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Valosin containing protein (VCP) interacts with macrolide antibiotics without mediating their anti-inflammatory activities

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ABSTRACT

In addition to antibacterial activity, some macrolide antibiotics, such as azithromycin and clarithromycin, also exhibit anti-inflammatory properties in vitro and in vivo, although the targets and mechanism(s) of action remain unknown. The aim of the present study was to identify protein targets of azithromycin and clarithromycin which could potentially explain their anti-inflammatory effects. Using chemical proteomics approach, based on compound-immobilized affinity chromatography, valosin containing protein (VCP) was identified as a potential target of the macrolides. Validation studies confirmed the interaction of macrolides and VCP and gave some structural characteristics of this interaction. Cell based assays however, including the use of gene silencing and the study of VCP specific cellular functions in J774.A1 (murine macrophage) and IB3-1 (human cystic fibrotic epithelial) cell lines, failed to confirm an association between the binding of the macrolides to VCP and anti-inflammatory effects. These findings suggest the absence of an abundant high affinity protein target and the potential involvement of other biological molecules in the anti-inflammatory activity of macrolides.

1. Introduction

Macrolide antibiotics are 14-, 15- and 16-membered macrocyclic compounds with one or two sugar moieties. They are bacteriostatic compounds and inhibit protein synthesis by reversibly binding to the 50S subunit of the bacterial ribosome (Schlunzen et al., 2001). In addition to antimicrobial activity, certain macrolide antibiotics, such as azithromycin and clarithromycin, also have immunomodulatory effects demonstrated in many in vitro and in vivo studies (Culic et al., 2001; Ianaro et al., 2000; Shinkai et al., 2008; Vanaudenaerde et al., 2008). They have shown a variety of beneficial effects, mainly in chronic inflammatory lung diseases such as diffuse panbronchiolitis, chronic obstructive pulmonary disease, chronic rhinosinusitis, cystic fibrosis, asthma, bronchiolitis obliterans and bronchiectasis (Lopez-Boado and Rubin, 2008).

Based on studies in vitro, these macrolide antibiotics decrease the production of various cytokines, chemokines, adhesion molecules, mucus and the oxidative burst; increase epithelial integrity, differentiation of monocytes to macrophages and the switch from M1 to M2 macrophages; and also modulate proliferation and apoptosis in many cells involved in inflammation (neutrophils, macrophages, lymphocytes, epithelial cells, dendritic cells, fibroblasts, smooth muscle cells, eosinophils and mast cells) (Halldorsson et al., 2010; Ito et al., 2009; Kohyama et al., 1999; Morikawa et al., 1996, 2002; Reato et al., 2004; Sato et al., 2001; Shimane et al., 2001; Stamatiou et al., 2009; Sugiyama et al., 2007; Vanaudenaerde et al., 2007; Yoshida et al., 2005; Zimmermann et al., 2009). Such numerous effects on the functions of cells involved in the process of inflammation highlight the immunomodulatory properties of azithromycin and clarithromycin and offer explanations for the beneficial effects of these macrolides on airway inflammation in chronic inflammatory lung diseases.

Despite all these effects in various in vivo and in vitro studies, the mode of action of the mentioned macrolides at the molecular level is unknown. Eukaryotic protein targets which might explain all or some of the mentioned immunomodulatory effects of macrolides have not been identified. It is described that macrolides interact with the motilin receptor (Peeters et al., 1989) and are substrates for ABC transporters

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(Seral et al., 2003), but these interactions cannot explain their immunomodulatory effects. Macrolides have been reported to indirectly inhibit the activation of NFκB and AP-1, transcription factors which regulate the expression of many proteins involved in inflammation (Desaki et al., 2000). Inhibition of ICSBP (interferon consensus sequence binding protein), a transcription factor which regulates expression of IL-12, has been shown in macrophages (Yamauchi et al., 2009). In various cellular models, macrolides inhibited activation of ERK 1/2 (Shinkai et al., 2006), phospholipase D (Abdelghaffar et al., 1997), phospholipase A1 (Van et al., 1996), decreased the concentrations of intracellular Ca^{2+} (Kondo et al., 1998) and cyclic AMP and consequently the activation of CREB transcription factor (Abeyama et al., 2003).

Chemical proteomics approaches employ a variety of techniques for the characterization of changes in proteomes after compound treatment or in the identification of compound-protein interactions (Bantscheff et al., 2009; Rix and Superti-Furga, 2009). Affinity chromatography with an immobilized ligand is well described and an efficient chemical proteomics approach for the target and off-target identification of bioactive molecules (Leslie and Hergenrother, 2008; Terstappen et al., 2007). After affinity chromatography with the relevant protein extract bound proteins are separated by electrophoresis and identified by mass spectrometry. Interaction of protein(s) with the compound and the relevance of this interaction for the activity of the compound are then confirmed in a validation phase employing different biochemical, cellular or in vivo assays. Identification of the target or off-target is important for elucidating the mode of action of a compound, a mechanism of disease, for rational drug design, for the design of more potent compounds, the identification of potential side effects and for patient stratification.

