Anti-Inflammatory Properties and Regulatory Mechanism of a Novel Derivative of Artemisinin in Experimental Autoimmune Encephalomyelitis

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Ethyl 2-[4-[(12-β-arterisininioxy)phenoxylpropionate (SM933) is a novel derivative of artemisinin, an herbal compound approved for the treatment of malaria. In this study, we show that SM933 has unique anti-inflammatory properties through regulation of signaling pathways, leading to amelioration of experimental autoimmune encephalomyelitis. The anti-inflammatory properties of SM933 were characterized by inhibition of encephalitogenic T cell responses that were altered to exhibit a Th2 immune deviation and reduced activity and concentration of NO and inducible NO synthase. The observed effect of SM933 was mediated through regulatory mechanisms involving the NFκB and the Rig-G/JAB1 signaling pathways. SM933 was found to inhibit the activity of NFκB by up-regulating IκB, which accounted for various downstream anti-inflammatory actions. Furthermore, it up-regulated Rig-G through the action of IFN-α and prevented JAB1, a master cell cycle regulator, from entering the nucleus to promote p27 degradation, resulting in down-regulation of CDK2 and cyclin A and cell cycle progression. Regulation of the Rig-G/JAB1 pathway by SM933 led to altered cell cycle activity of encephalitogenic T cells as a result of its selective effect on activated, but not resting, T cells. The study indicates that SM933 is a novel anti-inflammatory agent acting through defined signaling mechanisms and provides regulatory mechanisms required for effective drug targeting in treatment of autoimmune disease and inflammation. The Journal of Immunology, 2007, 179: 5958–5965.

Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; iNOS, inducible NO synthase.

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as exemplified in experimental autoimmune encephalomyelitis (EAE), an animal model for MS. Artemisinin, or qinghaosu, is the active component of Artemisia annua L. and is approved worldwide for the treatment and prevention of malaria (11, 12). It is a sesquiterpene trioxane lactone, containing an endoperoxide bridge essential for activity (13). There are preliminary indications in the Chinese medical literature that it has undefined anti-inflammatory and antitumor activities (14). The study was designed to probe how SM933 regulates autoimmune responses through specific interaction with various signaling pathways involved in proliferation and inflammatory properties of encephalitogenic cells. The results presented here provide an important example of the manner in which Chinese herbal medicine can be explored to identify effective anti-inflammatory compounds as well as specific signaling targets that are required for effective blocking of inflammatory processes in pathological conditions.

**Materials and Methods**

**SM333**

An artemisinin derivative, SM933, was synthesized chemically by coupling dihydroartemisinic acid with 2-(4-hydroxyphenyl)propiolic acid ethyl ester (15). The resulting compound used in this study demonstrated a purity of 99% and was dissolved in DMSO to provide stock solution.

**Induction and evaluation of EAE**

Male C57BL/6 mice (6–8 wk; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were immunized s.c. with a synthetic peptide (300 μg) of myelin oligodendrocyte glycoprotein (MOG residues 35–55). The amino acid sequence of the peptide was Met-Glu-Asn-Gly-Lys and displayed a purity of 95% (BioAsia Biotechnology). Immunization was performed by mixing MOG peptide in CFA containing 90 μg/ml heat-killed H37Ra, strain of Mycobacterium tuberculosis (Difco Laboratories). Two hundred nanograms of pertussis toxin (List Biological Laboratories). Two hundred nanograms of pertussis toxin (List Biological Laboratories) in PBS were administered i.v. on the day of immunization and 48 h later. For the treatment protocol, SM933 or vehicle was administered at 400 μg per mouse i.p. daily from day 8 onwards. The prevention protocol differed from the treatment protocol only in the start of SM933 administration (done 3 days before immunization and continuing as given in the treatment protocol). Mice were examined daily and scored for disease severity using the standard scale: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund or death. The animal protocol was approved by the institutional review board of the Institute of Health Sciences.

