Macrolide maintenance treatment for bronchiectasis
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CHAPTER 3

Immunomodulatory effects of macrolide antibiotics – part 1: biological mechanisms.

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Abstract
Macrolide antibiotics are well known for their antibacterial and anti-inflammatory properties. This article provides an overview of the biological mechanisms through which macrolides exert this ‘double effect’. Their anti-bacterial effect consists of inhibition of bacterial protein synthesis, impaired bacterial biofilm synthesis and attenuation of other bacterial virulence factors. Apart from these direct anti-microbial effects, macrolides are known for their modulating effect on many components of the human immune-system. By influencing the production of cytokines, they have a dampening effect on the pro-inflammatory response. Furthermore, the majority of cells, involved in the immune-response, are, one way or the other, influenced when macrolide antibiotics are administered. Having such an obvious effect on the various aspects of the immune system macrolides seem to be exceptionally suited for the treatment of chronic inflammatory diseases.

Introduction
Since their discovery in 1952, many beneficial effects have been attributed to antibiotics belonging to the macrolide family, originally isolated from cultures of Streptomyces erytriae. Macrolide antibiotics were named after their main characteristic; a macrocyclic lactone ring which can contain up to 23 atoms [1]. The most commonly used macrolides have 14 (e.g. erythromycin, clarithromycin, roxithromycin) or 15 (e.g azithromycin) atoms attached to their macrocyclic rings and are therefore defined as 14- or 15-membered ring macrolides. Over the last decades macrolide antibiotics have been used as a treatment for common infectious diseases like pneumonia, bronchitis, pharyngitis or skin infections, possessing a moderately broad spectrum of antibacterial activity.

An accumulating body of evidence has emerged, indicating that 14- and 15 membered ring macrolides possess modes of action independent of their antimicrobial activity. This became first known in 1987, when Kudoh and colleagues [2] reported a spectacular decrease in symptoms and increase in life expectancy in patients with diffuse panbronchiolitis (DPB) when they were treated with the macrolide erythromycin. Until then, DPB had been a rapidly progressive and debilitating inflammatory airway disorder carrying a very poor prognosis. After 1987, when erythromycin was introduced as standard therapy for DPB, an impressive increase of 10-year survival was seen; from 10-20% to more than 90% [3-6].

The unexpected success was attributed to a previously unknown anti-inflammatory effect of erythromycin. This theory was supported by the fact that serum levels of erythromycin in these DPB-patients were well below minimal inhibitory concentrations (MIC) for the detected pathogens and the known lack of susceptibility of most gram-negative organisms to erythromycin.

Exhaustive evidence has shown that macrolides indeed have a direct anti-microbial effect, but, more importantly, also modulate many components of the immune-response. Because of this anti-inflammatory or ‘immune modulating’ effect, macrolide antibiotics have been widely used as maintenance treatment for various chronic inflammatory pulmonary diseases. Chronic inflammatory diseases generally feature a distorted inflammatory response. Instead of protecting the human body against exogenous attacks, the cascade of anti-inflammatory responses fails, damaging cells and making them more vulnerable to new attacks. In this article we aim to clarify the biological mechanisms through which macrolides exert their immune-modulating and anti-bacterial effect. These mechanisms are shown schematically in figure 1.
Biofilm

A biofilm is an aggregate of micro-organisms immersed in a polysaccharide matrix, adherent to each other and to the airway mucosa. Biofilm-forming bacteria are protected from phagocytosis, antimicrobial agents and the ciliary action of the airway epithelial cells. Furthermore, micro-organisms gathered in a biofilm develop significantly different genetic properties, compared to planktonic species. Research on biofilm effects of macrolides mainly focuses on *Pseudomonas aeruginosa* (PA), being one of the more virulent biofilm-forming micro-organisms with a natural resistance to macrolides.

