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Review Article

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Immunotherapy of Autoimmune Diseases with Nonantibiotic Properties of Tetracyclines

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Abbreviations
AD, Alzheimer's disease; APC, Ag presenting cell; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; COX-2, cyclooxygenase-2; DC, dendritic cell; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalitis; GO, Graves' orbitopathy; iNOS, inducible nitric oxide synthase; Mino-DC, DC generated from bone marrow cells in the presence of minocycline; MMP, matrix metalloproteinase;

ABSTRACT

Tetracyclines, which have long been used as broad-spectrum antibiotics, also exhibit a variety of nonantibiotic activities including anti-inflammatory and immunomodulatory properties. Tetracyclines bind to the 30S ribosome of the bacteria and inhibit protein synthesis. Unlike antimicrobial activity, the primary molecular target for the nonantibiotic activity of tetracycline remains to be clarified. Nonetheless, the therapeutic efficacies of tetracyclines, particularly minocycline and doxycycline, have been demonstrated in various animal models of autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis, and asthma. In this study, we summarized the anti-inflammatory and immunomodulatory activities of tetracyclines, focusing on the mechanisms underlying these activities. In addition, we highlighted the on-going or completed clinical trials with reported outcomes.

Keywords: Minocycline; Doxycycline; Anti-inflammatory activity; Immunomodulatory activity; Autoimmune disease; Clinical trial

INTRODUCTION

Tetracyclines are a group of broad spectrum antibiotics active against a wide range of pathogenic bacteria, including *Chlamydia*, *Rickettia*, and *Mycoplasma* (1). Tetracyclines bind to and inhibit the functions of both prokaryotic 70S and eukaryotic 80S ribosomes. However, they inhibit the growth of bacterial cells, but not that of animal cells, because in bacteria they penetrate using an efficient and specific transport system, whereas in animal cells their internal concentration never attains the inhibitory levels (1). Chlortetracycline and oxytetracycline, purified from the fermentation broth of *Streptomyces* sp., are classified as natural tetracyclines and are the first members of the tetracycline family introduced as clinical antibacterial chemotherapeutics. Later, numerous semisynthetic derivatives, including minocycline and doxycycline, have been developed and introduced for clinical use (2).

In addition to their well-known antibiotic activities, tetracyclines, especially minocycline and doxycycline, exhibit great therapeutic potential in various animal models of neurological diseases, including stroke, spinal-cord injury, Parkinson's disease, and Huntington's disease (3-5). Tetracyclines are also beneficial in the treatment of various autoimmune disorders,

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MS, multiple sclerosis; NO, nitric oxide; OA, osteoarthritis; PGE2, prostaglandin E2; PLA2, phospholipase A2; RA, rheumatoid arthritis; RRMS, relapsing remitting multiple sclerosis; SRBC, sheep red blood cell.

Conflict of Interest
The authors declare no potential conflicts of interest.

Author Contributions
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such as multiple sclerosis (MS), rheumatoid arthritis (RA), and asthma (6-8). Recently, doxycycline has even been proposed as a potential anti-cancer drug based on its inhibitory effects on cancer cell proliferation and metastasis (9).

Although the mechanism of action for the antibiotic activities of tetracyclines is well known, the exact mechanism of action underlying their nonantibiotic activities is yet to be fully understood. For instance, multiple mechanisms have been proposed for the nonantibiotic activity of minocycline. These include the inhibition of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (10-12), inhibition of proinflammatory enzymes such as inducible nitric oxide synthetase (iNOS) and matrix metalloproteinases (MMPs) (13,14), downregulation of MHC class II expression in microglia and macrophages (15), suppression of T cell proliferation and activation (16,17), and induction of tolerogenic dendritic cells (DCs) (18,19).

In this review, we summarized the anti-inflammatory and immunomodulatory activities of tetracyclines, especially minocycline and doxycycline, and the clinical trials that are being carried out to treat autoimmune disorders with these antibiotics.

ANTI-INFLAMMATORY AND IMMUNOMODULATORY ACTIVITIES

Inhibition of T cell proliferation and cytokine production

The suppressive effects of tetracyclines on T cells have been described earlier. Tetracyclines, in particular doxycycline, have potent suppressive effects on delayed-type hypersensitivity (DTH) response to sheep red blood cells (SRBCs) in mice, although they have no significant suppressive effects on the Ab production to SRBC (20). Minocycline suppresses the mitotic response of lymphocytes in whole blood cultures with phytohemagglutinin (21). Minocycline inhibits TCR/CD3-induced IL-2, IFN-γ, and TNF-α production by $CD4^+$ synovial T cell clones derived from the synovium (22). The production of TNF-α and IFN-γ by stimulated PBMCs are also inhibited by minocycline (16). Further, minocycline suppresses NFAT1-mediated transcriptional activation in human $CD4^+$ T cells (23).