**Immunohistochemistry**

For H&E and Luxol fast blue staining, spinal cords from mice transcardially perfused with 4% paraformaldehyde were postfixed overnight. Paraffin-embedded tissue was sectioned at 4 μm with a cryostat, and the sections mounted upon slides, air dried, and stained with hematoxylin and eosin (H&E) or Luxol fast blue or H&E and then examined by light microscopy. For immunochemistry, tissue was formalin fixed for 10 min with 100% acetone. After blocking with 3% BSA, the sections were incubated overnight at 4°C with specific primary Abs: anti-IFNγ; anti-IL-6; anti-IL-10; anti-IκBα; anti-VEGFR1 (Genetex, Inc.). Isotype-matched Abs were used as negative controls. The degree of infiltration of CD4+ and CD8+ T cells in spinal cord tissue was quantified by cell count on an average of 216 fields of view per mouse from 3 mice. The results were expressed as the number of infiltrating CD4+ cells and CD8+ cells per 106 spinal cord cells.

**Immunoblot analysis**

Protein extracts were loaded onto 10% or 12% SDS-polyacrylamide gels and subjected to electrophoresis. Immuoblot analysis was performed by initial transfer of proteins onto Immobilon-P membrane (Millipore) using a Mini Trans-Blot apparatus (Bio-Rad). After 2 h of blocking, the membranes were incubated overnight at 4°C with the specific primary Abs: anti-inducible NO synthase (iNOS; Upstate), anti-IκBα (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology), anti-cyclin A (Santa Cruz Biotechnology), anti-p27kip1 (Cell Signaling Technology), anti-iC Domain 2 (Santa Cruz Biotechnology), anti-IFNα (clone F8; Hyctult Biotechnology b.v. and clone RMAA-1; PBL Biomedical Laboratories), or anti-JAB1 (Sigma-Aldrich). After washing and subsequent incubation with a goat anti-rabbit (Sigma-Aldrich) or anti-rat Ab (Jackson ImmunoResearch) conjugated with HRP for 1 h at room temperature and extensive washing, signals were visualized with ECL substrate (Pierce).

**Table 1. Specific primers used in real-time PCR analysis**

| Name (bp) | Primer | Sequence (5′→3′) |
|-----------|--------|------------------|
| β-Actin   | FW*    | TGGTACACCTTCCAGACAGATG |
|           | RV     | AGCTTGAAAGCTTCGAGCTCAG |
| INOS      | FW     | GCCACACAACTGTCGAGCAAA |
|           | RV     | GTATCGGAGATGCTGTAAGA |
| RIG-G     | FW     | CAGGCCATTGAGTGGATACCC |
|           | RV     | TTAATCACCGAAGCTTCCTAG |
| IFNα-1    | FW     | ATGTCCTGGCGGCGTGAGT |
|           | RV     | ACCAGAATGTCAAGGCTTCTT |
| IFNα-11   | FW     | TCTTCTCTGATGATCCTGATT |
|           | RV     | CCTCAAGGGCTCTTCGTCAG |
| IL-13     | FW     | AGTCAGTCGCTGAGCTGTA |
|           | RV     | TGGGTGGATATGCAATGTAG |
| IL-6      | FW     | TTTCATTCCAGTGCTTCT |
|           | RV     | TTGTTGAGGGCTTCTCCTG |
| IL-17     | FW     | CCAGAATAGGACCTCCCTAGA |
|           | RV     | TCACTGTGGTTCGAGCTTC |
| CCL9      | FW     | GTGTCTTCCAACGGCGATT |
|           | RV     | TUTACTCACGCTTGACATAAGT |
| CCR1      | FW     | TGGCAGGAGCCATGCATGT |
|           | RV     | AACAGCCTGGCAAGCTTCCTC |
| CXCL9     | FW     | TCGGTCTTCTCAAGGAGA |
|           | RV     | GCACGAGGCGTTTCTCCAGT |
| CCRX4     | FW     | TCCCCCCCTTGCCGAGTTG |
|           | RV     | AACGCTGCTGTAGAGGTCACA |
| IL-18     | FW     | CAGGCTGACATTCCTGCA |
|           | RV     | ACACTGCGAGCATTGTCAGT |
| IL-3      | FW     | TGGTACACCTTCCAGACAGATC |
|           | RV     | TTTCGGAGACCTTCAATCTC |
| MAP2K3    | FW     | GTCGTGTCTCCAGCCGGCTCA |
|           | RV     | GACAACCATCGCTTGGCTTTT |
| MAPK14    | FW     | CAGCCAGTCGCTAGAAAGCT |
on day 8 or a prevention protocol (and scored daily as described in FIGURE 2.

