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Characterization of a new mPGES-1 inhibitor in rat models of inflammation☆

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ABSTRACT

Microsomal prostaglandin E synthase-1 (mPGES-1) inhibition has been proposed as an alternative to cyclooxygenase (COX) inhibition in the treatment of pain and inflammation. This novel approach could potentially mitigate the gastro-intestinal and cardiovascular side effects seen after long-term treatment with traditional non-steroidal anti-inflammatory drugs (NSAIDs) and Coxibs respectively. Several human mPGES-1 inhibitors have been developed in the recent years. However, they were all shown to be considerably less active on rodent mPGES-1, precluding the study of mPGES-1 inhibition in rodent models of inflammation and pain. The aim of this study was to characterize the new mPGES-1 inhibitor compound II, a pyrazolone that has similar potency on rat and human recombinant mPGES-1, in experimental models of inflammation. In cell culture, compound II inhibited PGE_2 production in synovial fibroblasts from patients with rheumatoid arthritis (RA) and in rat peritoneal macrophages. *In vivo*, compound II was first characterized in the rat air pouch model of inflammation where treatment inhibited intra-pouch PGE_2 production. Compound II was also investigated in a rat adjuvant-induced arthritis model where it attenuated both the acute and delayed inflammatory responses. In conclusion, compound II represents a valuable pharmacological tool for the study of mPGES-1 inhibition in rat models.

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1. Introduction

Microsomal prostaglandin E synthase (mPGES)-1 is an enzyme belonging to the superfamily of membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) [1]. As a part of the prostanoid synthesis cascade, mPGES-1 is responsible for the conversion of prostaglandin (PG) H_2, produced by the cyclooxygenases (COX) enzymes, into PGE_2, a powerful mediator of inflammation and fever. It is also the most efficient PGE synthase known. mPGES-1 expression, similar to COX-2 expression, is inducible under pro-inflammatory conditions [2,3] and these two enzymes are predominantly functionally coupled to generate the outburst of PGE_2 seen under inflammatory conditions [4]. In humans, enhanced mPGES-1 expression and PGE_2 production have been demonstrated in several pathologies including rheumatoid arthritis [5], osteoarthritis [6], myositis [7], atherosclerosis [8], inflammatory bowel disease [9], cancer [10,11], and Alzheimer's disease [12].

Moreover, gene deletion studies have confirmed the involvement of mPGES-1 in various animal models of disease including collagen-induced arthritis [13], atherosclerosis [14], lipopolysaccharide-induced pyresis [15], pain hypersensitivity [16], experimental autoimmune encephalomyelitis [17] and infection-induced neonatal hypoxia [18].

NSAIDs and Coxibs are well-recognized anti-inflammatory drugs that target the prostanoid synthesis cascade at the COX enzymes level. They thereby shut down the synthesis of all prostanoids at once which results in severe side-effects. On one hand, traditional NSAIDs, which inhibit both isoforms of COXs,

Abbreviations: AIA, adjuvant-induced arthritis; COX, cyclooxygenase; DA, dark agouti; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; FA, formic acid; FBS, fetal bovine serum; HRP, horseradish peroxidase; h-PGDS, hematopoietic prostaglandin D synthase; LLOQ, lower limit of quantification; l-PGDS, lipocalin-type prostaglandin D synthase; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; LPS, lipopolysaccharide; MDA, malone dealdehyde; mPGES, microsomal prostaglandin E synthase; MRM, multiple reaction monitoring; NSAIDS, non steroidal anti-inflammatory drugs; PG, prostaglandin; PGIS, prostacyclin synthase; RA, rheumatoid arthritis; TBA, thiobarbituric acid; TX, thromboxane; TXAS, thromboxane A synthase.

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cause gastro-intestinal ulceration from their interference with COX-1 derived gastroprotective functions. On the other hand, Coxibs, which selectively inhibit COX-2, trigger an imbalance in the blood prostacyclin/thromboxane (TX) ratio which results in a predisposition to myocardial infarction [19–21]. Novel strategies targeting the prostanoid cascade thus aim at dissociating anti-inflammatory activity from potentially harmful side-effects. One approach to resolve this problem is to increase treatment specificity by individually targeting the enzymes downstream of the cyclooxygenases in the cascade. As inducible PGE_2 is the most prominent prostaglandin in inflammation, mPGES-1 becomes a prime candidate for this strategy. In 2007, the first selective mPGES-1 inhibitors with *in vivo* potency were published. These inhibitors showed good efficacy in inflammatory models. However, they exhibited inter-species discrepancies in potency, hinting at key structural differences between human and rodent mPGES-1 homologs [22].

In this study, we characterized the pyrazolone compound II as a dual rat/human mPGES-1 inhibitor. It showed potency on recombinant mPGES-1, in cell systems as well as *in vivo* in rat models of acute inflammation and arthritis. Compound II thus constitutes a useful pharmacological tool that will allow studies of mPGES-1 inhibition in rat models of human diseases.

