vehicle EAE mice (Fig. 4C). All of these data suggest that adenanthin suppresses the stimulatory capacity of APCs.

Adenanthin suppresses the stimulatory capacity of APCs

APCs are important for the full activation of T cells by Ag presentation through the tricomplex of the MHC, Ag, and TCR together with the expression of costimulatory molecules, such as CD80, CD86, and PD-L1 (25). Compared with naïve mice, the vehicle-treated EAE mice presented substantially increased expressions of costimulatory molecules CD80 and CD86 and decreased PD-L1 but without significant changes of MHC class II in both splenic CD11b+ and CD11c+ APCs, the important APCs for T cell activation (26, 27). Upon adenanthin treatment, MHC class II and CD80, but not CD86, were substantially decreased, whereas PD-L1 was markedly upregulated as compared with those of vehicle-treated EAE mice (Fig 4A). Next, purified splenic CD11b+ or CD11c+ APCs from adenanthin- or vehicle-treated EAE mice were cocultured with MOG35–55-reactive CD4+ T cells derived from EAE mice in the presence or absence of 20 μg/ml MOG35–55, followed by detection of T cell proliferation. The results demonstrated that both CD11b+ and CD11c+ APCs from adenanthin-treated EAE mice led to significantly reduced MOG35–55-reactive CD4+ T cell proliferation (Fig. 4B). Additionally, splenic CD11b+ and CD11c+ APCs from adenanthin-treated mice expressed fewer cytokines involved in shaping Th1 and Th17 differentiation (28), including IL-1β, IL-6, IL-12, and IL-23, compared with those of vehicle EAE mice (Fig. 4C). All of these data suggest that adenanthin suppresses the stimulatory capacity of APCs.

Adenanthin is a potent inhibitor of NF-κB signaling

There is increasing evidence showing the involvement of NF-κB activation in T cell activation (29–31), functions of APCs (32, 33), and induction of CNS damage in EAE (34–36). To elucidate whether adenanthin potentially regulates the NF-κB signaling pathway, we transfected an NF-κB response element–containing luciferase reporter plasmid to THP-1 (THP-1NF-κB), followed by the stimulation of TNF-α and LPS, respectively. The results showed that adenanthin significantly inhibited TNF-α– or LPS-stimulated NF-κB response element–driven luciferase expression in a dose-dependent manner (Fig. 5A). As is well known (37, 38), the canonical NF-κB activation in response to TNF-α and LPS proceeds sequentially through activation of IKK, phosphorylation, ubiquitination, and degradation of inhibitor of NF-κB (IκBα), followed by the release of NF-κB to nucleus, where it binds with the promoter of the targeted genes. EMSA with nuclear extracts of THP-1 cells, which were preincubated with or without adenanthin and followed by TNF-α stimulation, showed that adenanthin dose-dependently antagonized TNF-α-stimulated NF-κB DNA probe complex formation (Fig. 5B). Immunofluorescent staining and fractionation-based immunoblots also showed that adenanthin significantly blocked nuclear translocation of NF-κB induced by TNF-α or LPS (Fig. 5C, 5D, Supplemental Fig. 3A). Further analysis showed that
Adenanthin blocked TNF-α– or LPS-triggered IKK phosphorylation, and thus IκBα phosphorylation and degradation. Moreover, adenanthin also inhibited TNF-α– or LPS-induced phosphorylation of p65, whereas the expressions of IKKβ, p65, or p50 proteins were unaffected (Fig. 5E, Supplemental Fig. 3B). Notably, adenanthin significantly suppressed TNF-α– and LPS-induced upregulation of the NF-κB–dependent genes involved in immunomodulatory action (Fig. 5F).

