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ORIGINAL ARTICLE

Non-antibiotic 12-membered macrolides: design, synthesis and biological evaluation in a cigarette-smoking model

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The 14-membered macrolide erythromycin A expresses three distinct biological properties, including antibacterial activity, gastrointestinal motor-stimulating activity and anti-inflammatory and/or immunomodulatory effects. Although low-dose, long-term therapy using 14- and 15-membered macrolides displaying anti-inflammatory and/or immunomodulatory activity effectively treats diffuse panbronchiolitis and chronic sinusitis, bacterial resistance may emerge. To address this issue, we developed the 12-membered non-antibiotic macrolide (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (EM900) that promotes monocyte to macrophage differentiation, a marker for anti-inflammatory and/or immunomodulatory effects, without possessing antibacterial activity. In this article, we report that the new macrolide derivative (8*R*,9*S*) -de(3'-*N*-methyl)-3'-*N*-(*p*-chlorobenzyl)-de(3-*O*-cladinosyl)-3-dehydro-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A 12,13-carbonate (EM939) exhibited stronger promotive activity for monocyte to macrophage differentiation than that of the parent compound EM900 in addition to reduced cytotoxicity toward THP-1 cells and antibacterial inactivity. In a cigarette-smoking model used to simulate chronic obstructive pulmonary disease (COPD), the EM900 derivatives significantly attenuated lung and alveolar inflations, functionally and histologically, via oral administration. Because of these marked therapeutic effects, non-antibiotic EM900 derivatives may become central to the treatment of chronic inflammatory diseases such as COPD.
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INTRODUCTION

The 14-membered macrolide erythromycin A (EMA) displays antibacterial activity (Figure 1); however, undesirable physicochemical properties under acidic conditions have restricted its applicability. To overcome this problem and increase its antibacterial activity and spectrum, semisynthetic derivatives, such as clarithromycin and azithromycin, have been developed.[1] In addition to their unique antibacterial activity, these 14- and 15-membered macrolides exhibit gastrointestinal motor-stimulating activity, such as motilin-like agonism,[2–4] as well as anti-inflammatory and/or immunomodulatory effects.[5–8] The motiline agonist *N*-demethyl-*N*-*iso*propyl-8,9-anhydroerythromycin A 6,9-hemiacetal (**EM574**, Figure 1),[9–12] or motilide, was chemically synthesized to fabricate a molecule that exclusively displays gastrointestinal motor-stimulating activity without antibacterial activity. Although anti-inflammatory and/or immunomodulatory effects of EMA were first recognized in the low-dose, long-term therapy of diffuse panbronchiolitis at the clinical level, as reported by Kudoh *et al.*[5–7] In particular, 14- and 15-membered macrolides exhibit a broad spectrum of pharmacological effects in humans and animals and influence several pathways of the inflammatory process such as neutrophil migration,[13] oxidative burst in phagocytes[14] and proinflammatory cytokine production.[15] EMA, clarithromycin and roxithromycin promote the differentiation of human peripheral blood monocytes or human monocytic THP-1 cell line to macrophages[16] and inhibit human T-cell proliferation.[17] These macrolide antibiotics have exhibited immunomodulatory properties resulting in immune response stimulation and suppression. For instance, macrolide treatment has stimulated the production of some cytokines, such as monocyte chemoattractant protein-1 and interleukin 12 (IL-12), but reduced the production of IL-8, tumor necrosis factor-α and IL-1β in an early phase before allowing its decrease.[18,19] These immunomodulatory effects are considered to normalize dysregulated immune systems.

Despite the absence of clear mechanisms, the interaction between macrolides and leukocytes has been suggested to have an important role in anti-inflammatory and/or immunomodulatory effects. Indeed, macrolide antibiotics accumulate into polymorphonuclear leukocytes,[20] which may in turn alter phagocyte functions that appear equally crucial for antibacterial defense and inflammatory processes. Some researchers have suggested that antioxidant properties affect the anti-inflammatory activity of macrolides.[21–23]