Clinical course and severity of EAE in mice treated with SM933. C57BL/6 mice were immunized with MOG35–55 peptide to induce EAE and inflammatory responses (Griess method (19, 20). Briefly, aliquots of culture supernatant (100 µl of Griess reagent at room temperature for 10 min. Optical density was measured at 540 nm in an automated microplate reader. The concentration of nitrite was determined by reference to a standard curve of sodium nitrite using culture medium as background control. Measurement of nitrite production

Splenocytes from EAE mice on day 14 after immunization were stimulated with the MOG peptide and cultured at 37°C with 10 µg/ml purified anti-mouse IFN-α Ab (PBL Biomedical Laboratories) or anti-mouse IFN-αR1 Ab (R&D Systems) or goat IgG as control (Boster) for 1 h. Cells were then cultured for 24 h in the presence of 1 µg/ml SM933 or DMSO control. Resulting cells were harvested and subjected to real-time PCR for Rig-G mRNA expression with actin as reference and immunoblotting for JAB1 protein levels as described in Real-time PCR.

Measurement of nitrite production

Splenocytes isolated from EAE mice were stimulated with the MOG peptide in complete DMEM in the presence of SM933 added at the indicated concentrations, along with the vehicle control. After 24 h of incubation, nitrite concentrations were measured in culture supernatants using the Griess method (19, 20). Briefly, aliquots of culture supernatant (100 µl) were mixed with 100 µl of Griess reagent at room temperature for 10 min. Optical density was measured at 540 nm in an automated microplate reader. The concentration of nitrite was determined by reference to a standard curve of sodium nitrite using culture medium as background control. cDNA array analysis

The expression profile of selected pro- and anti-inflammatory genes was analyzed using a validated cDNA array system that contained 364 cytokines and chemokines and their receptor genes all related to autoimmune and inflammatory responses (GEArray S Series, SuperArray Bioscience Corp.) according to the manufacturer’s instructions. The detailed gene list can be found at the manufacturer’s website (www.superarray.com). Briefly, splenocytes derived from EAE mice were stimulated with MOG peptide in the presence or absence of SM933 (1 µg/ml) or vehicle control for 24 h. Total RNA was extracted using TRizol Reagent (Invitrogen). Three micrograms of total RNA were reverse transcribed into cDNA in the presence or absence of SM933 and scored daily as described in Materials and Methods. Data are representative of three independent experiments.

FIGURE 1. Chemical structure of SM933.

Real-time PCR

Total RNA was isolated from cell pellets using an RNeasy Mini Kit (Qiagen) and first-strand cDNA was subsequently synthesized using Sensiscript RT Kit (Qiagen) according to the manufacturer’s instructions. mRNA expression of IL-17, iNOS, Rig-G, and selected pro- or anti-inflammatory molecules was determined by real-time PCR using SYBR Green master mix (Applied Biosystems). Nucleotide sequences of specific primers are listed in Table I. Thermocycler conditions comprised of an initial holding at 95°C for 2 min and subsequently at 95°C for 10 min, which was followed by a two-step PCR program consisting of 95°C for 15 s, and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). Mouse β-actin gene was used as an endogenous control for sample normalization. Results were presented relative to the expression of β-actin.