**Adenanthin inhibits NF-κB signaling in vivo**

The above findings prompted us to investigate whether adenanthin inhibits NF-κB activity in EAE mice. The HLL transgenic mice, in which luciferase expression is driven by the NF-κB–dependent portion of the HIV-1 long terminal repeat (16), were immunized with MOG35–55 peptide and received 20 mg/kg adenanthin according to the prevention and treatment protocols as described above. Compared with naive mice, the vehicle EAE mice exhibited significantly increased phosphorylation of IκBα together with decreased IκBα and increased phosphorylation of p65 in LNs of EAE mice at day 18 after immunization, which were remarkably antagonized by adenanthin treatment (Fig. 6A). Furthermore, immunofluorescent staining also showed that adenanthin administration in the prevention and treatment protocols remarkably blocked IκBα phosphorylation on Ser32 in spinal cords of EAE mice at day 18 after immunization (Fig. 6B, top panel). Notably, DAPI staining also supported the above-mentioned fact that the infiltrating MNCs of spinal cords in EAE mice were dramatically reduced by adenanthin treatment in vivo (Fig. 6B, bottom panel).
FIGURE 3. Effects of adenanthin (Ade) on MOG35-55-reactive T cell responses. (A) MNCs of spleen and LNs were derived from Ade- or vehicle-treated EAE mice at day 18 after immunization for the treatment protocol and cell viability percentages were evaluated by trypan blue exclusion assay. (B) Splenocytes derived from Ade- or vehicle-treated EAE mice at day 18 after immunization were examined ex vivo for proliferation in the presence of different concentrations of MOG35-55 peptide measured by [3H]thymidine incorporation. (C) Splenocytes derived from EAE mice at day 18 after immunization were treated with different concentrations of Ade and cell viability was measured by Cell Counting Kit-8 assay. (D) Splenocytes derived from EAE mice at day 18 after immunization were incubated with different concentrations of Ade in the challenge of MOG 35-55 peptide (20 μg/ml), followed by the detection for proliferation by [3H]thymidine incorporation. (E) MOG35-55-reactive splenic CD4+ T cells purified from EAE mice at day 18 after immunization were pretreated with Ade or vehicle, followed by coculture with splenic APCs of EAE mice in the presence or absence of MOG 35-55 peptide (20 μg/ml). Cell proliferation was then examined by [3H]thymidine incorporation. (F) Intracellular cytokine staining of IL-17, IFN-γ, and Foxp3 in splenic CD4+ T cells from Ade- or vehicle-treated EAE mice restimulated with 20 μg/ml MOG35-55. The values are representative data of an independent experiment. (G) Sera was harvested from Ade- or vehicle-treated EAE mice at day 18 after immunization for the cytokine Ab array analysis. *p < 0.05 versus vehicle-treated EAE mice.

The in vivo bioluminescence imaging clearly showed that adenanthin inhibited NF-κB activation in the CNS of EAE mice at day 18 after immunization for both the prevention and treatment protocols (Fig. 6D, 6E). In line with this, the inhibition of NF-κB activation by adenanthin treatment could also be observed in purified splenic CD4+ T cells and CD11b+ APCs from adenanthin-treated EAE mice (Fig. 6F). Several NF-κB-dependent proinflammatory cytokines and chemokines were also transcriptionally downregulated in splenocytes of adenanthin-treated EAE mice (Supplemental Fig. 3C).

To exclude the possibility that the in vivo actions of adenanthin on NF-κB are secondary to the overall diminished disease rather than a direct effect of the drug itself, we also examined NF-κB activation before the onset of EAE (day 6 after immunization) in response to the prevention protocol. As shown in Fig. 6G, MNCs of LNs from adenanthin-treated EAE mice exhibited the suppressed NF-κB activation compared with those of vehicle EAE mice. The inhibitory effect of adenanthin on NF-κB activation could also be confirmed by the in vivo bioluminescence imaging at the manifestation phase (day 12 after immunization) for both the prevention and treatment protocols (Supplemental Fig. 3D). Additionally, we treated splenocytes from vehicle-treated EAE mice at day 8 after immunization (the onset of EAE disease) with different concentrations of adenanthin in the presence of MOG35-55 peptide, and the results showed that MOG35-55 restimulation induced NF-κB activation, which could be inhibited by adenanthin in a dose-dependent manner (Fig. 6H).