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The paper is dedicated to Professor Amos B Smith III on the occasion of his 70th birthday.
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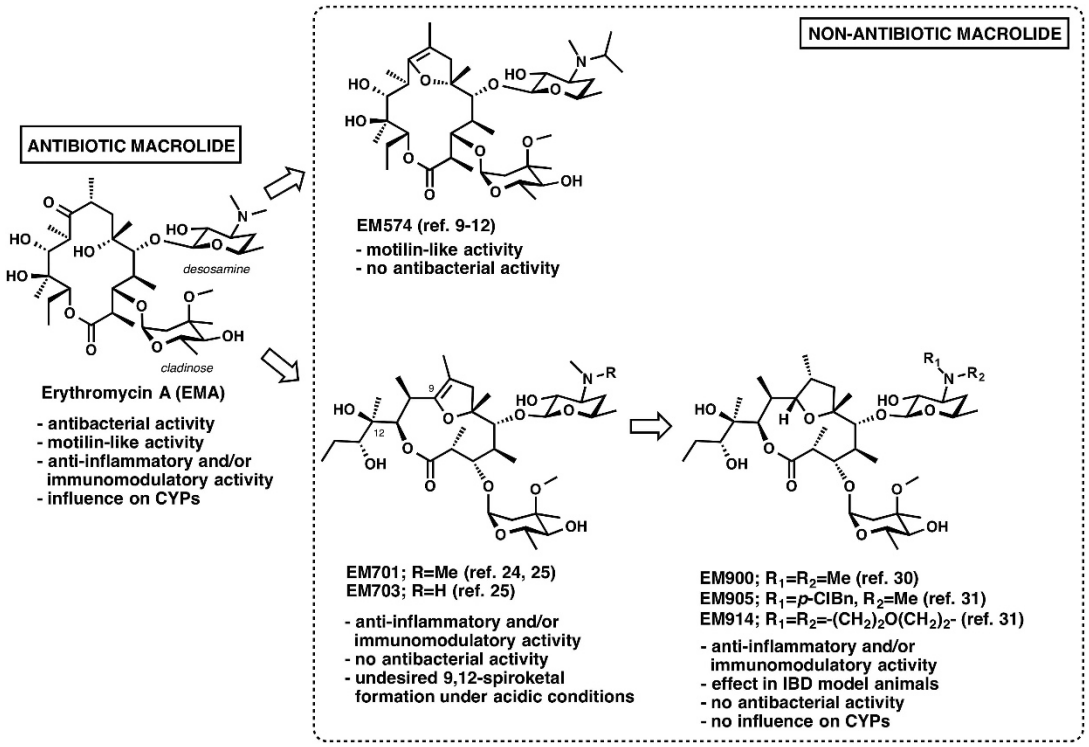


Figure 1 Antibiotic and non-antibiotic macrolides.

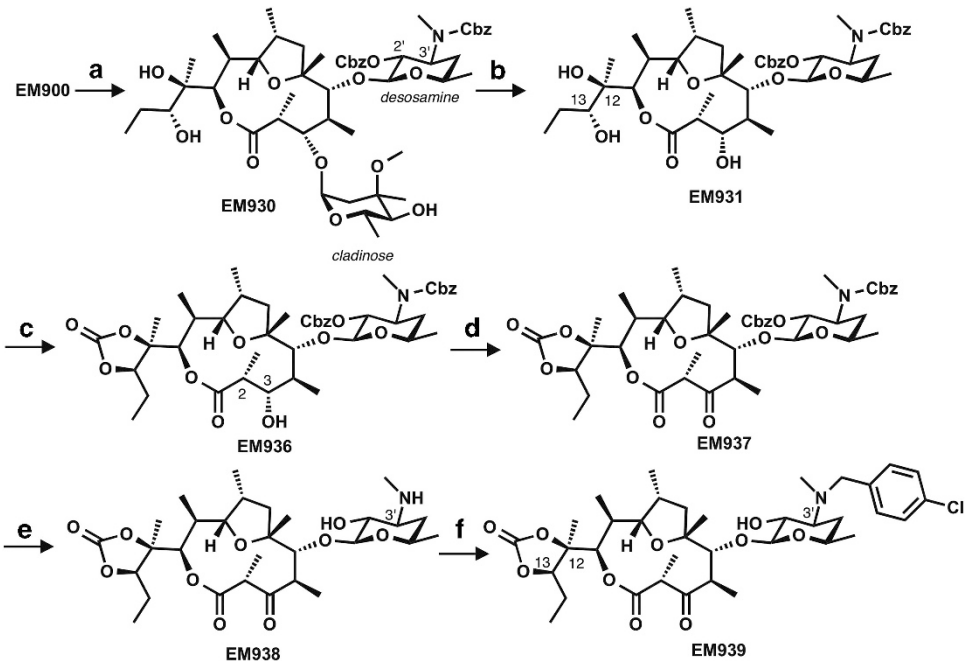
Macrolide therapies for chronic inflammatory status control are clinically well established, especially in diffuse panbronchiolitis[6] or chronic sinusitis. However, the appearance of resistant bacteria resulting from long-term antibiotic dosage is problematic. To address this concern, our continuing research efforts have been focused on creating non-antibiotic macrolides, leading to 12-membered EMA analogs, such as 8,9-anhydropseudoerythromycin A 6,9-hemiketal (**EM701**)[24] and its de-*N*-methyl derivative (**EM703**, Figure 1).[25,26] These macrolides have exhibited better promotive activity for monocyte to macrophage differentiation,[16] a marker for anti-inflammatory and/or immunomodulatory effects, than EMA, without antibacterial activity. In addition, **EM701** and **EM703** have presented weak or no motiline-like activity, respectively. In addition, **EM703** has shown preventive effects for experimental bleomycin-induced acute lung injury in rats,[27] suppressed nuclear factor-κB activation and IL-8 production[28] and inhibited macrophage-tropic HIV-1 replication in macrophages.[29]