Suppression of Ag presentation capacity

Tetracyclines, in particular minocycline, inhibit the processing and presentation of tetanus toxoid by human peripheral blood Ag presenting cells (APCs) to the human T cells. This inhibition was not observed when tetanus toxoid was first incubated with APCs before the addition of minocycline, indicating that minocycline inhibits the intracellular processing of tetanus Ag (24). Recently, we showed that DCs generated from the bone marrow cells in the presence of minocycline together with GM-CSF and IL-4 (Mino-DCs) demonstrated the characteristics of regulatory DCs. Mino-DCs were impaired in MHC class II-restricted exogenous Ag presentation, inflammatory cytokine production, and alloantigen-specific T cell priming. However, they increased in the production of IL-10 and expansion of $CD4^+CD25^+Foxp3^+$ T regulatory cells (18,19).

Inhibition of microglial cell function

Numerous studies have reported tetracyclines to inhibit the activation and proliferation of microglial cells, macrophage-like cells located throughout the brain and spinal cord. Inflammation induced and aggravated by microglial cells is one of the reasons

responsible for the ischemic neuronal death. Thus, compounds inhibiting the activation and proliferation of microglial cells may have a therapeutic value as anti-ischemic drugs. Previous studies reported the therapeutic efficacy of tetracyclines in a rat model of transient middle cerebral artery occlusion and in a gerbil model of forebrain ischemia (25,26). Since then, the protective effects of tetracycline have been explored in various models of central nervous system diseases, including MS (17,27), Parkinson's disease (28,29), nerve injury-induced neuropathic pain (30), and neuroinflammation (31). Minocycline appears to exert neuroprotective activity via the inhibition of microglial activation and proliferation (26,29,32).

Inhibition of the production of proinflammatory cytokines

One of the major nonantibiotic activities of tetracyclines is the inhibition of inflammatory cytokine production by the immune cells. Numerous studies have shown that tetracyclines, especially minocycline and doxycycline, suppress the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (18,33-36). The inhibitory effect of doxycycline on proinflammatory cytokine production has also been demonstrated in various disease models, including neonatal rat hypoxia-ischemia (36), murine experimental autoimmune encephalitis (EAE) (18), and murine polymicrobial sepsis (37). The *in vivo* efficacy of doxycycline has also been demonstrated in patients with dengue hemorrhagic fever (38). Since proinflammatory cytokines play an essential role in the process of inflammation, blocking their production using tetracyclines provide the theoretical basis for the clinical application of tetracyclines in the treatment of inflammation-based diseases.

Inhibition of MMPs

The best-characterized nonantibiotic activity of tetracyclines is their ability to inhibit MMPs. In 1983, minocycline has been reported to inhibit tissue collagenolytic enzyme activity in gingiva of diabetic rats by a mechanism unrelated to its antibacterial efficacy (39). Later, it was confirmed that some of the tetracyclines, including doxycycline, inhibit MMPs. MMPs are a family of zinc-dependent protease, and involves in the metabolism of connective tissue and in the process of cell formation (40,41). MMPs can be subdivided based on the substrate specificity into collagenases, gelatinases, stromelysins, matrilysins, membrane-bound MMPs, and other MMPs (42). MMPs are secreted by many cells including fibroblasts, vascular smooth muscle, and leukocytes, and are involved in various physiological processes such as inflammation, immunity, neurite growth and bone remodeling (43). Increased MMP activity is associated with the pathophysiology of various immune disorders, including RA, inflammatory bowel diseases, and some neurodegenerative diseases (27,44). MMPs that are secreted from activated T cells or tissues play an important role in the pathogenesis of EAE and MS (45). While tetracyclines affect a number of MMPs, minocycline and doxycycline are especially effective in inhibiting MMP-2 and MMP-9 (6).