2. Materials and methods

2.1. Synthesis of derivatives for affinity chromatography

Azithromycin and clarithromycin were purchased from PLIVA. Derivatives of azithromycin and clarithromycin for affinity chromatography were synthesized as described (Alihodzic et al., 2006, 2009; Bright et al., 1988; Culic et al., 2006; Farmer et al., 2005; Mercep et al., 2004, 2006; Stepanic et al., 2011).

2.2. Immobilization of compounds to solid support

Affi-Gel 10 (Bio-Rad) matrix was used for immobilization of macrolide derivatives and affinity chromatography. Compounds dissolved at various concentrations in dimethyl sulfoxide (DMSO) were incubated with matrix for 2 h at room temperature. Compound to matrix ratio was adjusted to achieve appropriate compound density on matrix beads. Afterwards, 1 M ethanolamine was added to block free reactive groups on the matrix and incubated for an additional 1 h at room temperature. The control matrix was incubated with 1 M ethanolamine only. Matrices were washed with and stored in lysis buffer (50 mM HEPES, 150 mM sodium chloride, 1% (v/v) Triton X-100, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 17 μg/ml PMSF, 0.2 mM sodium orthovanadate, 2 mM sodium fluoride, 4 mM β-glycerophosphate, 0.4 mM sodium pyrophosphate, pH 7.4) overnight at +4 °C.

2.3. High performance liquid chromatography–mass spectrometry (HPLC–MS)

For determination of immobilization efficiency compounds were analyzed by HPLC–MS (Waters Alliance 2690) using XBridge C18 2.1×50 mm column (Waters) before and after immobilization to solid support. The samples were applied to the column and compounds were eluted with increasing concentrations of acetonitrile with a flow rate of 0.5 μl/min. Compounds were ionized at 2500 V and analyzed by mass spectrometry (Micromass Quattro, Waters).

For protein identification, peptides were separated by HPLC (μ1100, Agilent) using C18 75 μm column and eluted with increasing concentrations of acetonitrile with a flow rate of 0.25 μl/min. Separated peptides were ionized and analyzed by mass spectrometry (qTOF 2, Micromass). Proteins were identified using the X! Tandem search engine.

2.4. J774A.1 lysate preparation

J774A.1 cells (ATCC, USA) were grown in Dulbecco's modified Eagle medium supplied with 10% of fetal bovine serum and 1% GlutaMAX. 1×10^8 of J774A.1 cells were washed with phosphate buffered saline, scraped and centrifuged at 1200 rpm for 10 min at room temperature. They were lysed in 5 ml of lysis buffer on ice for 30 min and then centrifuged at 10,000 xg for 10 min at +4 °C. The supernatant was stored at −20 °C until use in affinity chromatography.

For Western blot analysis, 5×10^6 of J774A.1 cells were seeded in petri dishes (BD Falcon, 100×20 mm) in the same medium. The cells were treated with azithromycin (50 μM) or tunicamycin (6 μM, Sigma) for 6 h for analysis of endoplasmic reticulum stress or for 2 h with azithromycin and an additional 20 min with lipopolysaccharide (LPS, from E.coli, Sigma, 1 μg/ml) for analysis of signal transduction. The cells were lysed in 100 μl of lysis buffer as described.

2.5. Lysate preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from human buffy coats using Ficoll-Paque. Fifty milliliters of buffy coat was mixed with 150 ml of phosphate buffered saline and added to 15 ml of Ficoll-Paque. The samples were centrifuged at 400×*g* for 30 min at room temperature. Peripheral blood mononuclear cells were collected from Ficoll-Paque, mixed with phosphate buffered saline and centrifuged at 300×*g* for 10 min at room temperature. For lysis of erythrocytes, the pellet was mixed with 50 ml of ammonium chloride buffer (150 mM ammonium chloride, 10 mM sodium chloride, 1 mM EDTA, pH 7.4). After 3 min, cells were again centrifuged at 200×*g* for 10 min. The pellet was lysed in 10 ml of lysis buffer as described for J774A.1 cells.

2.6. Murine lung homogenate preparation

All procedures on animals were approved by the ethics committee of GlaxoSmithKline Research Centre Zagreb Limited, and performed in accordance with the European Economic Community Council Directive 86/609. Four lungs from BALB/cJ mice (Charles River) were homogenized in 20 ml of lysis buffer for 1 min on ice using a homogeniser (IKA Labortechnik, T25 basic). After 30 min on ice the homogenate was centrifuged at 10,000×*g* for 10 min at +4 °C and the supernatant was stored at −20 °C until use in affinity chromatography.

2.7. Determination of protein concentration

Protein concentration was determined by the BCA (bicinchoninic acid) method according to the manufacturer's instructions (Pierce, USA) in microtiter plates. Two milligrams per milliliter of bovine serum albumin solution was used as a standard and lysis buffer as a negative control. Absorbance was read at 562 nm (SpectraMax 190, Molecular Devices) and protein concentration was calculated from a standard curve.

2.8. Affinity chromatography

Four milligrams of total proteins from different protein extracts were incubated with 40 μl of affinity (with immobilized macrolide compounds) and control matrices in 1.5 ml Eppendorf tubes for 60 min at +4 °C with constant mixing. Matrices were washed five