On the other hand, the aglycone of **EM701** has displayed some undesirable chemical properties under acidic conditions. To solve this issue, the 12-membered macrolide (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM900**, Figure 1), which does not undergo 9,12-spiroketal formation under acidic conditions and promotes monocyte to macrophage differentiation, was developed.[30] In addition, **EM900** displays some beneficial properties such as antibacterial inactivity and weak interaction with the cytochrome P450 enzyme CYP3A4. Its derivatives, (8*R*,9*S*)-de(3'-*N*-methyl)-3'-*N*-(*p*-chlorobenzyl)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM905**) and (8*R*,9*S*)-de(3'-*N, N*'-dimethylamino)-3'-morpholino-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM914**, Figure 1), have proven effective against inflammatory bowel disease in a mouse model, even at considerably low doses,[31] making them promising lead candidates for the development of new therapeutic drugs for chronic inflammatory diseases. However, no **EM900** derivative exhibiting strong anti-inflammatory and/or imunomodulatory effects as well as lower cytotoxicity ($IC_{50} > 100\ \mu M$) in THP-1 cells has been developed to date. Therefore, this study aims to create such molecules based on structure–activity relationship maps. Herein, we report the design, synthesis and structure–activity relationship study of (8*R*,9*S*)-de(3'-*N*-methyl)-3'-*N*-(*p*-chlorobenzyl)-de(3-*O*-cladinosyl)-3-dehydro-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A 12,13-carbonate **EM939**. This newly synthesized macrolide strongly promotes monocyte to macrophage differentiation, indicative of anti-inflammatory and/or immunomodulatory effects and exhibits reduced cytotoxicity against THP-1 cells without antibacterial activity. Moreover, orally administered **EM905**[31] and **EM939** show *in vivo* curative effects in cigarette smoke exposure-induced emphysema model animals, demonstrating that non-antibacterial macrolides are effective in chronic inflammatory disease model animals.

RESULTS

Chemical synthesis and biological properties

Our initial studies[30,31] highlighted several structure–activity relationships for the promotive activity for the monocyte to macrophage differentiation (ED_{50}) and cytotoxicity (IC_{50}) of **EM900**-type macrolides. (1) The *p*-chlorobenzyl group in **EM905** enhanced the macrolide promotive activity ($ED_{50} = 2.7\ \mu M$) but increased their cytotoxicity at the same time ($IC_{50} = 30\ \mu M$). (2) The removal of cladinose decreased the cytotoxicity but reduced the promotive activity as well. Therefore, our strategy focused on introducing a *p*-chlorobenzyl group, instead of a methyl group, in the desosamine *N*-dimethylamino substitutent and removing cladinose to decrease the macrolide cytotoxicity toward THP-1 cells while retaining their promotive activity for the monocyte to macrophage differentiation. At the outset, treatment of **EM900** with



Scheme 1 Synthesis of **EM939**. Reagents and conditions; (**a**) CbzCl, $NaHCO_3$, EtOAc, 70 °C, 94%. (**b**) 1 N HCl aq., MeCN, rt, 95%. (**c**) triphosgene, pyridine, CH_2Cl_2, –78 °C to rt, 90%. (**d**) Dess–Martin periodinane, CH_2Cl_2, rt, 97%. (**e**) H_2, $Pd(OH)_2$, EtOH, rt, 97%. (**f**) *p*-ClBnBr, *N,N*-diisopropylethylamine, $CHCl_3$, rt, 93%. Cbz = benzyloxycarbonyl. rt = room temperature.

benzyl chloroformate simultaneously replaced one methyl substituent of the dimethylamino group and chemoselectively protected the 2'-hydroxyl group by benzyloxycarbonyl (Cbz) groups in the desosamine moiety,[32] leading to **EM930** in 94% yield (Scheme 1). This chemoselective acylation at C2' position is reported owing to the neighboring effect of dimethylamino group as an intramolecular catalyst.[33] Cladinose removal of **EM930** under acidic conditions (**EM931**, 95% yield) and subsequent cyclic carbonate formation at the C12,13 positions produced **EM936** in 90% yield. Oxidation at the C3 position in the presence of Dess–Martin periodinane gave the β-ketolactone[34] **EM937** in 97% yield without any epimerization at the C2 position. Finally, Cbz deprotection via palladium-catalyzed hydrogenation (**EM938**, 97% yield) followed by introduction of the *p*-chlorobenzyl group on the desosamine moiety provided **EM939** in 93% yield.