Inhibition of iNOS

Inhibition of iNOS activity is also a common feature of most tetracyclines. Overproduction of nitric oxide (NO) has been implicated in a variety of inflammatory and autoimmune diseases including RA, systemic lupus erythematosus, ulcerative colitis, and Crohn's disease (46). NO is the major mediator of tissue injury in arthritis (47). Doxycycline and minocycline inhibit iNOS in LPS-stimulated murine macrophages (48,49) and also in cultures of cartilage cuts obtained from patients with advanced osteoarthritis (OA) undergoing knee replacement surgery, which spontaneously release NO sufficient to cause cartilage damage (48). In cultures of cartilage cuts, doxycycline or minocycline inhibits iNOS activity through the

inhibition of *NOS* mRNA expression. In a murine model of dextran sodium sulfate-induced acute colitis, treatment with minocycline significantly diminished the mortality rate and attenuated the severity of the disease (50). Mechanistic investigation showed that minocycline administration suppresses the iNOS expression along with the inhibition of proinflammatory cytokine secretion.

Inhibition of inflammation-associated molecules

Minocycline and doxycycline inhibit secretory phospholipase A_2 (PLA_2), an enzyme with an important role in inflammatory processes. Structural analysis showed that minocycline binds to the hydrophobic cleft located at the entrance of the active site in the calcium (Ca^{2+}) binding loop of PLA_2 (51,52). Thus, the binding of the substrate molecules to the active site is inhibited, resulting in the inhibition of enzymatic activity (52).

Cyclooxygenase-2 (COX-2), increased during inflammation, leads to the synthesis of prostaglandins. Minocycline inhibited COX-2 expression and subsequently production of prostaglandin E_2 (PGE_2) in a rat model of transient middle cerebral artery occlusion (26). A recent study showed that minocycline significantly reduces the expression of COX-2 and PGE_2 in rats with systemic LPS-induced spinal cord inflammation (53). However, there is a report showing tetracyclines to enhance the PGE_2 production in RAW 264.7 cells (54).

The mechanisms for the nonantibiotic activity of tetracyclines, especially minocycline and doxycycline, are summarized in **Fig. 1**. Unlike the antimicrobial activity, multiple mechanisms have been proposed for the nonantibiotic activity of tetracyclines.

CLINICAL TRIALS BASED ON ANTI-INFLAMMATORY AND IMMUNOMODULATORY ACTIVITY OF TETRACYCLINES

Based on the successful therapeutic outcomes of minocycline or doxycycline in animal models, various clinical trials have been completed or ongoing in patients with autoimmune diseases such as MS, RA, asthma, Alzheimer's disease (AD), and type 2 diabetes. Clinical trials ongoing or with reported outcomes that have been registered on ClinicalTrials.gov portal are summarized in **Table 1**.

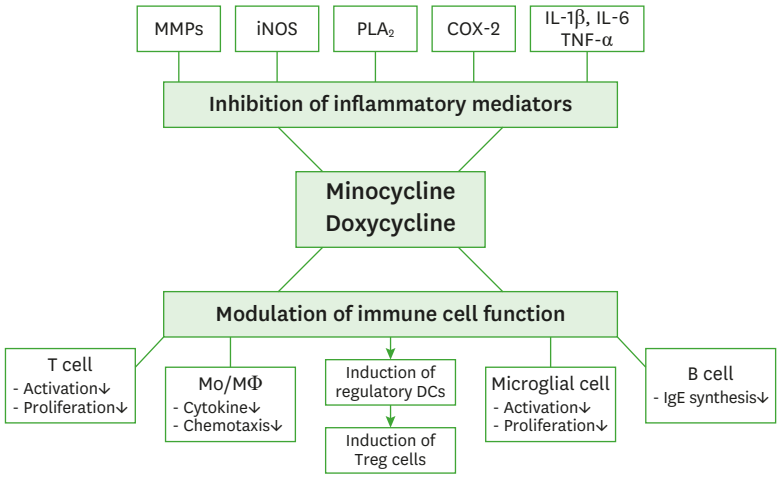

Figure 1. Mechanisms for the nonantibiotic activities of minocycline and doxycycline.