Adenanthin targets the p65 subunit of NF-κB and IKKβ

Previously we reported that adenanthin targets and inactivates Prx I/II proteins for its antileukemic activities (15, 39). To assess whether Prx I/II proteins account for adenanthin-mediated inhibition of NF-κB activation, we knocked down the expression of Prx I protein by two pairs of specific small interfering RNAs (siRNAs; siPrx I/no. 1 and siPrx I/no. 2) with the scrambled negative siRNA as a control in THP-1 NF-κB cells, which are naturally absent of Prx II protein (Fig. 7A). The results showed that siPrx I/no. 1 significantly reduced Prx I expression, whereas siPrx I/no. 2 and the control had no effect on Prx I expression (Fig. 7B). However, Prx I knockdown failed to inhibit TNF-α–induced NF-κB activation (Fig. 7B), which excluded the possibility that Prx I protein contributes to adenanthin-induced suppression of NF-κB activation. Thus, we used synthetic biotin-tagged adenanthin (biotin-adenanthin) as a chemical probe to identify whether adenanthin might directly act on the NF-κB signaling. For this, THP-1 cell lysates were incubated with biotin-adenanthin or free biotin. The mixtures were then precipitated with streptavidin agarose beads and the precipitates were further blotted with Abs against p65, p50, IKKα, IKKβ, or IκBα. As shown in Fig. 7C, biotin-adenanthin effectively pulled down p65 and IKKβ, which could be competitively inhibited by higher concentrations of unlabeled adenanthin, indicating that p65 and IKKβ might be adenanthin-bound proteins. To determine whether adenanthin directly inhibits the DNA binding activity of NF-κB, we transfected p65-
expressing plasmids into HEK293T cells, followed by TNF-α (10 ng/ml) treatment for 30 min. Then, its nuclear extracts were incubated with different concentrations of adenanthin for EMSA assay. The results demonstrated that adenanthin suppressed the DNA binding activity of recombinant p65 protein in a dose-dependent manner (Fig. 7D). EMSA assay with the nuclear extracts of TNF-α–activated THP-1 cells incubated with adenanthin at different concentrations also demonstrated that adenanthin suppressed the DNA binding of NF-κB in a dose-dependent manner (Fig. 7E). Notably, adenanthin was shown to have no effect on DNA binding of OCT-1, indicating that the effect of adenanthin-mediated inhibition of NF-κB binding was specific (Fig. 7D, 7E). Furthermore, we incubated the recombinant human active IKKβ with adenanthin at different concentrations in vitro and then examined the kinase activity through a DELFIA assay. The result showed that adenanthin directly inhibited the activity of IKKβ in vitro in a dose-dependent manner (Fig. 7F). The suppressed kinase activity was also observed when TNF-α–activated THP-1 cell lysates were incubated with adenanthin at different concentrations (Supplemental Fig. 3E).

Discussion
Given the application of the plant I. adenanthus as a medicinal herb for enteritis and dysentery in the Yunnan Province of China, we investigated the role of adenanthin in inflammatory diseases such as the autoimmune disease MS. Our previous pharmacokinetic studies showed that adenanthin, when it was given as a single dose of 5 mg/kg in adult mice by tail vain injection, had a relatively high plasma clearance, although a mean maximum plasma drug concentration of 6.36 μM could be reached (15). Therefore, in this study we used higher doses of adenanthin to explore its potential effects on EAE. Our results showed that adenanthin exerted both preventive and therapeutic effects on EAE, as evidenced by the marked improvement of clinical scores and weight loss as well as the reduced infiltration of inflammatory cells and demyelination in the CNS.

To our knowledge, this is the first report on the efficacy of adenanthin in EAE. We also found that adenanthin treatment displayed no significant toxicity and did not reduce the cell viability of T cells in vivo and in vitro, suggesting that the effects of adenanthin on EAE were not caused by the nonspecific cytotox-
icity. Studies of MOG35–55-reactive T responses in spleen showed that adenanthin inhibited MOG35–55-reactive T cell proliferation, decreased Th1/Th17 differentiation, and increased Treg populations. Recently, the critical role of a Th17/Treg imbalance has been highlighted in the pathogenesis of T cell–mediated autoimmune disease such as rheumatoid arthritis (40), and the shift in predominance from Th17 to Tregs has attracted great clinical interest (41, 42). This warrants further study to elucidate how adenanthin affects CD4 T cell differentiation and especially Th17/Treg differentiation via a direct or indirect effect.

Because adenanthin significantly delayed the onset of EAE, we proposed that adenanthin might inhibit the early events of T cell activation. Given that the proper functions of APCs are necessary for the activation and differentiation of T cells and have been implicated in the pathogenesis of MS/EAE (43), we showed that adenanthin treatment significantly reduced the stimulatory power of EAE-activated APCs.

