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Title: Targeting P2 receptors - current progress in treating musculoskeletal diseases

Ning Wang, Alison Gartland*

The Mellanby Centre for Bone Research, Department of Human Metabolism, The University of Sheffield, Sheffield, UK

*** Corresponding author and person to whom reprint requests should be addressed:**

Dr Alison Gartland,

The Mellanby Centre for Bone Research

Department of Human Metabolism

The University of Sheffield

Beech Hill Road

Sheffield, S10 2RX

UK

Phone: (+44) 0114 226 1435

Fax: (+44) 0114 271 1711

Email: a.gartland@sheffield.ac.uk

Abstract

It is widely recognized that purinergic signalling, extracellular nucleotides acting at purinergic receptors, is the most primitive and ubiquitous signalling system participating in numerous biological processes in almost all tissue types. The P2 receptors, including P2X and P2Y purinoceptor subtypes, have been proposed to play important roles in the musculoskeletal systems since the early 1990s. During the past five years, significant progress in this field has been made; this review will summarize these most recent developments and highlight the pharmaceutical potential from these findings.

Introduction

Musculoskeletal diseases cause patients considerable morbidity and even mortality, resulting in a huge financial burden to the health service systems around the globe. For example, around 20% of all postmenopausal women in western countries would meet the WHO criteria for osteoporosis, one of the most common forms of musculoskeletal diseases. Treating osteoporosis and the associated 1.5 million fragility fracture in the US alone costs approximately $18 billion/year, while in the UK this number is £1.7 billion/year [1,2]. Arthritis, another common musculoskeletal disease, affects about 50 million people in the US and 8 million in the UK [3]. Due to the ageing of the world's population, the prevalence of musculoskeletal diseases is expected to rise further. For example in US alone, one in two adults over age 50 is expected to be at risk of osteoporosis by 2020 [1]. Therefore, finding efficient and economical treatments for musculoskeletal diseases is still a pressing and rewarding task.

It has long been recognized that extracellular nucleotides such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), acting via purinoceptors including the P1 nucleoside and P2 nucleotide receptors, play important roles in many pivotal events of biological processes, such as neurotransmission and controlling cellular functions [4]. After it was first recognised in the early 1990s, the field of purinergic signalling in the musculoskeletal system has been rapidly expanding. Evidence has shown that all of the seven P2X ion channel receptor subtypes (P2X1-7) and eight P2Y G protein-coupled receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) are expressed in bone and cartilage cells. Extracellular nucleotides bind to these cell surface P2 receptors, trigger the

intracellular calcium signalling cascades, direct the fate of bone or cartilage cells, and ultimately control the homeostasis of the skeleton [5]. For more extensive historical reviews, please refer to Burnstocket al., 2013, Rumney et al., 2012 and Jorgensen et al., 2013 [4-6].

This review will discuss the most recent progress in the field of P2 receptors in musculoskeletal system. We will highlight the potential pharmacological benefits of targeting P2 receptors signalling in treating various musculoskeletal diseases including metabolic bone diseases such as osteoporosis, joint diseases such as rheumatoid arthritis (RA), and cancer induced bone disease.

P2 receptors in bone and cartilage

Bone is the primary specialized connective tissue that not only provides support and protection for the human body but also performs a metabolic function via working as a major source of inorganic ions such as calcium and phosphate, and actively participating in calcium homeostasis in the body [7]. To fulfil these functions, bone continues to turn over throughout life even after growth ceases. This predominantly involves osteoblast controlled bone formation activities and osteoclast controlled bone resorption activities, with the coordinating activities of osteocytes, bone lining cells, and stem cells in a process known as bone remodelling [8]. A fine balance between bone formation and resorption is critical for a functional skeleton, while disruption of this balance is one of the most common pathological reasons of many musculoskeletal diseases such as osteoporosis and Paget's disease.

In the bone microenvironment, nucleotides (mainly ATP) are locally released and regulate bone remodelling as extracellular signalling molecules via P2 receptors [9]. Osteoblasts have been shown to be the main source of ATP release [10,11]. Recent evidence shows that osteocytes, osteoclasts and even mesenchymal stem cells (MSC) can also release ATP upon mechanical stimulation [12-15]. Extracellular nucleotides are then able to act as mitogens for osteoblasts and osteoclasts through P2 receptors which couple to signal transduction cascades and in turn activate various osteogenic or osteoclastogenic signalling such as *c-fos*, osteopontin (OPG), runt-related transcription factor 2 (RUNX2), and receptor activator of nuclear factor kappa-B ligand (RANKL). Recent studies analysing the bone phenotype of P2X7,

$P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{13}$ receptor knock-out rodent models have added more details to the expression and function profile of P2 receptor in osteoblasts and osteoclasts [16-23] and will be detailed below; for a historical review of studies before 2010 please refer to Orriss et al., 2010 [24]. Further progress has also been achieved in elucidating the role of P2 receptor activation and ATP release in osteocyte mechanotransduction. For example, P2X2, P2X7, $P2Y_2$ and/or $P2Y_4$ and $P2Y_{12}$ and/or $P2Y_{13}$ receptors were found to be expressed in MLO-Y4 osteocyte models and ATP release induced upon mechanical stimulation, cell rupture and nucleotide (UTP) stimulation [25,26].