The antibacterial activity of these synthetic compounds was investigated along with their promotive activity for the differentiation of monocytic THP-1 cells to macrophages and cytotoxicity (Table 1). No antibacterial activity was observed against 27 types of strains for all derivatives. In terms of promotive activity for the monocyte to macrophage differentiation and cytotoxicity, **EM930** ($ED_{50} = 10.4\ \mu M$) showed slightly better activity and lower cytotoxicity ($IC_{50} > 100\ \mu M$) than **EM900** ($ED_{50} = 17.1\ \mu M$), suggesting that the benzylcarbamate group enhances activity. The cladinosyl-free compound **EM931** exhibited a markedly reduced activity ($ED_{50} > 100\ \mu M$) compared with **EM930** while retaining a weak cytotoxicity ($IC_{50} > 100\ \mu M$), indicating that cladinose or physical property by cladinose influences the promotive activity for the monocyte to macrophage differentiation. Interestingly, the introduction of the cyclic carbonate restored the promotive activity of the macrolide (**EM936**, $ED_{50} = 9.7\ \mu M$). Ketone **EM937** ($ED_{50} = 36.2\ \mu M$) exhibited a slightly lower activity than **EM900** and Cbz deprotection (**EM938**, $ED_{50} = 32.8\ \mu M$) led to similar activity. As expected, the introduction of the *p*-chlorobenzyl group on the desosamine enhanced the activity 13-fold (**EM939**, $ED_{50} = 1.3\ \mu M$) compared with that of **EM900** ($ED_{50} = 17.1\ \mu M$). In addition, this step did not increase the cytotoxicity (**EM939**, $IC_{50} > 100\ \mu M$), suggesting that cladinose removal and cyclic carbonate formation impact cytotoxicity.

Effects of EM905 and EM939 on cigarette smoke-induced emphysematous changes

After exposing guinea pigs to cigarette smoke or air for 4 weeks, the average functional residual capacity amounted to 5.48 ± 0.24 ml in the control group exposed to air and increased to 6.90 ± 0.26 ml ($P < 0.01$) in the untreated smoke-exposed group. This increase in functional residual capacity was limited to 6.23 ± 0.29 and 5.68 ± 0.27 ml ($P < 0.05$) in the presence of 10 and 30 mg kg^{-1} **EM905**, respectively. On the other hand, functional residual capacity values were 6.37 ± 0.44 and 5.93 ± 0.18 ml for animals treated with 10 and 30 mg kg^{-1} **EM939**, respectively. The control group exhibited a residual volume value of 0.63 ± 0.32 ml, which significantly increased to 2.52 ± 0.31 ml ($P < 0.01$) for the untreated smoke-exposed group. This smoke-induced increase in residual volume was reduced to 1.61 ± 0.35 and 1.22 ± 0.28 ml ($P < 0.05$) in animals treated with 10 and 30 mg kg^{-1} **EM905**, respectively. Residual volume values amounted to 1.21 ± 0.38 ($P < 0.05$) and 1.22 ± 0.27 ml ($P < 0.05$) on treatment with 10 and 30 mg kg^{-1} **EM939**, respectively (Figure 2).

Histopathologic examinations showed that smoke exposure resulted in significant alveolus enlargement and intra-alveolar accumulation of foamy/macrophage-like cells (Figure 3). On the other hand, no intra-alveolar cellular infiltration of neutrophilic or eosinophilic material was observed in this model. The mean alveolar length significantly increased from 39.18 ± 1.07 μm in the control group to 47.78 ± 1.29 μm ($P < 0.01$) in the untreated smoke-exposed group. This smoke-induced increase was reduced upon treatment with **EM905** (10 mg kg^{-1}: 41.95 ± 1.55 μm, $P < 0.05$; 30 mg kg^{-1}: 43.50 ± 1.25 μm,

Table 1 Biological properties of EM900 derivatives

Strains/compounds	*EMA*	*EM900*	*EM905*[a]	*EM930*	*EM931*	*EM936*	*EM937*	*EM938*	*EM939*
ED_{50} (μM)[b]	—	17.1	2.7	10.4	>100	9.7	36.2	32.8	1.3
Cytotoxicity (IC_{50}, μM)	—	100	30	>100	>100	>100	>100	>100	>100
MIC μg ml^{-1}									
S. aureus FDA209P[c]	⩽0.5	>128	>128	>128	>128	>128	>64	>128	>64
S. aureus Smith[c]	⩽0.5	>128	>128	>128	>128	>128	>64	>128	>64
MRSA KUB853[d]	>256	>128	>128	>128	>128	>128	>64	>128	>64
MRSA KUB854[d]	>256	>128	>128	>128	>128	>128	>64	>128	>64
MRSA 70[d]	>256	>128	>128	>128	>128	>128	>64	>128	>64
MRSA 92-1191[d]	>128	>128	>128	>128	>128	>128	>64	>128	>64
S. aureus KUB857[e]	>256	>128	>128	>128	>128	>128	>64	>128	>64
S. aureus KUB858[f]	>256	>128	>128	>128	>128	>128	>64	>128	>64
S. aureus KUB859[g]	64	>128	>128	>128	>128	>128	>64	>128	>64
S. aureus KUB860[h]	>256	>128	>128	>128	>128	>128	>64	>128	>64
S. epidermidis KUB795[i]	⩽0.5	>128	>128	>128	>128	>128	>64	>128	>64
M. luteus ATCC9341[j]	⩽0.5	128	>128	>128	>128	>128	>64	>128	>64
E. faecalis ATCC29212[j]	1	>128	>128	>128	>128	>128	>64	>128	>64
E. faecalis NCTC12201[k]	>256	>128	>128	>128	>128	>128	>64	>128	>64
E. faecium NCTC12203[l]	>256	>128	>128	>128	>128	>128	>64	>128	>64
E. coli NIHJ JC-2[j]	64	>128	>128	>128	>128	>128	>64	>128	>64
C. freundii ATCC8090[j]	256	>128	>128	>128	>128	>128	>64	>128	>64
K. pneumoniae NCTC9632[j]	32	>128	>128	>128	>128	>128	>64	>128	>64
P. mirabilis IFO3849[j]	>256	>128	>128	>128	>128	>128	>64	>128	>64
P. vulgaris OX-19[j]	256	>128	>128	>128	>128	>128	>64	>128	>64
M. morganii IID Kono[j]	>256	>128	>128	>128	>128	>128	>64	>128	>64
S. marcescens IFO12648[j]	128	>128	>128	>128	>128	>128	>64	>128	>64
E. cloacae IFO13535[j]	256	>128	>128	>128	>128	>128	>64	>128	>64
E. aerogen NCTC10006[j]	128	>128	>128	>128	>128	>128	>64	>128	>64
P. aeruginosa 46001[j]	>256	>128	>128	>128	>128	>128	>64	>128	>64
P. aeruginosa E-2[j]	>256	>128	>128	>128	>128	>128	>64	>128	>64
A. calcoaceticus IFO12552[j]	4	>128	>128	>128	>128	>128	>64	>128	>64