IFN-α Ab blocking experiments

Splenocytes from EAE mice on day 14 after immunization were stimulated with the MOG peptide and cultured at 37°C with 10 µg/ml purified anti-mouse IFN-α Ab (PBL Biomedical Laboratories) or anti-mouse IFN-αR1 Ab (R&D Systems) or goat IgG as control (Boster) for 1 h. Cells were then cultured for 24 h in the presence of 1 µg/ml SM933 or DMSO control. Resulting cells were harvested and subjected to real-time PCR for Rig-G mRNA expression with actin as reference and immunoblotting for JAB1 protein levels as described in Real-time PCR.

cDNA array analysis

FIGURE 2. Clinical course and severity of EAE in mice treated with SM933. C57BL/6 mice were immunized with MOG35–55 peptide to induce EAE and were administered daily i.p. injections of SM933 (400 µg, ○) or vehicle control (○) using a treatment protocol (A) in which daily injections were begun on day 8 or a prevention protocol (B) in which daily injection began 3 days before immunization. Each group consisted of 12–15 mice and was monitored and scored daily as described in Materials and Methods. Data are representative of three independent experiments.

EMSA

MOG-reactive splenocytes treated with SM933 or vehicle control were collected and washed with PBS. Nuclear protein extraction was prepared as described previously (21). 5'-AGTTGAGGGGACTTTCCCAGGC-3' and its complementary strand were annealed and labeled as probes with [γ-32P]dATP (Amersham) using T4 polynucleotide kinase (Promega). Fifteen micrograms of nuclear protein were incubated with 2.5 ng of the probes for 30 min at 4°C. The mixture was electrophoresed on a non-denaturing 4% polyacrylamide gel in Tris-buffered EDTA. After electrophoresis, the gel was dried and autoradiographed by overnight exposure to x-ray film.

Apoptosis analysis

Analysis for apoptosis was performed using an annexin V-FITC apoptosis detection kit (BD Biosciences). Briefly, cells were treated with SM933 (1 µg/ml) or vehicle for 24 h. Resulting cells were washed and incubated with 5 µl of annexin V-FITC and 5 µl of propidium iodide for 15 min at room temperature. Stained cells were analyzed subsequently using a FACSARia instrument (BD) within 1 h.

Statistics

A Student t test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student t test. A p value of <0.05 was considered statistically significant.

Results

Suppression of disease severity in EAE by SM933

The natural form of artemisinin was initially found in our study to moderately suppress EAE (<30% inhibition of the disease severity), prompting us to explore its chemical modifications. One of the fat-soluble derivatives of artemisinin, ethyl 2-[4-(12-artemisini-noxy)]phenoxypropionate, termed SM933 (Fig. 1), showed...
improved efficacy in suppressing EAE and minimum toxicity compared with that of unmodified artemisinin. This novel compound had a low LD_{50} of 1.25 g/kg when administered to mice by i.p. injection. As illustrated in Fig. 2, when administered daily from day 8 onwards (treatment protocol) or 3 days before immunization and followed by daily injections (the prevention protocol), SM933 showed a significant inhibitory effect on the severity of EAE as compared with a vehicle control. The effect became visible at the time of disease onset (day 10 or day 12) and persisted over the entire course of EAE. Histological analysis of spinal cord tissue sections from EAE mice treated with SM933 exhibited markedly reduced inflammation and demyelination as compared with those from untreated controls. This observation was consistent with the decreased infiltration of CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T cells in affected spinal cord lesions of treated mice observed by immunohistochemistry (Fig. 3).

Regulatory effects of SM933 on encephalitogenic T cell responses

The significant treatment effect of SM933 in EAE prompted us to investigate in detail potential regulatory mechanisms of the compound as they affected encephalitogenic T cell responses and to identify the target molecules through which SM933 might regulate the immune system. To this end, splenocytes were isolated from
SM933 treated and control EAE mice and characterized for T cell reactivity and cytokine profile in response to in vitro challenge by the disease-eliciting MOG peptide. The results revealed that the encephalitogenic T cell response was significantly decreased as compared with that of untreated controls ($p < 0.05$; Fig. 4A). Furthermore, when challenged in vitro with the MOG peptide, encephalitogenic T cells derived from treated mice displayed a markedly altered cytokine profile from that of control mice. The treated profile was characterized by significantly reduced production of IFN-$\gamma$ and increased production of Th2 cytokines, i.e., IL-4, IL-5, IL-10, and IL-13 (Fig. 4B), representing a Th2 immune deviation, whereas IL-17 production was not significantly changed. However, SM933 did not affect the expression of T-bet and GATA-3, two transcription factors known to regulate Th1 and Th2 immune responses (data not shown), thus pointing to alternative explanations.