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Table 1. Clinical trials ongoing or with reported outcomes that have been registered on ClinicalTrials.gov

Disease	Antibiotics	Primary purpose of study	Phase	Status (last update posted)	NCT number	Major study results and related publications
Multiple sclerosis	Minocycline	Determine that oral minocycline improves the efficacy of glatiramer acetate in RRMS.	2	Completed (Apr. 12, 2011)	NCT00203112	The risk of relapse tended to be lower in the minocycline-glatiramer combination group (55).
		Determine that oral minocycline reduces the conversion from a first demyelinating event (also known as CIS) to MS.	3	Completed (Feb. 27, 2017)	NCT00666887	The risk of conversion from a CIS to MS was significantly lower with minocycline than with placebo over 6 months but not over 24 months (56).
		Confirm the benefits of minocycline in MS patients who have had a first demyelinating event in the past 180 days and have at least 2 brain T2 lesions of minimum 3 mm in diameter.	3	Recruiting (Mar. 2, 2020)	NCT04291456	No results posted.
	Doxycycline	Evaluate the efficacy, safety, and tolerability of combination therapy with intramuscular IFN-β1a and oral doxycycline in patients with RRMS having breakthrough disease activity.	4	Completed (Apr. 4, 2018)	NCT00246324	Combination of intramuscular IFN-β1a and oral doxycycline treatment was effective, safe, and well tolerated (57).
Rheumatoid arthritis	Minocycline	Determine if a combination of methotrexate and minocycline works better than methotrexate alone in early RA.	3	Completed (Jan. 17, 2018)	NCT00579644	No results posted.
	Doxycycline	Determine the efficacy of doxycycline in the treatment of RA when used in combination with methotrexate.	NA	Completed (May 18, 2020)	NCT03194204	In patients with early seropositive RA, initial therapy with methotrexate plus doxycycline was superior to treatment with methotrexate alone (62).
Osteoarthritis	Doxycycline	Determine whether doxycycline decreases the severity or rate of progression of OA in the knee.	3	Completed (Apr. 30, 2013)	NCT00000403	Doxycycline lowers the rate of JSR in woman with moderate unilateral knee OA, but has no effect on the rate of JSR in the contralateral knee (63).
Asthma	Minocycline	Trial effects of minocycline as add-on therapy to adults with asthma with a history of requiring at least one episode of oral steroid therapy to control the disease.	2	Completed (Apr. 18, 2017)	NCT00456677	No results posted.
		To test the hypothesis that minocycline can be used in the treatment of asthma. Evaluates the benefit of minocycline as add-on therapy for adults with asthma.	2	Completed (Mar 10, 2020)	NCT00536042	No results posted.
Chronic obstructive pulmonary disease	Doxycycline	Demonstrate that doxycycline reduces neutrophilic airway inflammation in patients with COPD.	4	Unknown (Mar. 6, 2009)	NCT00857038	A 3-wk course of doxycycline did not influence the sputum levels of myeloperoxidase, nor any of the other inflammatory sputum and systemic markers (65).
Autism	Minocycline	To test the effectiveness of minocycline in treating regressive autism. A medicine with anti-inflammatory properties may be beneficial for children with regressive autism.	4	Completed (Dec. 11, 2015)	NCT00409747	In this small group of children, no clinical improvements were observed during or after the 6 months of minocycline administration (66).
		Evaluate the effect of minocycline on microglial activation in adults with ASD, and provide information regarding the relationship between levels of brain inflammation, cognitive and behavioral function in ASD.	1	Recruiting (Dec. 22, 2018)	NCT03117530	No results posted.
		To determine if minocycline shows initial evidence of efficacy, safety, and tolerability in youth with ASD aged between 12 and 22 years.	1	Recruiting (Apr. 24, 2020)	NCT04031755	No results posted.
Alzheimer's disease	Doxycycline	Examine the cerebrospinal fluid of patients with AD for biomarkers of inflammation and their response to the antibiotics, doxycycline and rifampicin.	3	Unknown (Feb. 4, 2009)	NCT00715858	A 3-month course of doxycycline and rifampicin reduced the cognitive worsening at 6 months of follow-up in patients with mild to moderate AD (67).

(continued to the next page)

Table 1. (Continued) Clinical trials ongoing or with reported outcomes that have been registered on ClinicalTrials.gov

