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The transition state analog inhibitor of Purine Nucleoside Phosphorylase (PNP) Immucillin-H arrests bone loss in rat periodontal disease models

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ABSTRACT

Purine nucleoside phosphorylase (PNP) is a purine-metabolizing enzyme that catalyzes the reversible phosphorolysis of 6-oxypurine (deoxy)nucleosides to their respective bases and (deoxy)ribose-1-phosphate. It is a key enzyme in the purine salvage pathway of mammalian cells. The present investigation sought to determine whether the PNP transition state analog inhibitor (Immucillin-H) arrests bone loss in two models of induced periodontal disease in rats. Periodontal disease was induced in rats using ligature or LPS injection followed by administration of Immucillin-H for direct analysis of bone loss, histology and TRAP staining. *In vitro* osteoclast differentiation and activation of T CD4+ cells in the presence of Immucillin-H were carried out for assessment of RANKL expression, PNP and Cathepsin K activity. Immucillin-H inhibited bone loss induced by ligatures and LPS, leading to a reduced number of infiltrating osteoclasts and inflammatory cells. *In vitro* assays revealed that Immucillin-H could not directly abrogate differentiation of osteoclast precursor cells, but affected lymphocyte-mediated osteoclastogenesis. On the other hand, incubation of pre-activated T CD4+ with Immucillin-H decreased RANKL secretion with no compromise of cell viability. The PNP transition state analog Immucillin-H arrests bone loss mediated by T CD4+ cells with no direct effect on osteoclasts. PNP inhibitor may have an impact in the treatment of diseases characterized by the presence of pathogens and imbalances of bone metabolism.

Introduction

Bone mass is maintained due to the balanced activities of bone forming cells (osteoblasts) and bone reabsorbing cells (osteoclasts) [1]. Osteoclasts are large multinucleated cells derived from the monocyte/macrophage lineage that are responsible for the dissolution of inorganic hydroxyapatite and the cleavage of organic collagen fibers in bone matrix [2]. Chronic inflammatory conditions provide signals required for proliferation and differentiation of osteoclast precursor cells that cause bone loss [3]. In this process, activated T cells play a key role in osteoclast formation and bone resorption due to the ability to synthesize osteoclast-differentiating molecules [1]. Persistent inflammation and activation of the immune response lead to increased differentiation of osteoclast precursor cells that cause pathological reduction of bone mass [4]. Among osteoclast mediators, RANKL/RANK plays a key role in the activation and differentiation of osteoclasts [5,6].

Purine nucleoside phosphorylase (PNP) is a purine-metabolizing enzyme that catalyzes the reversible phosphorolysis of 6-oxypurine (deoxy)nucleosides such as (deoxy)inosine and (deoxy)guanosine (dGuo) to their respective bases and (deoxy)ribose-1-phosphate [7]. It is a key enzyme in the purine salvage pathway of mammalian cells where inosine and guanosine derived mainly from ribonucleotides hydrolysis, and 2′-deoxyguanosine derived from DNA degradation are its main substrates [8]. Genetic deficiency of PNP leads to a selective depletion of T-cells, with normal to elevated B-cell function [9]. T-cells rely heavily on PNP activity to maintain its functions and are particularly sensitive to PNP deficiency, which is attributed to a relatively high level of kinase and low level of nucleotidase activity compared to other cells [7]. PNP has recently become a potential target of drug development since analogs of the PNP transition state have been synthesized and tested with promising results [10]. Immucillin-H (BCX-1777) is a transition-state analog that inhibits

Abbreviations: PNP, Purine nucleoside phosphorylase; CEJ, Cemento-enamel junction; TRAP, Tartrate resistant acid phosphatase; RANKL, Receptor activator of NF-kB ligand; RANK, Receptor activator of NF-kB; CK, Cathepsin K; dGuo, Deoxyguanosine; LPS, Lipopolysaccharide; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; dGuo, (deoxy)guanosine; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide; Con A, Concanavalin A.

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PNP activity [11-13]. It has been shown to cause dGTP accumulation, suppressing growth in rapidly dividing T cells [14]. Moreover, this compound can inhibit human T cell malignancies in the presence of dGuo [7,13] due to its selectivity for human T lymphocytes [10]. PNP activity has been detected in biological fluids [15], and we have recently demonstrated that PNP activity is increased in sites affected by periodontal disease, and that periodontal treatment has effectively decreased PNP activity in chronic and aggressive periodontitis [16]. Periodontal disease is a biofilm-triggered process that produces gingival inflammation and destruction of the supporting structures of the teeth, leading to bone resorption and, eventually, tooth loss [17,18]. The host immune response to bacteria and its products, such as lipopolysaccharide (LPS), plays a key role in the establishment and progression of periodontal tissue destruction leading to bone loss [6,19].

Since periodontal disease is characterized by bacteria-induced bone loss mediated by the innate and acquired immunity, we hypothesized that inhibition of the PNP enzyme could arrest events that lead to bone resorption. Here we show that Immucillin-H, a PNP transition state analog inhibitor, blocks bone resorption in two different *in vivo* models of periodontal disease. Noteworthy, culture and co-culture assays revealed that Immucillin-H affects differentiation of osteoclasts mediated by T CD4+, but has no direct effect over the activity of resident osteoclasts or osteoclast precursor cells.