**Inhibition of NF-$\kappa$B activity and the production of proinflammatory molecules in MOG-reactive splenocytes after SM933 treatment**

Because the NF-$\kappa$B signaling pathway is known to play a critical role in inflammatory processes involved in EAE (22–24), we examined whether SM933 had an inhibitory effect on the activity of transcriptional factor NF-$\kappa$B. We were particularly interested in the possibility that SM933 might impair the activity of NF-$\kappa$B by preventing its degradation through up-regulation of its inhibitory protein I$\kappa$B-$\alpha$ as is commonly seen in other situations (25–28). Indeed, as shown in Fig. 5, SM933 appeared to markedly affect the activity of NF-$\kappa$B in EMSA (Fig. 5A), which correlated with increased expression of I$\kappa$B-$\alpha$ in immunoblot experiments (Fig. 5B). Furthermore, it is known that the iNOS gene is regulated by NF-$\kappa$B and that its product catalyzes the generation of NO, a molecule critically involved in inflammation (29). Our experiments showed that in vitro treatment of MOG-reactive splenocytes with SM933 resulted in a significant reduction in the expression of iNOS (Fig. 6A) and the production of NO (Fig. 6B) in a dose-dependent manner. Similar effect was also seen in purified CD11b$^+$ cells (data not shown).

It was of interest, then, to evaluate the scope of the anti-inflammatory properties induced by SM933 in MOG-reactive splenocytes. To this end, a validated cDNA array system containing 364 genes, all related to inflammation and autoimmune disease, was used to profile the effects of SM933. The results revealed that, with few exceptions, treatment of MOG-reactive splenocytes

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**FIGURE 6.** Inhibition by SM933 of iNOS expression and NO production in MOG-reactive splenocytes. Splenocytes isolated from EAE mice 14 days postimmunization were stimulated in vitro with the MOG peptide in the presence or absence of SM933 at the indicated concentrations for 24 h. A, iNOS mRNA expression levels were analyzed by real-time PCR using $\beta$-actin as a reference. iNOS protein levels were measured using immunoblot analysis (inset). B, Concentrations of nitrite in supernatants of abovementioned cell cultures were measured by the Griess reaction. Results are expressed as means $\pm$ SD. Data are representative of three independent experiments.

**FIGURE 7.** Expression profiling of pro- and anti-inflammatory genes of MOG-reactive splenocytes in response to SM933 treatment. Splenocytes isolated from EAE mice were stimulated with the MOG peptide in the presence of SM933 (1 $\mu$g/ml) or vehicle control for 24 h. Expression profiles of 364 genes selected for their relevance in autoimmune and inflammatory responses were analyzed using a validated cDNA array system. A representative experiment is shown in dot blots (A). Digital data were analyzed using GEArray Expression Analysis Suite and expressed as ratios of significant change ($\geq$2.0) in gene expression (SM933 vs control) (B). The results are representative of three independent experiments using different EAE mouse splenocyte samples. C, The effect of SM933 on the expression of selected pro- and anti-inflammatory genes was confirmed by real-time PCR. D, MOG-reactive splenocytes treated with SM933 were subjected to immunoblot analysis for determination of IFN-$\alpha$ protein levels.
with SM933 led to selective up-regulation of the expression of anti-inflammatory genes (e.g., CTLA-4, IL-13, and IL-6) and the inhibition of proinflammatory genes (e.g., CXCR4, IL-18, MAPK, VCAM-1, etc.) as illustrated in Fig. 7, A and B. The regulatory effect of SM933 determined by the cDNA array on the expression of some of the critical pro- or anti-inflammatory genes was then confirmed quantitatively by real-time PCR (Fig. 7C). Interestingly, gene expression of IFN-α (Ifna1 and Ifna11) was markedly up-regulated in SM933-treated cells when compared with other related genes. Increased production of IFN-α protein in cells treated with SM933 was further confirmed by immunoblot analysis (Fig. 7D).