2. Methods

2.1. Materials

Compounds I and II (NovaSAID AB, Sweden). Dulbecco's modified eagle medium (DMEM), RPMI-1640 medium, Penicillin-streptomycin cocktail, Sodium pyruvate and Glutamine (Invitrogen AB, Sweden). Heparin LEO (LEO Pharma AB, Sweden). Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), dimethyl sulfoxide (DMSO), NS-398, SC-560, MK-886, lambda carrageenan, light mineral oil, Tween-80, Lipopolysaccharide (LPS) (from *S. minnesota* strain Re-595) and polyclonal anti-β-actin antibody (A1978) (Sigma–Aldrich, St. Louis, MO). polyclonal anti-GAPDH antibody (ab9485) (Abcam, Cambridge, UK). Bovine serum albumin (BSA) (PAA, Austria) IL-1β and TNF-α (R&D Systems, UK). M. Tuberculosis (DIFCO, USA). Tissue protein extraction reagent (T-PER) (Thermo Scientientific, Rockford, IL). Complete protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Hoseradish peroxidase (HRP)-coupled anti-mouse and anti rabbit antibodies and Amersham ECL Plus (GE Healthcare Bio-Sciences AB, Sweden). Arachidonic acid (cat. # 90010.1), U51605 (cat. # 16465), HQL-79 (cat. # 10134), polyclonal anti-mPGES-1 antibody (cat. # 140160), polyclonal anti-COX-2 antibody (cat. # 160106 and 160112), lipocalin-type Prostaglandin D synthase (l-PGDS) (human, recombinant, cat. # 10006788), hematopoietic Prostaglandin D synthase (h-PGDS) (human, recombinant, cat. # 10006593), COX-1 (ovine, isolated, cat. # 60100), COX-2 (human, recombinant, cat. # 60122), COX Inhibitor Screening Assay Kit (cat. # 560131), TXB_2 EIA Kit (cat. # 519031) as well as Prostaglandin E_2 EIA Kit – Monoclonal (cat. # 514010) were purchased from Cayman Chemicals. The expression vectors containing the coding sequence of human prostacyclin synthase (PGIS) and mPGES-2 were kind gifts from Dr. Lee-Ho Wang, University of Texas-Houston, USA and Dr. Kikuko Watanabe, Division of Life Sciences, University of East Asia, Japan respectively.

2.2. Animals

Dark Agouti (DA) female rats (8–12 weeks old, 100–130 g) were purchased from Scanbur BK, Sollentuna, Sweden. Lewis male rats (8 weeks old, 200–220 g) were obtained from Charles Rivers USA, Inc. The animals' health status was monitored according to guidelines from the Swedish Veterinary board (SVA) and reported free from screened pathogens. Rats were maintained under climate-controlled conditions with a 12-h light/dark cycle and fed standard rodent chow and water *ad libitum*. The ethical committee of Stockholm, North, Sweden, granted the ethical approval.

2.3. Protein expression and subcellular fractionation

Membrane fractions containing rat and human mPGES-1 and human mPGES-2 were produced in-house in an *E. coli* expression system as described in [23]. PGIS was expressed, and membrane fractions were prepared as described in [23], except for an optimized incubation time of 20 h after isopropyl-β-D-1-thiogalactopyranoside induction.

2.4. MDA-TBA enzyme activity assay

PGH_2 converting activity of mPGES-1, mPGES-2, l-PGDS, h-PGDS and PGIS was determined as previously described [24] and inhibitor potency for these enzymes was measured over a significant range of inhibitor concentrations. Briefly, (30 μg/ml total protein) of recombinant mPGES-1 was incubated for 90 s at room temperature in the presence of 10 μM PGH_2 and inhibitors or vehicle (1% DMSO) control. The reaction was carried out in 0.1 M sodium phosphate buffer, pH 8.0, supplied with 2.5 mM glutathione. Inhibitors were furthermore tested for cross-reactivity on mPGES-2 and PGIS in the same assay. In the case of mPGES-2, the cytosolic fraction, diluted to 150 μg/ml total protein was used, and the reaction was carried out in 0.1 M potassium phosphate buffer in the presence of 0.5 mM dithiothreitol (DTT). In the case of PGIS, 100 μg/ml PGIS membrane fraction from bacterial extracts was used and the reaction was carried out in 0.1 M sodium phosphate buffer, pH 8.0, without any reducing agent.

For all enzymes, instant reaction stop was achieved by substrate depletion after the incubation time. Unmetabolized PGH_2 was converted to 12-(S)-hydroxy-8, 10-*trans*-5-*cis*-heptadecatrienoic acid (12-HHT) and malone dealdehyde (MDA) by addition of an excess of $FeCl_2$. Thiobarbituric acid (TBA) was subsequently added and the reaction mixture was heated to 80 °C for 30 min to allow formation of the fluorescent MDA-TBA conjugate. Conjugate concentration was analyzed at excitation 485 nm/emission 545 nm. The reference inhibitors used for each enzyme were: MK-886 for mPGES-1, HQL-79 for h-PGDS and U-51605 for PGIS.

2.5. COX inhibitor screening assay

Inhibitors were screened for cross-reactivity against COX-1 (ovine, isolated) and COX-2 (human, recombinant) using the Cayman COX Inhibitor Screening Assay and following the manufacturer's instructions with an 80% reduction of reaction volumes. The reference inhibitors used for each enzyme were: SC-560 for COX-1 and NS-398 for COX-2.

2.6. Thromboxane synthesis inhibition assay

To assess inhibitor specificity, inhibition of thromboxane synthesis was evaluated in human platelets. Previously developed methods for platelet isolation and induction of thromboxane synthesis were used after modification [25,26]. Briefly, blood was drawn from healthy volounteers (no NSAIDs one week prior) into acid-citrate dextrose (ACD) vacutainer vials (BD, Sweden). The vials were centrifuged at 120 g for 5 min and the platelet-rich plasma (PRP) was collected and transferred to new tubes. The PRP was then centrifuged at 1100 g to pellet platelets. The platelet poor plasma (PPP) was discarded and platelets were washed in Tyrode's solution

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