Abbreviations: EMA, erythromycin A; MIC, minimum inhibitory concentration.
[a]see ref 31.
[b]ED_{50} values were determined to evaluate the promotive activity for the monocyte to macrophage differentiation and compared with EMA results at 100 μM. All data are the mean values for three independent experiments.
[c]*Staphylococcus aureus* FDA209P and Smith: MSSA; susceptible strains.
[d]MRSA KUB853, MRSA KUB854, MRSA 70 and MRSA 92-1191: MRSA strains isolated from clinical patients.
[e]*S. aureus* KUB857: inducibly macrolide resistant strain, encoded by *erm* gene.
[f]*S. aureus* KUB858: constitutively macrolide resistant strain, encoded by *erm* gene.
[g]*S. aureus* KUB859: encoded by *erm* gene.
[h]*S. aureus* KUB860: encoded by *erm* and *mef* gene.
[i]*S. epidermidis* KUB795: strains isolated from clinical patients.
[j]Standard strain.
[k]*Enterococcus faecalis* NCTC12201: encoded by *van A* gene.
[l]*E. faecium* NCTC12203: encoded by *van A* gene.

Figure 3b) and **EM939** (10 mg kg^{-1}: 41.05 ± 1.02 μm, $P<0.01$; 30 mg kg^{-1}: 41.76 ± 1.24 μm, $P<0.01$, Figure 3b). Therefore, oral administration of **EM900** derivatives markedly inhibited the development of cigarette smoke-induced alveolar enlargement but decreased the intra-alveolar cell accumulation less effectively.

These findings indicate that **EM905** and **EM939** dose-dependently inhibited hyperinflation and degradation of elastic recoil in cigarette smoke-exposed lungs. **EM905** and **EM939** ameliorated alveolar enlargement in smoke-exposed lungs, a typical emphysematous histopathological finding; however, the dose dependency was unclear.

DISCUSSION

This study demonstrated that **EM939** (ED_{50} = 1.3 μM) exhibited potent promotive activity for the monocyte to macrophage differentiation, indicative of anti-inflammatory and/or immunomodulatory effects, as well as weak cytotoxicity toward THP-1 cells (IC_{50} > 100 μM), satisfying our long-term goal (vide supra). In addition, structure–activity relationships were elucidated. (1) The introduction of a *p*-chlorobenzyl group on the desosamine moiety simultaneously increased the promotive activity of macrolides for the monocyte to macrophage differentiation and their cytotoxicity toward THP-1 cells. (2) The incorporation of a cyclic carbonate enhanced their promotive activity but not their cytotoxicity.

Previous investigations of biological properties, such as anti-inflammatory and/or immunomodulatory effects, have revealed that **EM900** reduced the expression of inflammatory cytokines, such as IL-8, IL-1β and cachexin, in the IL-1β-stimulated cultured airway A549 epithelial cell line.[35] In addition, **EM900** inhibited vascular endothelial growth factor production in cultured fibroblasts from nasal polyps under hypoxia more potently than conventional macrolides (H Ishinaga and S Matsune, *et al.*, unpublished data). Among many biological characters related to anti-inflammatory and/or

