EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Suppression of NFAT5-mediated Inflammation and Chronic Arthritis by Novel κB-binding Inhibitors

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ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form 17 March 2017
Accepted 27 March 2017
Available online xxxx

Keywords:
NFAT5 suppressor
κB inhibitor
Small molecules
High-throughput drug screening
Chronic arthritis

ABSTRACT

Nuclear factor of activated T cells 5 (NFAT5) has been implicated in the pathogenesis of various human diseases, including cancer and arthritis. However, therapeutic agents inhibiting NFAT5 activity are currently unavailable. To discover NFAT5 inhibitors, a library of >40,000 chemicals was screened for the suppression of nitric oxide, a direct target regulated by NFAT5 activity, through high-throughput screening. We validated the anti-NFAT5 activity of 198 primary hit compounds using an NFAT5-dependent reporter assay and identified the novel NFAT5 suppressor KRN2, 13-(2-fluoro)-benzylberberine, and its derivative KRN5. KRN2 inhibited NFAT5 upregulation in macrophages stimulated with lipopolysaccharide and repressed the formation of NF-κB p65-DNA complexes in the NFAT5 promoter region. Interestingly, KRN2 selectively suppressed the expression of pro-inflammatory genes, including *Nos2* and *Il6*, without hampering high-salt-induced NFAT5 and its target gene expressions. Moreover, KRN2 and KRN5, the latter of which exhibits high oral bioavailability and metabolic stability, ameliorated experimentally induced arthritis in mice without serious adverse effects, decreasing pro-inflammatory cytokine production. Particularly, orally administered KRN5 was stronger in suppressing arthritis than methotrexate, a commonly used anti-rheumatic drug, displaying better potency and safety than its original compound, berberine. Therefore, KRN2 and KRN5 can be potential therapeutic agents in the treatment of chronic arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by synovial hyperplasia, causing cartilage and bone destruction (Firestein, 1996). The synovial tissues of RA patients contain diverse innate and adaptive immune cells activated by self or non-self antigens (Firestein, 2003). In particular, synovial macrophages are activated by the stimulation of a variety of inflammatory mediators secreted from surrounding inflammatory cells or via cell-to-cell contact. Activated macrophages, in turn, release matrix metalloproteinases and pro-inflammatory cytokines and chemokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), granulocyte/macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein (MCP-1), thereby contributing to chronic inflammation (Firestein, 2003; Kinne et al., 2000). Moreover, the number of synovial macrophages, but not the number of lymphocytes, correlates with the progression of RA (Mulherin et al., 1996). In sum, previous studies suggest that macrophages are the major cell type responsible for RA pathology.

Nuclear factor of activated T cells 5 (NFAT5), also known as tonicity-responsive enhancer-binding protein (TonEBP), is a transcription factor whose DNA binding domain shares structural homology with NF-κB and other members of the NFAT family (Lopez-Rodriguez et al., 1999). In response to osmotic stress, NFAT5 is activated via p38 mitogen-activated protein kinase (MAPK) signaling to protect cells from hypertonic stimulation (Ko et al., 2002). Therefore, NFAT5 has important roles in different

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http://dx.doi.org/10.1016/j.ebiom.2017.03.039

tissues normally exposed to hypertonicity, such as kidney, skin, and eye (Miyakawa et al., 1999; Go et al., 2004; Neuhofer, 2010; Sawazaki et al., 2014). It has also been implicated in several physiologic and pathologic conditions, including cancer cell proliferation and invasion (Kuper et al., 2014; Jauliac et al., 2002). Recently, evidence has emerged that NFAT5 is activated by isotonic stimuli. For example, NFAT5 induces the expression of toll-like receptor (TLR)-mediated inflammatory genes in macrophages in a tonicity-independent manner (Buxadé et al., 2012; Kim et al., 2013). High salt and TLR ligation activate distinct sets of downstream target genes in a NFAT5-dependent manner (Kim et al., 2014). While ROS are essential for this, their source differs depending on the context: mitochondria for high salt and xanthine oxidase for TLR (Kim et al., 2014). Moreover, the two pathways are mutually suppressive (Kim et al., 2013). Therefore, to apply anti-NFAT5 therapies to chronic inflammatory diseases, it may be necessary to selectively inhibit its inflammatory effects without affecting its osmotic effects since the latter are involved in cellular homeostasis and cytoprotection (Miyakawa et al., 1999).

In a previous study, we firstly uncovered the crucial role of NFAT5 in the development of autoimmune disease by demonstrating that NFAT5-deficient mice showed a marked reduction of antibody-induced arthritis (Yoon et al., 2011). Moreover, we have demonstrated that NFAT5 is highly expressed in the synovia of RA patients and regulates synoviocyte proliferation and angiogenesis (the so-called pannus formation), the pathologic hallmark of RA (Yoon et al., 2011). We also identified a significant decrease in the incidence of TLR-induced chronic arthritis in NFAT5 haplo-insufficient mice as compared to their wild-type littermates (Kim et al., 2014). Taken together, our previous results strongly suggest that NFAT5 is crucial in RA pathogenesis and should be further studied for the development of RA treatment. Despite the pivotal role of NFAT5 in the pathogenesis of cancer and arthritis (Kuper et al., 2014; Jauliac et al., 2002; Yoon et al., 2011; Kim et al., 2014), specific drugs that inhibit NFAT5 activity, especially those selectively targeting the inflammatory effects of NFAT5, are currently unavailable.