Cartilage is a type of dense connective tissue, composed of chondrocytes and cartilage extracellular matrix. The chondrocyte is a unique cell type that mediates synthesis, assembly, and degradation of the cartilage matrix. Although many P2 receptor subtypes have been shown to be expressed by chondrocytes, their exact roles in cartilage physiology still require further investigation. This is mainly due to the conflicting role of extracellular nucleotides in cartilage metabolism. For example, ATP was reported to stimulate the production of cartilage inflammatory mediators such as nitric oxide (NO) and prostaglandins (PGE) [27]. More recently chondrocyte differentiation, pre-chondrogenic condensation, and accumulation of cartilage proteoglycan and collagen have been shown to be facilitated by activation of P2 receptors, including P2X4, by extracellular ATP directly released from chondrocytes under physiological joint loading and articular cartilage compression [28-30].

P2 receptor and musculoskeletal diseases

Osteoporosis

Osteoporosis is the most common bone disease with unbalanced bone remodelling characterised as higher resorption and lower formation inducing bone loss, bone fragility and high fracture risk. In terms of P2 receptors in relation to the treatment of osteoporosis, most of the recent developments were achieved from a European Framework 7 funded collaboration called “ATPBone: Fighting osteoporosis by blocking nucleotides: purinergic signalling in bone formation and homeostasis”. Among these studies, the P2X7 receptor has received the most attention and brought exciting progress involving human patient data. The P2X7 receptor gene (*P2RX7*) is highly polymorphic with 26 non-synonymous single nucleotide polymorphisms (SNPs) listed on the NCBI database (Build 131). A series of studies

have shown that loss of function *P2RX7* SNPs are associated with an increased risk of osteoporosis fracture [31,32]. For example, Gartland et al. revealed that the c.946A (p.Arg307Gln) SNP was significantly associated with low bone mineral density (BMD) in patients from the Aberdeen Prospective Osteoporosis Screening Study (APOSS) [31]. These results were confirmed in a study using the Danish Osteoporosis Prevention Study (DOPS) cohort [13]. These studies provided strong evidence that P2X7 receptor agonists [ie, 2',3'-*O*-(4-benzoylbenzoyl)ATP (BzATP)] could be valuable for osteoporosis treatment and screening for *P2RX7* SNPs may represent a future early diagnostic tool to manage treating or preventing osteoporosis.

Studies of other P2 receptor subtypes have also made considerable progress and provide further potential alternatives to treat osteoporosis. For example, studies using a cohort of Dutch fracture patients showed SNPs of P2X4 and $P2Y_2$ receptor gene are associated with low BMD and osteoporosis risk [33,34]. The role of the $P2Y_6$ receptor in facilitating osteoclast survival, formation and activity has recently been confirmed as characterisation of the $P2Y_6$ receptor knock-out mouse revealed a bone phenotype of increased cortical bone thickness [16]. In another study, again using the knock-out mouse model, depletion of $P2Y_{13}$ receptor was shown to reduce bone remodelling rate, protect mice from ovariectomy induced bone loss, and enhance the osteogenic response to mechanical loading [17,21]. In addition, the $P2Y_{13}$ receptor was shown to play an important role in MSC differentiation as genetic inhibition of $P2Y_{13}$ receptor led MSC to differentiate towards adipocytes instead of osteoblasts [23]. These findings confirm the previous reports that ADP, the preferred agonist of $P2Y_{13}$ receptor, is a powerful osteolytic agent [35]. These data thus present potential treatments for osteoporosis with antagonists for $P2Y_6$ receptor or antagonists for $P2Y_{13}$ receptor combined with exercise.