Effect of SM933 on activated T cells through regulation of cell cycle activity

Another important finding relevant to the role of SM933 was that it significantly inhibited the proliferation of encephalitogenic T cells. This effect was not specific for MOG-reactive T cells as the same effect was seen with nonspecific T cells stimulated with anti-CD3 Ab (Fig. 8A). Our initial experiments ruled out the possibility that this effect of SM933 was caused by direct cell killing or apoptosis by flow cytometry (6% apoptotic cells in both SM933-treated and control preparations). With the discovery of markedly increased production of IFN-α induced by SM933 and the known biological activity of IFN-α in relation to the cell cycle (30), we hypothesized that SM933 might affect T cell proliferation by up-regulating Rig-G, an IFN-α target gene. Rig-G interacts with JAB1, which prevents JAB1 from entering the nucleus, where it inhibits the degradation of p27kip1, a critical negative regulator of cell cycle. Our results showed that treatment of MOG-reactive splenocytes with SM933 significantly increased the expression of Rig-G (Figs. 8B), which correlated with a decreased level of nuclear JAB1 as demonstrated using immunoblot analysis (Fig. 8C). Results of immunofluorescence staining confirmed that the amount of JAB1 that colocalized with the nuclear stain 4',6-diamidino-2-phenylindole was markedly decreased in SM933-treated splenocytes (Fig. 8D). It was evident that SM933 treatment altered the expression of signaling molecules associated with cell cycle progression, including p27kip1, cyclin A, and CDK2 (Fig. 8C). Furthermore, we examined whether these effects were specifically mediated by IFN-α using blocking Abs directed at IFN-α or IFN-α receptor under the same experimental conditions. The results showed that Ab blockade of IFN-α or its receptor significantly abolished the effects of SM933 on Rig-G up-regulation by real-time PCR (Fig. 8E) and JAB1 nuclear translocation by immunoblot (Fig. 8F), further confirming a pivotal role of the IFN-α/Rig-G pathway in SM933 regulation of the cell cycle. Our parallel experiments suggested that the antiproliferative effect of SM933 was independent of the MAPK pathway, given that the level of ERK, p38 and JNK was not significantly altered by SM933 (data not available).
Regulatory mechanisms of Artemisinin-Derived Agent

Regulations of cell cycle in activated T cells by SM933. Activated T cells from a human myelin basic protein-specific T cell line were compared with CD4⁺ T cells (resting T cells) freshly isolated from PBMC of a healthy individual. A. Surface expression of CD25 in activated and resting T cells was determined by flow cytometry. B. Expression of cyclin A, CDK2, p27kip1, was determined by immunoblot analysis in activated or resting T cell preparations treated with SM933 or vehicle control for 24 h.

Discussion

The results described here indicate that SM933, a novel derivative of artemisinin, composed of dihydroartemisinic acid and 2-(4-hydroxyphenoxyp)propionic acid ethyl ester, has unique anti-inflammatory properties and therapeutic potential. The compound is more effective than artemisinin itself in reducing the severity of EAE and has an improved toxicity profile. The treatment effects are accompanied by significantly reduced infiltration of CD4⁺ T cells into spinal cord tissue and preservation of CNS myelin. This study has addressed several important issues. First, we demonstrated that SM933 had a marked treatment effect on the clinical course of EAE using either the treatment or the prevention protocols. However, the compound did not significantly delay the disease onset when it was applied using the preventive protocol. This finding implies that SM933 acts directly upon encephalitogenic T cells and is consistent with its mechanism of direct regulation of signaling molecules as described in this study. It may explain why the compound has a treatment but not a preventative property that would require memory cellular components modulated by a treatment agent. Other immunomodulatory agents used for MS or EAE, such as β-IFN and epigallocatechin-3-gallate, have similar characteristics (28, 31, 32).