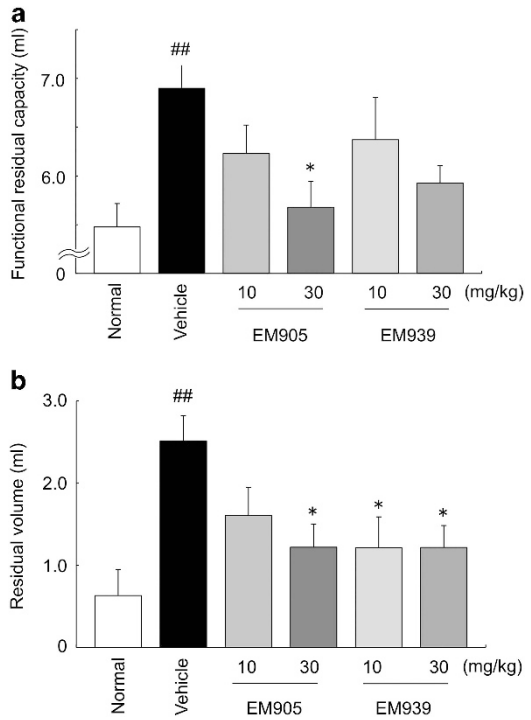


Figure 2 Effects of macrolide compounds **EM905** and **EM939** on pulmonary function in cigarette smoke-exposed guinea pigs. Pulmonary function tests were performed four weeks after cigarette smoke exposure. (**a**) Dose-dependent inhibition of cigarette smoke-induced increase in FRC. (**b**) Dose-dependent inhibition of cigarette smoke-induced increase in RV. Each value represents the mean ± standard error (s.e.) obtained for 8–10 animals. ##: $P<0.01$; significant difference from normal (Student's *t*-test or Aspin-Welch's *t*-test). *: $P<0.05$; significant difference from vehicle (Dunnett's multiple test).

immunomodulatory effects of EMA, the ability to promote monocyte to macrophage differentiation is unique. Phagocytic clearance of apoptotic granulocytes has a pivotal role in determining an inflammatory outcome, such as resolution or progression to a chronic state, associated with the development of fibrotic repair mechanisms or autoimmune responses.[36] Therefore, the promotion of monocyte to macrophage differentiation by EMA and its derivatives may markedly augment the capacity for phagocytosis of apoptotic neutrophils at inflamed sites, leading to resolution of inflammation. The enhanced anti-inflammatory and/or immunomodulatory properties of **EM900** derivatives visibly modified several inflammatory processes more effectively, resulted in curative effects against many inflammatory conditions and treated systemic inflammatory diseases such as chronic airway or bowel disease. For example, **EM905** and **EM914** exhibited similar beneficial impact on inflammatory bowel disease treatment in rats and required significantly lower dosages than therapeutic sulfa drugs such as sulfasalazine.[31]

Chronic obstructive pulmonary diseases (COPD), such as emphysema (characterized by airspace enlargement and destruction of lung parenchyma) and chronic bronchitis, have turned into a rapidly increasing global health problem. Defined by the Global Initiative for Chronic Obstructive Lung Disease, this disease is characterized by progressive, not fully reversible flow limitation associated with an abnormal inflammatory response of the lungs.[37] Therefore, chronic inflammatory response, which occurs throughout the airways and parenchyma and participates in the progression and exacerbation of this disease, has been attributed a central role. Cigarette smoke has been identified as the most important risk for the development of COPD. Therapeutic approaches to COPD mainly rely on supportive and symptomatic techniques such as the use of bronchodilators. The chronic inflammatory response is sometimes modulated using glucocorticoids, although these drugs have proven largely ineffective in attenuating inflammation in COPD patients,[38] demonstrating a need for new agents capable of suppressing this inflammatory response. Compounds such as 14-,15-membered macrolides may prevent the progression of this disease. Seemungal *et al*.[39] have observed a significant reduction of COPD exacerbations by long-term EMA or clarithromycin therapy.[39,40] Similarly, long-term azithromycin therapy have reportedly prevented these exacerbations.[41]

Here, **EM900** derivatives, such as **EM905** and **EM939**, functionally and histologically exhibited a marked attenuation of lung and alveolar inflations in cigarette smoke-exposed guinea pigs. Cigarette smoke exposure of guinea pigs led to lung responses that, at least in part, mimicked lung inflammatory and structural changes observed in COPD.[42] This knowledge of the effectiveness of **EM900** derivatives is crucial because few studies have reported the discovery of therapeutic agents that ameliorated smoke-induced emphysematous changes.[43] Although its precise molecular mechanism is currently not known, the suppression of the emphysematous inflammatory response may stem from the synergistic anti-inflammatory and/or immunomodulatory effects and antioxidative properties of **EM900** derivatives. These derivatives are expected to become leading agents for controlling chronic inflammatory airway disease, where existing therapeutic approaches are unsatisfactory.

The inhibitory effects of **EM900** derivatives on inflammatory development in COPD and inflammatory bowel disease animal models[31] indicate that these compounds are promising candidates for clinical chronic inflammatory disease treatment. Similar to conventional macrolides, these derivatives show various immunomodulatory actions such as the modulation of cytokine production or inflammatory cell infiltration around inflamed tissues. However, the action that primarily promotes the therapeutic effect of **EM900** derivatives on chronic inflammatory diseases remains unknown. In the absence of a detailed molecular mechanism for these anti-inflammatory and/or immunomodulatory effects, the chemical structure responsible for these effects needs identification. Further investigation of this molecular mechanism is also necessary for the development of potential therapeutic agents for diseases with poor prognosis.