Effects of macrolides however, were also demonstrated on biofilm formation in *H influenzae* and *S epidermidis* [9,10]. Macrolides were shown to alter the structure and architecture of the bacterial biofilm [11-13]. Results of Japanese in vitro studies indicate that azithromycin and clarithromycin change the structure of bacterial biofilms via inhibition of polysaccharide synthesis [12,14]. An insufficient biofilm allows for enhanced phagocytosis and clearance of bacteria by alveolar macrophages [11,15].

Quorum sensing

During infection, bacteria employ mutual communication (quorum sensing [QS]) to coordinate the expression of genes, e.g. genes encoding for tissue-damaging factors [16]. Through production of auto-inducer molecules, genes can be switched on or off, depending on local pathogen density. Furthermore, activation of the QS cascade is claimed to promote biofilm formation and to stimulate IL-8 production, causing enhanced neutrophil influx at the site of infection [6]. Several authors suggest that suppression of QS-systems, through reduced transcription of QS-genes is also one of the mechanisms of macrolide action [16-18].

Bacterial adherence

In vitro and in vivo evidence suggests that PA bacilli, when cultured in the presence of low levels of macrolides, e.g. erythromycin, are less adherent to cells of the airway epithelium [19-21]. Since adherence of bacteria to mucosal surfaces is an important initial event in the pathogenesis of most bacterial infectious diseases, this could help explain the clinical efficacy of low-dose macrolide therapy in patients colonized with PA.
Mobility

The effect of macrolides on PA is accompanied by impairing the mobility of this micro-organism. *Pseudomonas* spp are mobile thanks to two distinctive modalities; flagella, tail-like structures that project from the cell body and move in a whip-like manner; and type IV pili (fimbriae), that provide twitching motility.

Exposure to sub-MIC concentrations of macrolide antibiotics results in loss of mobility, partly due to the inhibition of flagellin production [22-24] -the principal constituent of bacterial flagella- and partly because some macrolides alter the assembly of type IV pili [13,25]. This loss of mobility facilitates easier phagocytosis and killing of bacteria by alveolar macrophages.

Bacterial toxins

Cytotoxic enzymes, produced by bacteria when causing infection, including exotoxin A, alkaline protease, elastase and and phospholipase C, are important factors in bacterial virulence. Erythromycin, and, more recently, also azithromycin, have been shown to suppress the production of those enzymes and, consequently, to diminish bacterial virulence [23] [26-28].

Intracellular effects

Macrolides accumulate and show a prolonged retention in human cells after oral or IV administration, an effect that is augmented when macrolide treatment is given for a longer period of time [29-32]. In CF-patients treated with azithromycin (500mg daily) for at least 35 consecutive days, the concentration of azithromycin in neutrophils appeared to be up to 3000 times higher as compared to the concentration in plasma (wilms). Macrolides have also been shown to accumulate in alveolar macrophages [30,33].

The above suggests that tissue and intracellular concentrations may be more useful for assessing the antibacterial activity of azithromycin than serum concentrations [34,35]. Because intracellular concentrations of macrolide antibiotic often exceed the minimal inhibitory concentration (MIC) of phagocytized pathogens, macrolides have also been demonstrated to be effective against micro-organisms with in vitro macrolide resistance [35,36]. The excellent intracellular penetration of macrolides also appears to explain their effectiveness against intracellular pathogens [34],

2. Effects on airway epithelial cells and mucus properties

Besides inhibiting production of pro-inflammatory cytokines by bronchial epithelial cells [37,38], macrolides distinctly modulate features of bronchial epithelium, making it better armed against exogenous attacks. The bronchial epithelium is critically important in lung defense. In addition to being a mechanical barrier, it regulates electrolyte content of the airway surface liquid, by means of its tight junctions between adjacent cells. In vitro studies demonstrate that azithromycin increases transepithelial electrical resistance of human airway epithelium by changing the processing of tight junction proteins, as such preventing excess leakage of electrolytes and ameliorating mucus properties [39].