**FIGURE 5.** In vitro inhibition of NF-κB activation by adenanthin (Ade). (A) THP-1NF-κB and RAW264.7NF-κB cells were pretreated with Ade for 4 h at different concentrations followed by stimulation with TNF-α and LPS, respectively, for 30 min. The transcriptional activity was determined by measuring the luciferase activity. (B–D) THP-1 cells were treated with Ade for 4 h at different concentrations followed by TNF-α stimulation for 30 min. (B) Nuclear extracts were incubated with biotin-labeled NF-κB consensus oligonucleotides and analyzed for NF-κB DNA binding activity by EMSA. Nonspecific band. (C) Cell samples were fixed and analyzed for the distribution of NF-κB by immunofluorescence staining using anti-p65 Ab. Scale bars, 7.5 μm. (D) p65 and p50 proteins were detected by immunoblotting in whole cell extracts (total), fractionated, nucleus and cytoplasm with β-actin/lamin B as loading controls. (E) Cell lysates of THP-1 cells pretreated with Ade for 4 h followed by TNF-α stimulation for different times were collected and analyzed by immunoblotting using Abs against the indicated proteins. (F) mRNA levels of genes indicated were determined by quantitative real-time PCR in THP-1 and RAW264.7 cells pretreated with Ade for 4 h followed by stimulation with TNF-α and LPS, respectively, for 30 mins. All experiments were repeated three times with similar results.
potential of both CD11b+ APCs and CD11c+ APCs, which were correlated with suppressed expressions of MHC class II and the costimulatory CD80 as well as increased expression of the negative costimulatory signal PD-L1. Based on the current view of the immunoregulatory effect of adenanthin in vivo, we propose that adenanthin might possess synergistic properties in vivo by modulating T cell responses and APC functions and it may represent a novel strategy for the development of highly effective agents for the treatment of autoimmune diseases and transplant rejection. However, it remains to be determined whether adenanthin could also affect other inflammatory cells involved in T cell activation (e.g., B cells, NK cells).

The dysregulation of NF-κB signaling involving induced transcription of proinflammatory target genes (e.g., cytokines, chemokines, and adhesion molecules) has been linked to the pathogenesis of human inflammatory disorders, including MS (4, 6). Various efforts have been made to clarify the predominant role of NF-κB in the pathogenesis of MS/EAE and to prove the NF-κB signaling pathway as a highly attractive target for therapeutic development (6, 31). Our in vitro investigations showed that adenanthin inhibited NF-κB activation of cell lines induced by TNF or LPS stimulation in a dose-dependent manner. Adenanthin also inhibited NF-κB activation at different phases of EAE disease in vivo, supporting that the amelioration of adenanthin on EAE was closely associated with suppression of NF-κB activation.

Previously, we reported that a denanthin directly targets and inhibits the peroxidase activities of Prx I/II to induce leukemic cell differentiation (15, 39). Prx I has recently been shown to be secreted and to act as a proinflammatory factor mainly through binding to TLR4 (44), and extracellular Prx immunoreactivity is independent of its peroxidase activity in most cases (45). However, Prx I knockdown failed to inhibit TNF-α–induced NF-κB activation, which excluded the possibility that Prx I protein contributes to adenanthin-induced suppression of NF-κB activation. On the contrary, using synthetic biotin-tagged adenanthin as a chemical probe, we found that adenanthin bound with the p65 subunit of NF-κB and IKKβ, and this binding inhibited the DNA binding ability of NF-κB as well as the kinase activity of IKKβ.

FIGURE 6. In vivo inhibition of NF-κB activation by adenanthin (Ade). MOG35–55-immunized HLL transgenic mice were treated by adenanthin as described in Fig. 1. (A) Proteins of LNs isolated from Ade- or vehicle-treated HLL EAE mice were subjected to immunoblot analysis for determination of NF-κB signaling. (B) Representative staining with anti–p-IκBα (Ser32) Ab in spinal cord sections from each group. High magnification images are shown in bottom panels. Scale bars, 100 μm. (C) Integral OD (IOD) in white matter was calculated for three sections in each group by Image-Pro Plus software. (D) HLL mice of different groups were analyzed for NF-κB activity by measuring the luciferase activity through bioluminescence imaging. (E) Quantitative results were analyzed for five mice of each group and expressed as total photon values per second (Total Flux [p/s]). (F) Purified CD4+ and CD11b+ splenocytes at day 18 after immunization were analyzed for NF-κB activity by measuring the luciferase activity. (G) MNCs from LNs isolated from Ade- or vehicle-treated EAE mice at day 6 after immunization in the prevention protocol were subjected to immunoblot analysis for determination of NF-κB signaling. (H) Splenocytes derived from vehicle-treated EAE mice at day 8 after immunization were treated with or without Ade at different concentrations for 12 h in the presence or absence of 20 μg/ml MOG35–55 and subjected to immunoblot analysis for determination of NF-κB signaling. Data are representative of three independent experiments. *p < 0.05 versus vehicle-treated EAE mice, #p < 0.05 versus naive mice.
in vitro. Taken together, these results strongly suggest that adenanthin can target several proteins for its different functions, and it can serve as a novel NF-κB inhibitor by targeting p65 and IκBα.