MATERIALS AND METHODS

Chemicals

All 12-membered macrolide derivatives **EM900**, **EM930**, **EM931**, **EM936**, **EM937**, **EM938** and **EM939** (Scheme 1) were synthesized at the Kitasato Institute for Life Sciences, Kitasato University. All other chemicals were of analytical grade. Physicochemical properties of all new compounds (**EM930**, **EM931**, **EM936**, **EM937**, **EM938** and **EM939**) are reported in the Supplementary Information.

General methods

Analytical and preparative thin-layer chromatography separations were performed using precoated silica gel plates with a fluorescent indicator (Merck 60 F254). Flash column chromatography was performed using Kanto Chemical (60N, spherical neutral, 0.040–0.050 mm, Cat. No. 37563-84) or Merck silica gel (60N, 230–400 mesh ASTM 0.040–0.063 mm, Cat. No. 109385). ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, using a JEOL ECA-500 spectrometer (500 MHz). Chemical shifts are expressed in ppm using internal solvent peaks for $CDCl_3$ (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.0

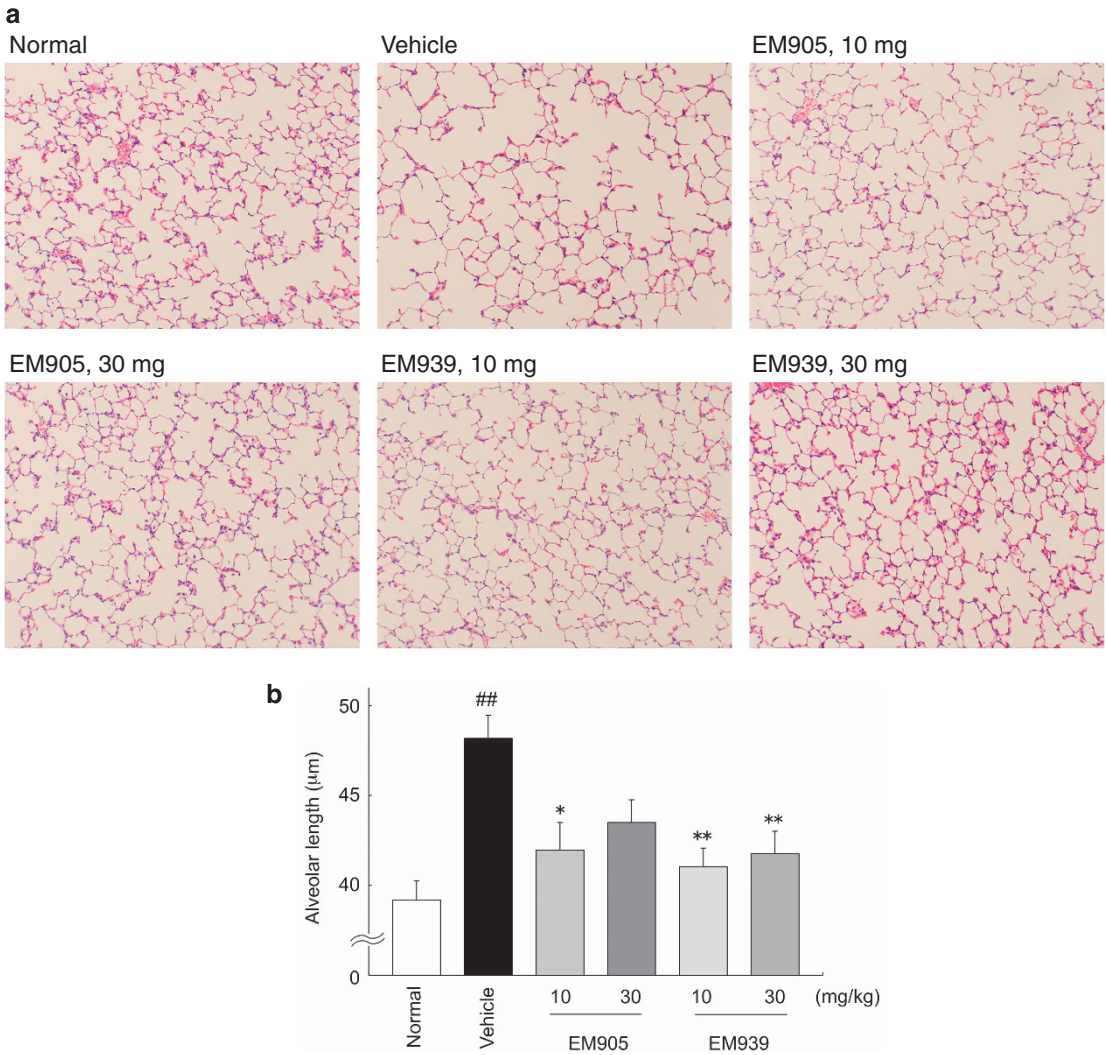


Figure 3 Effects of macrolide compounds **EM905** and **EM939** on alveolar length in cigarette smoke-exposed guinea pigs. Histological examinations were performed 4 weeks after cigarette smoke exposure. (**a**) Representative photomicrographs (original magnification: ×25) of hematoxylin-eosin-stained sections of smoke-exposed and control guinea pig lung tissues. (**b**) Inhibition of cigarette smoke-induced increase in alveolar length. Each value represents the mean ± s.e. obtained for 8–10 animals. ##: $P<0.01$; significant difference from normal (Student's *t*-test). *: $P<0.05$, **: $P<0.01$; significant difference from vehicle (Dunnett's multiple test).