Conventional disease-modifying anti-rheumatic drugs (DMARDs) must be discontinued within the second year in two-thirds of RA patients due to drug toxicity or therapy-independent relapse (Smolen and Aletaha, 2015; van der Kooij et al., 2007). Thus, new therapeutic agents with different modes of action are required for optimal treatment of RA. In the present study, we attempted to identify NFAT5 inhibitors with this in mind. Using high-throughput screening (HTS), > 40,000 compounds were screened with a cell-based inhibition assay for the induction of nitric oxide (NO), a target of NFAT5. We validated the anti-NFAT5 activity of the primary hit compounds using an NFAT5-specific reporter and identified novel NFAT5 suppressors, KRN2 and its derivative KRN5, the latter of which exhibits high oral bioavailability and metabolic stability. KRN2 reduced the expression of inflammatory NFAT5-target genes, including *Nos2*, *Il6*, *Tnf*, and *Csf2*, in TLR4-stimulated RAW 264.7 macrophages. We also found that the suppressive effect of KRN2 on NFAT5 expression was mediated through inhibition of NF-κB p65 binding to the promoter region of the *Nfat5* gene. Interestingly, high salt-induced NFAT5 and its target genes, including *Ar*, *Bgt* and *Smit*, were unaffected by KRN2. Finally, using in vivo models of chronic arthritis, we showed that KRN2 and KRN5 ameliorated arthritis severity, decreasing proinflammatory cytokine production. Therefore, KRN2 and KRN5 demonstrate potential of NFAT5 inhibitors as possible therapeutic agents to treat chronic inflammatory arthritis, including RA.

2. Materials and Methods

2.1. Synthesis of KRN2 and KRN5 From Berberine

Detailed methods are described in the Supplemental methods section.

2.2. High-throughput Screening (HTS)

RAW 264.7 (murine macrophage/monocyte) cells were grown in modified DMEM, and nitrate was measured using Griess reagent as previously reported (Kim et al., 2013, 2014). Briefly, cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well and left overnight. Compound (1 μM) and LPS (100 ng/ml) were added to the cells, which were then incubated at 37 °C, 5% CO_2 and 95% humidity for 21 h. Equal volumes of supernatant and Griess reagent (Sigma) were mixed and incubated at room temperature for 15 min. Absorbance was measured at 540 nm with an Envision Multilabel Reader (PerkinElmer). Since there are no reported NFAT5 inhibitors, 1,4-PBIT (*S*,*S*′-1,4-phenylene-bis[1,2-ethanediyl]bis-isothiourea) (Cayman), an iNOS enzyme inhibitor, was used as a reference in HTS setup and performance using Biomek FX and ORCA robot systems (Beckman Coulter).

2.3. Isolation and Cell Culture of Murine Macrophages and Splenocytes

Murine peritoneal macrophages were obtained from C57BL/6 mice peritoneally injected with 3% thioglycollate for 3 days. The cells were incubated in RPMI 1640 medium with 10% fetal bovine serum (FBS, Gibco BRL) at 37 °C in a 5% CO_2 atmosphere. To isolate splenocytes, lipopolysaccharide (LPS) was peritoneally injected into C57BL/6 mice for 24 h and their spleens were then dissected. After red blood cell (RBC) lysis, isolated splenocytes were incubated in RPMI 1640 supplemented with 10% FBS. RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC) and were maintained in RPMI 1640 medium supplemented with 10% FBS.

2.4. Cloning of NFAT5 Expression Reporter

To construct an NFAT5 expression reporter system, mouse genomic DNA, encompassing base-pair positions −3000 to +1 relative to the start codon of *Nfat5*, was cloned by PCR using primers (5′-catatgcatacaataaggca-3′ and 5′-tcgactcgcagctcgacccagcc-3′) containing restriction enzyme sites of *Nde*I and *Sal*I. The 3 kb *Nfat5* promoter region, which includes two NF-κB consensus sequences, was transferred into the pEGFP-N1 vector (Clontech). Then the cytomegalovirus promoter was removed using *Ase*I and *Sal*I restriction enzymes (BioLabs). The recombinant reporter gene construct was verified by sequencing (Cosmo Genetech). To analyze the transcriptional activity of NFAT5, the *Nfat5* consensus sequence with tandem repeats (3 copies of TGGAAAATTACCG) was inserted into the pEGFP-N1 vector (Clontech) and the pDsRed-Express-N1 vector (Clontech) as described previously (Kim et al., 2013, 2014). To construct the cells that are highly expressed with NFAT5 reporter genes, RAW 264.7 macrophages were seeded to 40–50% confluence in 12-well plates and then transduced with a GFP-NFAT5 promoter reporter using Lipofectamine 2000 (Invitrogen). After 2–3 days, cells were reseeded and then selected with 50 μg/ml geneticin (Invitrogen) for 3 weeks, as previously described (Kim et al., 2013, 2014).

2.5. Flow Cytometry Analysis

Green fluorescence protein (GFP) expression levels were detected using a FACS Canto II system (BD Biosciences). GFP intensity was analyzed using FlowJo software (Tree Star). Data are shown as percentage change in mean fluorescence intensity (% ΔMFI), which was calculated by the following formula: (MFI of treated sample − MFI of untreated sample) × 100 / MFI of untreated sample.

2.6. Quantitative Real-time PCR

Total RNA was isolated with an RNeasy Mini kit according to the manufacturer's protocol (Qiagen). Isolated RNA was reverse-transcribed to cDNA using reverse transcriptase (Takara, Shiga, Japan).

Please cite this article as: Han, E.-J., et al., Suppression of NFAT5-mediated Inflammation and Chronic Arthritis by Novel κB-binding Inhibitors, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.03.039