Furthermore, when airway epithelial cells are exposed to inflammatory mediators in vitro, macrolides display a protective effect against epithelial damage and ciliary dysfunction [40,41]. This positive effect on ciliary beat frequency, however, was not confirmed in in vivo studies in patients with chronic rhinosinusitis or bronchiectasis [42,43].

Airway mucus hypersecretion and the resulting excess sputum expectoration is an important characteristic of several chronic inflammatory pulmonary diseases. Mucus hypersecretion may lead to more exacerbations and poor health related quality of life (HRQL) [44].

Macrolides have been shown not only to reduce the quantity of expectorated sputum in vivo, for example in bronchiectasis, but also to change the composition of mucus, thereby enhancing mucus clearance [45-51].

3. Effects on the immune system

Innate immunity

Cytokine and chemokine response

Cytokines are hormone-like proteins that enable immune cells to communicate, and play an integral role in the initiation, perpetuation and subsequent down regulation of the immune response. Chemokines are cytokines with a particularly strong chemotactic capacity. Production of cytokines is effectuated by a variety of cell types, including alveolar macrophages, eosinophils, neutrophils and bronchial epithelial cells. Pro-inflammatory cytokines (such as interleukin (IL) 1, 2, 4, and 6, IFN-gamma, TNF-α, GM-GCSF) and – chemokines (such as IL-8, RANTES) amplify the immune response through positive feedback loops. Anti-inflammatory cytokines, e.g. IL-10, prostaglandins and Transforming Growth Factor (TGF) –β, attenuate the immune response through a negative-feedback mechanism. In general, macrolides inhibit synthesis and/or secretion of pro-inflammatory cytokines
while increasing the release of anti-inflammatory cytokines [1]. Some recent research however, promotes a more discerned view in which macrolides can differentially modulate pro-inflammatory cytokine secretion. [37]. Changes in cytokine and chemokine production are probably related to an effect of macrolides on the activation of transcription factors: nuclear factor (NF)-κB and activator protein (AP)-1 [52]. Inhibition of the production of pro-inflammatory cytokines has been described in several in vivo studies in healthy subjects and patients with cystic fibrosis (CF), asthma or chronic rhinosinusitis [53-59].

**Alveolar macrophages**

Macrophages play a critical role in the phagocytosis of apoptotic cells and the removal of exogenous particles, such as bacteria. Recent studies prove that macrolides promote phagocytosis of apoptotic cells by alveolar macrophages, thus avoiding secondary necrosis and the release of cell contents that may induce further inflammation [60-62]. In addition, some authors propose that macrolides promote monocyte-to-macrophage differentiation, increasing the number of active macrophages [63,64]. Results of earlier research suggest that macrolides antibiotics also enhance other macrophage functions, including their cytoidal activity [65].

**Neutrophils**

Neutrophils are key players of the inflammatory response in patients with chronic airway disease [66]. They accumulate at the site of infection, responding to increased levels of chemokines and cytokines, primarily IL-8 and TNF-α. Macrolide antibiotics exert influence on several domains of neutrophil function.

**Reaction to chemokines**

Macrolide antibiotics cause a significant reduction in the chemotactic response of neutrophils to chemokines [67,68]. Together with the above described inhibition of chemotactic generation, this results in markedly decreased airway neutrophilia in patients with various inflammatory pulmonary diseases [8,51,59,69-72].

**Degranulation**

Upon activation, neutrophils release granules containing cytotoxic enzymes, such as elastase, a process called neutrophil degranulation or exocytosis. In general, macrolides seem to stimulate exocytosis, which may result in enhanced anti-bactericidal activity [1,73-76].

**Adhesion**

Leukocyte adhesion is a hallmark of the inflammatory cascade and cell adhesion molecules (CAM) are the mediators of this event [1]. Cultured bronchial epithelial cells treated with erythromycin show reduced levels of intercellular adhesion molecule (ICAM)-1 [38,77]. These findings suggest that reducing release of adhesion molecules in bronchial epithelial cells is another anti-inflammatory effect of macrolide antibiotics.