Secondly and more importantly, we demonstrated here that SM933 exerts anti-inflammatory actions in EAE through its unique regulatory properties affecting defined signaling pathways. It acts synergistically on encephalitogenic T cell responses by direct inhibition of T cell proliferation as well as T cell-mediated inflammation. The former action is achieved, at least partly, through the Rig-G/JAB1 pathway by the induction of IFN-α. SM933 has a unique property in inducing the production of IFN-α among other inflammation-related cytokines. IFN-α, as an initiator cytokine, up-regulates the expression of Rig-G, a cytoplasmic protein that traps JAB1 in the cytosol, thus preventing it from entering the nucleus. Consequently, reduced levels of JAB1 in the nucleus lead to accumulation of p27, a master cell cycle regulator, rescuing it from degradation and subsequent down-regulation of cyclin A and CDK2. These actions collectively result in direct inhibition of the proliferation of encephalitogenic T cells. Interestingly, activated but not resting T cells are more susceptible to the effect of SM933. This finding indicates that resting T cells characterized by the G0 phase are resistant to the signaling actions triggered by SM933. This selective property of SM933 may offer particular advantage in the treatment of autoimmune disease where an ideal agent is expected to target preferentially in vivo activated, rapidly dividing autoreactive and inflammatory T cells to reduce the threshold of inflammatory T cell activity without affecting the entire T cell pool.

In contrast, the anti-inflammatory actions of SM933 are seen to involve the NF-κB pathway through its interaction with IκB. SM933 has a sesquiterpene lactone (peroxide lactone) in its structure. Several sesquiterpene lactones are known to impair the activity of the transcriptional factor NF-κB, either by alkylating it or by preventing the degradation of its inhibitory protein IκB (33–35). In this regard, one important observation is that SM933 appears to be an interesting immune modulator, resulting in Th2 immune deviation as characterized by decreased production of Th1 cytokines and an overall increase of all major Th2 cytokines examined. This Th2 polarization was observed in both MOG-reactive T cells and naive T cells stimulated by TCR ligation (data not shown). Apparently, this characteristic effect of SM933 is not mediated through T-bet and GATA-3, the two key transcription factors controlling Th1 and Th2 immunity. It is likely that Th1 cells representing encephalitogenic T cells activated in EAE are rendered more susceptible to the direct action of SM933 through the Rig-G/JAB1 pathway whereas Th2 cells are not activated and thus relatively spared by the effect of SM933. Thus, the observed Th2 immune deviation is likely to reflect a new balance of Th1 and Th2 immunity achieved by selective inhibition of Th1 encephalitogenic T cells, tilting the balance preferentially toward a Th2 response. Nevertheless, this Th2 immune deviation may contribute to the overall treatment effect of SM933 as seen in many other situations (5, 8, 31). SM933 treatment induces a profound change in the cytokine milieu, as evidenced by the gene array expression profile induced by SM933 in MOG-reactive splenocytes, which is likely to have a critical impact on inflammation. Taken together, SM933 has promising anti-inflammatory properties and warrants further investigation to determine its therapeutic potential as a treatment for autoimmune inflammatory diseases such as MS.

There are other issues whose significance may go beyond the study of SM933 itself and have implications in the development of new anti-inflammatory drugs from natural compounds. First, an active chemical component, artemisinin, when singled out from natural compounds, although effective, may lack sufficient potency as a stand-alone treatment agent. However, such compounds provide excellent lead structures for further chemical modification to improve their pharmacological characteristics (e.g., fat or water solubility, tissue penetration, etc.) and, most importantly, their potency in the treatment of targeted disease. In this regard, SM933 provides an excellent example. The original unmodified structure of artemisinin is shown to have a moderate effect on the severity of EAE. However, when coupled with 2-(4-hydroxyphenoxyp)propionic acid ethyl ester, the resulting novel compound (SM933) has a markedly increased potency in reducing disease severity. Secondly, as described here, the treatment effect of SM933 is achieved through targeting of multiple signaling mechanisms/molecules critically related to inflammation. It is therapeutically more advantageous for an anti-inflammatory agent to act on multiple checkpoints within the inflammatory signaling cascade, with selectivity for activated pathogenic T cells, to create a synergistic treatment...
effect on disease activity. Our findings also provide important indications as to what molecules may be targeted to achieve potent anti-inflammatory effect. This study provides a novel example for the development of effective anti-inflammatory drugs from natural compounds.

Disclosures

The authors have no financial conflict of interest.

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