Materials and methods

Ligature and LPS-induced periodontal disease models

Experiments involving animals were fully approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) and were in accordance with the Guide for the Care and Use of Laboratory Animals. Periodontal disease was induced in Wistar rats using two different models; the ligature model was implemented by tying 4-0 cotton ligatures around the crowns of the mandibular 1st molars, bilaterally, just below the gum level. Four groups were examined ($n=4$ per group); test animals received Immucillin-H (4.4 mg/kg in PBS) intraperitoneally, starting 24 hours before ligature placement and administered every other day for 10 days. Control animals received intraperitoneal PBS following the same protocol, and a third group comprising animals not subjected to any treatment (untouched) was added as a reference of normal bone level. In another group of animals ($n=4$) periodontal disease was induced by directly injecting 20 μl of a 1 mg/ml stock of *E. coli* LPS (strain 055:B5, Sigma Aldrich, St. Louis, MO, USA) into the lingual aspect of the gum around the mandibular 1st molars, every other day starting the day after Immucillin-H was administered, for 10 days. Immucillin-H or PBS was administered following the protocol described for ligature-induced periodontal disease. A group of animals ($n=4$) received Immucillin-H alone as described above to assess potential effects of the compound on bone and soft tissues. Animals were euthanized after ten days by inhalation of isoflurane and the mandibles harvested for direct assessment of bone loss and histology.

Sample processing and direct assessment of bone loss

For direct analysis of bone loss, hemi-mandibles were incubated for 5 minutes in 50% H_2O_2 and immediately defleshed under a stereomicroscope with delicate scissors and pliers, until no remnants of soft tissue could be detected. After copiously washing with distilled water, the specimens were dried, stained with 1% methylene blue, washed, dried again and stored. To properly analyze direct bone loss, the dried mandibles were stabilized on a flat, yet moldable epoxy surface and analyzed under a stereomicroscope. Images were photographed with a digital camera assembled onto the stereomicroscope and exported to a computer. For direct linear measurements the captured images were analyzed with a software (Image Pro Suite version 6, Bethesda, USA) after calibration with a grid (1 mm^2). The distance between the cemento-enamel junction (CEJ) and the alveolar crest was registered for every sample in two different points at the furcation region area.

Histometric analysis and tartrate resistant acid phosphatase (TRAP) assay

Hemi-mandibles were harvested and fixed in 4% buffered formalin for 48 hours and decalcified in 15% EDTA pH 7.5 at room temperature for 4 weeks. The specimens were processed for paraffin embedding, and 5 μm serial sections cut and mounted in sialinized glass slides. Slides were stained with hematoxylin/eosin (HE) or Masson trichrome staining and analyzed with a light microscope (Olympus, Tokyo, Japan). For the TRAP assay, paraffin sections were stained using a commercial kit (Sigma Chem. Co., St. Louis, USA) according to the manufacturer's instructions. Slides were counter-stained with hematoxylin and analyzed as described above. Multinucleated TRAP-positive cells with three or more nuclei bordering the bone were counted under a magnification of 200× in the most coronal third of the bone crest of the furcation area by a single blinded examiner. Histometric analysis of the resorption area within the furcation region was carried out as previously reported [20]. Briefly, images (50× magnification) of tissue sections of the furcation area were captured using a digital camera assembled to a light microscope. Measurements were carried out directly on the digital images using an image analysis software (ImagePro 4, Media Cybernetics, USA). The area between the bone crest and the roof of the furcation was measured and expressed as bone loss area (μm^2). A single blinded examiner carried out all measurements.

Mouse T CD4 + cells activation and specific activity of PNP

T CD4+ memory cells were harvested from spleens of 3-month-old Swiss mice using negative selection with magnetic beads as described by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, single cell suspensions were obtained from mice spleens, washed in cold PBS and treated with a biotin-labeled antibody cocktail that recognized a series of markers found in T CD4+ cells. For magnetic depletion of naive T cells, CD8+ T cells, B cells, NK cells, γ/δ T cells, monocytes, DCs, granulocytes, platelets, and erythroid cells, PBMCs were incubated with a cocktail of biotinylated CD45RA, CD8, CD14, CD16, CD19, CD56, CD36, CD123, anti-TCRγ/δ, and CD235a (glycophorin A) antibodies. A monoclonal, microbead-conjugated antibody against biotin was then added, which retained non-T CD4+ cells within the magnet-assembled column, while the target T cells freely eluted through the column. Purity of the cells was assessed by flow cytometry using FITC-labeled anti-CD4 antibodies (approximately 98% enriched T CD4+ flowthrough, not shown). For each experimental condition 10^6 cell aliquots were transferred to 24-well plates containing RPMI 1640 media supplemented with 10% heat-inactivated FBS. Cells were incubated at 37 °C and 5% CO_2 in a humidified chamber and after 24 hours treated either with PBS (resting T cells) or Concanavalin A (ConA 5 μg/ml) alone or in association with Immucillin-H at different concentrations (80 nM, 100 nM, 150 nM, and 200 nM), and incubated for 4 hours; cells were then harvested and used to determine PNP activity. To this end cell aliquots were sonicated in cold Tris buffer pH 7.6 for 3 minutes, maintained on ice and processed for PNP specific activity using a modification of the phosphate assay as previously described [15]. In the presence of inorganic phosphate (Pi), the synthetic substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) (Molecular Probes, Carlsbad, USA) is enzymatically converted by PNP to 2-amino-6-mercapto- 7-methylpurine that leads to a shift in the maximum absorbance from 330 nm to 360 nm over a pH range of 6.5 to 8.5. For each sample collected, a reaction mix containing 50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.5, 200 μM KH_2PO_4, and 200 μM MESG was added to a 0.5 mL quartz cuvette; after 1 minute of incubation at 25 °C, 100 μL of total protein extract was added. The enzymatic conversion of MESG was immediately monitored at 360 nm for 60 seconds, at 5 s intervals