**Oxidative burst**

The production and release of reactive oxygen species by neutrophils to enhance their cytotoxic capability, is referred to as the ‘oxidative’ or ‘respiratory’ burst, a process mediated by NADPH-dependent oxidase. Contradictory data have been reported with regard to the effect of macrolides on the oxidative burst. Previously, evidence was presented showing an attenuation of oxidative burst capacity, but more recent studies disclosed an opposite effect or no effect at all [74,78-80].

**Apoptosis**

In the previous decade, it had already been proposed that apoptosis (programmed cell death) limits the ability of neutrophils to damage tissue while being involved in an inflammatory response [81,82]. Since then several in vitro studies demonstrated that macrolides shorten neutrophil survival by accelerating neutrophil apoptosis [74,79,82-84].

**Adaptive immunity**

The aforementioned research data indisputably show the existence of a direct modulating effect of macrolides on the innate immune system. Studies focusing on effects of macrolide antibiotics on cellular immunity also clearly demonstrate impact on T-cell regulation and antigen presentation.

Long-term use of macrolide antibiotics reduces the elevated number of lymphocytes in BAL-fluid of DPB patients to sub-normal levels [85,86]. In addition, 14- and 15 membered ring macrolides appear to be involved in the augmentation of apoptosis of activated lymphocytes and, as such, reduce inflammation [87]. Dendritic cells are the most important antigen-presenting cells and play a central role in the initiation and regulation of immune responses. Sugiyama et al [88] demonstrated that clarithromycin and azithromycin modulate the function of dendritic cells, each macrolide shows a different, immune-dampening effect. In addition, macrolides appear to have a suppressive effect on pro-inflammatory cytokine production by T-cells [89,90].
An early in vivo study in healthy volunteers showed a small but significant positive effect of azithromycin on the proliferative B-cell response of stimulated lymphocytes [91]. A more recent study in patients with bronchiectasis however, failed to confirm this finding [92], while research in vitro demonstrated an opposite effect [93].

Conclusion

Macrolide antibiotics are well known for their antibacterial and anti-inflammatory properties. They clearly possess an anti-bacterial effect, that consists of inhibition of bacterial protein synthesis, impaired bacterial biofilm synthesis, and attenuation of other bacterial virulence factors. Apart from these direct anti-microbial effects, macrolides are known for their modulating effect on many components of the human immune system. By influencing the production of cytokines, they have a dampening effect on the pro-inflammatory response. Furthermore, the majority of cells involved in both the innate and adaptive immune-response, are, one way or the other, influenced when macrolide antibiotics are administered. The most distinct effect of macrolides is found on neutrophils, the key players of the anti-inflammatory response. Among other things, neutrophil accumulation, adhesion and apoptosis are clearly reduced, which results in markedly decreased airway neutrophilia. Studies focusing on effects of macrolide antibiotics on cellular immunity also clearly demonstrate impact on T-cell regulation and antigen presentation.

Future perspectives

In the near future, clinicians might add new immunomodulatory drugs of the macrolide family to their armamentarium Immunomodulatory macrolide antibiotics devoid of anti-infective properties are developed by modifying the molecular structure of the atoms attached to the macrocyclic ring. These purely immunomodulatory macrolides would offer a way to circumvent bacterial resistance. This concept has been investigated for tetracyclines, an other group of antibiotics which also have anti-inflammatory properties. Chemically modified tetracyclines (CMT), without anti-bacterial capacity, induce an anti-inflammatory response by modulating cytokine and matrix metalloproteinase secretion [94-98]. However, only in vitro and animal studies have been performed investigating the effect of CMT. To our knowledge, no phase 1 studies are yet available describing the efficacy and safety of purely immunomodulatory drugs.

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