Disease	Antibiotics	Primary purpose of study	Phase	Status (last update posted)	NCT number	Major study results and related publications
Cystic fibrosis	Doxycycline	Determine the therapeutic potential of doxycycline in modulating host airway inflammation and its pharmacokinetics, pharmacodynamics, and safety in patients with CF.	4	Completed (Mar. 30, 2017)	NCT01323101	Doxycycline exhibited favorable oral absorption characteristics, with an overall pharmacokinetic profile similar to those in other patient populations (69).
		Examine the role of doxycycline in the treatment of CF patients who are hospitalized.	NA	Completed (Mar. 6, 2017)	NCT01112059	Doxycycline reduced the total sputum MMP-9 levels and improved the clinical outcome measures during CF inpatient exacerbation.
Graves' orbitopathy	Doxycycline	Evaluate the effects of a subantimicrobial dose doxycycline (50 mg/day), administered for 12 wks, for patients with active moderate-severe GO.	1, 2	Completed (Dec. 10, 2013)	NCT01727973	Subantimicrobial dose of doxycycline appeared to be effective and safe for the treatment of active and moderate-to-severe GO (71).
		Compare the efficacy and safety of prednisone versus sub-antimicrobial dose of doxycycline (50 mg/day) in the treatment of active and moderate-severe GO.	2, 3	Unknown (Dec. 10, 2013)	NCT01809444	No results posted.
		Evaluate the effects of a subantimicrobial dose of doxycycline (50 mg/day), administered for 12 wks, on patients with mild GO.	2	Unknown (Jul. 30, 2014)	NCT02203682	No results posted.
Type 2 diabetes	Doxycycline	To test the hypothesis that doxycycline will enhance the insulin sensitivity and decrease inflammation in obese participants with type 2 diabetes.	4	Completed (Jan. 13, 2020)	NCT01375491	Short-term treatment with doxycycline resulted in decreased inflammation and improved insulin sensitivity in type 2 diabetes patients (72).

ASD, autism spectrum disorders; CIS, clinically isolated syndrome; JSR, joint space narrowing, NA, not applicable.

MS

Three clinical trials have been completed with positive results and one clinical trial is ongoing. The first clinical trial for MS treatment examined the add-on effect of oral minocycline on subjects treated daily with Copaxone®, an injectable glatiramer acetate, in relapsing-remitting MS (RRMS). The combination treatment with minocycline and glatiramer acetate reduced the risk of relapse and was found to be safe and well tolerated (55). Another clinical trial determined whether oral minocycline reduced the conversion from a first demyelinating event, also known as clinically isolated syndrome, to MS. Results revealed that 100 mg of minocycline, administered orally twice daily, to significantly lower the conversion of clinically isolated syndrome to MS, as defined according to the 2005 McDonald criteria (56). Doxycycline was also examined to treat MS. The related study evaluated the efficacy, safety, and tolerability of combination therapy with intramuscular IFN-β1a and oral doxycycline in patients with RRMS having breakthrough disease activity. The clinical trial results showed combination of intramuscular IFN-β1a and oral doxycycline treatment to be effective, safe, and well tolerated (57). Currently, another trial is ongoing to confirm the benefits of minocycline in MS patients who have had a first demyelinating event in the past 180 days and at least possesses 2 brain T2 lesions of minimum 3 mm in diameter (NCT04291456). A similar clinical trial was performed with doxycycline by Iranian scientists. They examined the efficacy of doxycycline as an add-on to IFN-β1a in the treatment of MS and claimed that combination therapy can decrease the relapse rate and improve the expanded disability status scale scores (58). Collectively, the clinical trial results highlighted minocycline as an effective and safe antibiotic for treating MS.

RA

Although not listed in the ClinicalTrials.gov portal, several clinical studies performed worldwide have shown tetracyclines, especially minocycline and doxycycline, to significantly

reduce the severity of RA (59-61). In ClinicalTrials.gov portal, 2 clinical trials are listed as complete. One clinical trial aimed to determine whether a combination of methotrexate and minocycline works better than methotrexate alone in early RA (NCT00579644). The results of this study are awaited. The efficacy of doxycycline in the treatment of RA was also tested in combination with methotrexate. This study showed that initial therapy with methotrexate and doxycycline is superior to treatment with methotrexate alone in patients with early seropositive RA (62). Doxycycline was also tested to determine whether it could decrease the severity or rate of progression of OA in the knee. Results revealed doxycycline to lower the rate of joint space narrowing in women with moderate unilateral knee OA, but had no effect on the rate of joint space narrowing in contralateral knee (63).

Asthma

Two clinical trials are listed as complete in the ClinicalTrials.gov portal. Both the clinical trials examined the add-on effects of minocycline to standard asthma care regimen in adults with asthma (NCT00456677, NCT00536042). The results are not yet available. It is noteworthy that tetracycline, especially minocycline, in addition to its potent anti-inflammatory effects also decreases the production of IgE in an isotype-specific manner and suppresses the IgE-mediated responses (64).