Many drugs currently in use as anti-inflammatory and immunosuppressive therapeutics, such as glucocorticoids and nonsteroidal anti-inflammatory agents, have been shown to directly or indirectly interfere with the NF-κB pathway (46, 47). It might be possible to develop NF-κB inhibitory compounds with high efficacy by selectively targeting specific NF-κB subunits or signaling components that are crucially involved in a particular disease. Recently, increasing evidence has indicated the important role of IκBβ in the development of EAE (36, 48). Likewise, other studies have also shown that IκBβ is important in rheumatoid arthritis synovioctyes and that p65 is associated with inflammatory bowel disease (49, 50), which might lead to studies that explore the therapeutic potential of adenanthin on the animal models for these diseases. So adenanthin could be the template to generate NF-κB inhibitors with increased selectivity and high efficacy.

In summary, our present study demonstrates the promising anti-inflammatory properties of the natural product adenanthin and its therapeutic potentials for MS and possibly other autoimmune disorders.

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Disclosures

The authors have no financial conflicts of interest.

References

Fig. S1. The purity analysis of adenanthin and its $^1$H NMR spectrum.

Up panel: Adenanthin was purified by semipreparative HPLC, and then was repeatedly recrystallized to yield near 99.9 % purity. HPLC analysis was performed on an Agilent 1200 liquid chromatograph with a Zorbax SB-C$_{18}$, 4.6 mm × 25 cm
column (1 mL/min, detector UV $\lambda_{\text{max}}$ 238 nm, MeCN/H$_2$O 45:55, $t_R = 10.561$).

Bottom panel shows the $^1$H NMR spectrum of adenanthin.

Fig. S2. Amelioration of EAE by adenanthin in a dose-dependent manner. C57BL/6 mice were immunized with MOG$_{35-55}$ and were administered daily i.p. of different doses of adenanthin (Ade) or vehicle control and untreated control (normal saline injection) for the prevention protocol. Each group consisted of 8 mice. Mice were monitored and evaluated for clinical score daily. Data are representative of three independent experiments.
Fig. S3. Inhibition of NF-κB activation by adenanthin *in vitro* and *in vivo*. 

A, RAW264.7 cells were pretreated with adenanthin for 4 h at different concentrations followed by LPS activation for 30 mins. Cells were fixed and analyzed for the distribution of NF-κB by immunofluorescence staining using anti-p65 antibody. Scale bars = 7.5 μm. B, cell lysates were collected and analyzed by western blotting using antibodies against the indicated proteins. All experiments were repeated for 3 times with similar results. C, splenocytes from Ade- or vehicle-treated EAE mice at day 18 post immunization were analyzed for mRNA expression of the indicated cytokines by quantitative real-time PCR. D, HLL mice of different groups were analyzed for NF-κB activity by measuring the luciferase activity through bioluminescence imaging at the manifestation phase of disease (at day 12 post immunization). Data are representative of three independent experiments. Symbols * and # represent $p <0.05$ vs. vehicle-treated EAE mice and naïve mice, respectively. E, cell extracts of
TNF-α-activated THP-1 were incubated with adenanthin at different concentrations and phosphorylation of IκBα (ser32) was detected through immunoblotting.

**Supplementary Table : Primers used for real-time PCR assays**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
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<tr>
<td>hIL8</td>
<td>F: 5’-CAGAGACAGACAGACACACA-3’</td>
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<tr>
<td></td>
<td>R: 5’-TTCCCTGGGGGTCCAGACACA-3’</td>
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<td>hIκBα</td>
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<td>hβ-actin</td>
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<td>R: 5’-AGCCTGGATAAGCAACGTACATG-3’</td>
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<td>mTNF-α</td>
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<td></td>
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<td>mβ-actin</td>
<td>F: 5’-TGTCACCTCCAGCACAGATG-3’</td>
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<tr>
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<td>R: 5’-AGCTCAGTAAACAGTCCCGCTAG-3’</td>
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Notes: F, forward primers; R, reverse primers; h, human; m, mouse.