ppm) as references. *J*-values are given in hertz. Coupling patterns are expressed as s (singlet), d (doublet), dd (double doublet), ddd (double double doublet), dt (double triplet), t (triplet), q (quartet), m (multiplet) or br (broad). All IR spectra were measured using a Horiba FT-210 spectrometer. High- and low-resolution mass spectra were acquired using JEOL JMS-700 MStaiton and JEOL JMS-T100LP instruments. Melting points were determined using a Yanaco Micro Melting Point System MP-500P. HPLC analysis was performed on a model LC-2000 system (JASCO Corporation, Tokyo, Japan) equipped with a UV-2077 Plus detector (JASCO Corporation; Column, PEGASIL ODS SP100 (4.6ϕ × 250 mm, Senshu Scientific Co., Ltd., Tokyo, Japan): condition of HPLC; isocratic 75% MeCN/H_2O over 30 min, flow 1.0 ml min^{-1}, temperature 40 °C, detect 210 nm).

Promotive activity of monocyte to macrophage differentiation and cytotoxicity measurements

Promotive activities were determined by modifying a previously reported method.[16] The THP-1 cell line, derived from a monocytic leukemia patient, was supplied by the Health Science Research Resources Bank (Tokyo, Japan). THP-1 cells (2×10^4 per well in Roswell Park Memorial Institute 1640 medium (0.1 ml) containing 10% fetal bovine serum (GIBCO BRL, Kanagawa, Japan) were seeded in 96-well tissue culture microplates (IWAKI, Shizuoka, Japan) and cultured in the presence of 1 nM phorbol 12-myristate 13-acetate (Sigma, Tokyo, Japan) with each macrolide (1–100 μM) for 3 days at 37 °C under 5% CO_2 humidified air. The number and viability of adherent cells were measured by colorimetric determination using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT, Nacalai tesque, Kyoto, Japan) at 550 nm. To evaluate the promotion of monocyte to macrophage differentiation, ED_{50} values were determined and compared with the results obtained for EMA at 100 μM. All data are the mean values for three independent experiments. Cytotoxicity (μM) toward THP-1 cells was determined using cell count reagent SF (Nacalai tesque) according to manufacturer's instructions.

Antibacterial activity measurement

Antibacterial activities of **EM900** derivatives against *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Citrobacter freundii, Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris, Morganella morganii, Serratia marcescens, Enterobacter cloacae, Enterobacter aerogen, Pseudomonas aeruginosa* and *Acinetobacter*

calcoaceticus were investigated by the National Committee for Clinical Laboratory Standards method.[44]

Experimental emphysema in guinea pigs

Hartley guinea pigs (Japan SLC) were exposed either to room air or the smoke of 30 cigarettes (Hi-lite filter cigarettes: 17 mg of tar and 1.4 mg of nicotine) for 60 min per day, 5 days per week, for 4 weeks in an exposure chamber (Flow-past type nose-only inhalation chamber, NH06-CIG01A, MIPS Ltd., Japan, Tokyo). Six experimental groups were established as follows. Group 1: 10 mg kg^{-1} **EM905** treatment+smoke-exposed ($n=8$); group 2: 30 mg kg^{-1} **EM905** treatment+smoke-exposed ($n=9$); group 3: 10 mg kg^{-1} **EM939** treatment+smoke-exposed ($n=9$); group 4: 30 mg kg^{-1} **EM939** treatment+smoke-exposed ($n=8$); group 5: vehicle control+smoke-exposed ($n=8$); group 6: air exposed control ($n=10$). Test compounds were prepared as 2 or 6 mg ml^{-1} suspensions in 0.5% CMC-Na and were orally administered once daily to the animals via microtube throughout the experiment. At the end of the experiment, guinea pigs were endotracheally intubated under anesthesia (i.p. injection of 1.6 g kg^{-1} urethane) and their pulmonary functions were estimated using the BioSystem for Maneuvers PLY3115 (Buxco Electronics Inc., Wilmington, NC, USA) to obtain functional residual capacity or residual volume. Next, animals were killed and their lungs were fixed intratracheally with formalin (5%) at a pressure of 20 cm H_2O and subsequently stained with hematoxylin-eosin for histology and morphometry. Ten areas of hematoxylin-eosin-stained sections presenting a maximum number of alveoli were sampled. Photographs were captured at ×10 magnification using a microscope (CX21, Olympus, Tokyo, Japan) connected to a digital camera system. Images were directly collected using an image analysis software (WinROOF Ver.5.0, Mitani Corp., Tokyo, Japan), and individual alveolar lengths were measured.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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