Chronic obstructive pulmonary disease (COPD)

Based on the observation that doxycycline inhibits neutrophil-mediated inflammation, one clinical trial was performed to examine whether doxycycline reduces the neutrophilic airway inflammation in patients with COPD. Results revealed that doxycycline does not influence the markers of neutrophil inflammation in COPD (65).

Autism

Autism is a neurodevelopmental disorder that results in abnormalities concerning social and language development. Although there exists a strong evidence of heritability, the genes involved have not been identified. Based on the observation that minocycline is a powerful inhibitor of microglial activation and is neuroprotective in mouse models of amyotrophic lateral sclerosis and Huntington's disease, 3 clinical trials have been conducted to examine the effectiveness of minocycline in treating regressive autism. In a clinical trial involving a small group of children, no improvements were observed during or after 6 months of minocycline administration (66). Two other clinical trials are currently ongoing (NCT03117530, NCT04031755).

AD

Based on the observation that inflammatory responses and microglial activation are involved in the progression of AD, one clinical trial was registered and performed to examine whether combination therapy with doxycycline and rifampicin is useful for treating AD. Results revealed that a 3-month course of doxycycline and rifampicin reduces the cognitive worsening at 6 months of follow-up in patients with mild to moderate AD (67). Because rifampicin also exerts protective effects in various models of neurodegeneration and brain trauma (68), greater number of studies is required to conclude that minocycline has a therapeutic value in the treatment of AD.

Cystic fibrosis (CF)

CF is characterized by chronic neutrophilic inflammation and elevated MMP-9 expression in the airway tissues. One clinical trial determined the therapeutic potential of doxycycline

in modulating the host airway inflammation in patients with CF and also assessed its pharmacokinetics, pharmacodynamics, and safety. Results primarily revealed the oral absorption characteristics and pharmacokinetic profiles of doxycycline without any description on the therapeutic efficacy (69). Another clinical trial examined the therapeutic value of doxycycline in the treatment of hospitalized CF patients. In this case, the study results showed that doxycycline, administered over an 8-day period during hospitalization, reduces the total sputum MMP-9 levels and improves the clinical outcome measures during CF inpatient exacerbation (70).

Graves' orbitopathy (GO)

GO, also called Graves' ophthalmopathy, is an autoimmune disease of the retroocular tissues occurring in patients with Graves' disease. At least 3 clinical trials have been registered in the ClinicalTrials.gov portal. These studies evaluated the effects of a subantimicrobial dose of doxycycline (50 mg/day) in patients with active and moderate-to-severe GO. One clinical trial reported the subantimicrobial dose of doxycycline to be effective and safe for the treatment of active and moderate-to-severe GO (71).

Type 2 diabetes

Obesity has been recognized as a state of subclinical inflammation that results in loss of insulin receptor function and decreased insulin sensitivity. As a part of inflammation, expression levels and activity of MMPs are significantly elevated in the rodent models of obesity and obese humans. One clinical trial was performed to test the hypothesis that doxycycline enhances the insulin sensitivity and decreases the inflammation in obese people with type 2 diabetes. Results revealed that short-term treatment with doxycycline decreased inflammation and improved the insulin sensitivity in the type 2 diabetes patients (72). Since this study is a proof-of-concept clinical trial involving a small number of participants, greater number of studies with larger sample sizes is required to determine the therapeutic efficacy of doxycycline in treatment of type 2 diabetes.

CONCLUSION AND PERSPECTIVES

Tetracyclines, especially minocycline and doxycycline, are promising candidates for the treatment of diseases with inflammatory background. Tetracyclines are also beneficial in the treatment of autoimmune diseases, such as MA, RA, and asthma. These therapeutic efficacies of tetracyclines are independent of their antimicrobial activities. The quest is to identify the molecular targets for these nonantibiotic activities of tetracyclines; however, the precise molecular target remains to be clarified. Unlike the antimicrobial activity, multiple mechanisms have been proposed for the nonantibiotic activity of tetracyclines, including inhibition of the production of proinflammatory cytokines, inhibition of proinflammatory enzymes such as iNOS and MMPs, downregulation of MHC class II expression in microglia and macrophages, suppression of T cell proliferation and activation, and induction of tolerogenic DCs. The molecular targets for most of these activities have not been clarified yet, except for the binding of tetracyclines to iNOS and MMPs. Hence, future studies should consider elucidating the precise primary target for the nonantibiotic activities of tetracyclines to substantiate the clinically relevant therapeutic effects.

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