Recent studies investigating the role of the $P2Y_{12}$ receptor in bone have presented an interesting debate. Su *et al* [19] elegantly and comprehensively demonstrated that $P2Y_{12}$ receptor knock-out mice showed partial protection from both age-related or pathological bone loss. In addition, they treated mice with Clopidogrel (marketed as Plavix®, a selective antagonist for $P2Y_{12}$ receptor used for the treatment and prevention of coronary artery disease) and demonstrated increased bone mass due to the inhibition of osteoclast

formation. In contrast, Clopidogrel was found to be associated with osteoporotic fracture in a cohort of Danish patients prescribed it [36]. This association was biphasic, with the clinically recommended high doses being associated with higher fracture risk and low doses being associated with lower fracture risk [36]. The same group also found that Clopidogrel could inhibit osteoblast proliferation and differentiation *in vitro* and treating ovariectomized mice with 1mg/kg/d (corresponding to standard daily dosage for human patients) Clopidogrel significantly enhanced bone loss [37]. Clearly, further investigation is necessary to elucidate the mechanism of action of Clopidogrel/Plavix®, the second most sold pharmaceutical drug with worldwide sales of 6.8 billion $US in 2012, on bone cells and prevent potential risk of drug induced osteoporosis [38].

Rheumatoid arthritis

RA is a chronic systemic inflammatory disorder affecting the synovial lining of joints characterised by swollen joints, ligament damage, bone erosion, joint deformation and pain. Extracellular ATP has been long recognized as an immunomodulatory factor whilst the P2X7 receptor has been shown to be expressed in human rheumatoid synoviocytes and to induce the release of proinflammatory cytokines (e.g., interleukin-1β, interleukin-6, and prostaglandins) responsible for the pathophysiology of RA [39,40]. Recent studies from different cohorts of patients suggested that SNPs in *P2RX7* (e.g. c.489C>T (His155Tyr)) may contribute to the pathogenesis of RA [40,41]. In addition blocking P2X7 receptor signalling was shown to prevent peripheral inflammatory tissue damaging in animal models of RA [42]. Therefore, pharmaceutical companies are interested to develop new anti-inflammatory drugs based on P2X7 receptor antagonists for treatment of RA. Although previous selective drug-like P2X7 receptor antagonists have failed to pass phase IIb/III trials due to poor pharmacokinetics and pharmacodynamics, a new generation of P2X7 receptor antagonists with better drug-like properties are in early stage of clinic study [39,43]. Interestingly, a recent review suggested that bisphosphonates could work as novel anti-inflammatory drugs, producing a switch in P2X7 receptor signalling [39]. Combining P2X7 receptor antagonists with bisphosphonates could represents a more efficacious treatment regime for RA [39].

Osteoarthritis (OA)

OA is directly linked to the degeneration of articular cartilage and characterised as radiographic joint changes including marginal osteophytes, narrowing of the joint space,

subchondral degenerative cysts, and subchondral sclerosis, which leads to severe joint pain and disability [44]. The pain from OA is mainly due to inflammation; levels of ATP in the synovial fluid have been shown to be correlated to the pain intensity in OA patients [4,45]. Although understanding the role of P2 receptors in OA pathogenesis is currently still limited, the roles of P2 receptors (especially the P2X7 receptor) in inflammation generally are well established [46]. Therefore, we believe that the pharmaceutical potential to target P2 receptors when treating OA is still valuable. For example, the $P2Y_{13}$ receptor has been suggested to provide a negative feedback pathway for ATP release in osteoblast and other cells types [21]. Should this mechanism exist in cartilage, targeting the $P2Y_{13}$ receptor may provide a novel therapy to treat OA.

Cancer Induced bone diseases

P2 receptors are known to be expressed by most cancer types; with either activation or inhibition of selected P2 receptor subtypes inhibiting cancer cell survival or growth. These observations have led to increasing interest in the therapeutic potential of P2 receptor signalling for the treatment of cancer (see review [47]). However, knowledge of P2 receptor in cancer induced bone disease (CIBD), mainly cancer bone metastasis, is still limited. CIBD is a devastating clinical consequence affecting 1.5 million cancer patients worldwide each year, with the most common type of metastases from prostate and breast cancer. CIBD is the main reason, rather than the effects of the primary tumour, for the cause of morbidity and mortality in these patients. However, both preventing and treating bone metastasis are currently limited due to poor understanding of the mechanism leading to bone metastasis. Acquiring epithelial-mesenchymal transition (EMT)-like phenotype has been suggested as essential for these tumour cells going through the multi-step process to form metastases in bone [48]. Interestingly, a recent study in a breast cancer model has suggested that alteration in the expression of P2 receptors (e.g. P2X5 and $P2Y_{13}$) were involved in Epidermal Growth Factor (EGF)-induced EMT in MDA-MB-468 breast cancer cells [49]. This represents a novel mechanism in the initiation of cancer bone metastasis and highlights P2 receptors as possible treatment targets. In addition, pharmacologic or genetic inhibition of the $P2Y_{12}$ receptor were both shown to protect mice from tumour-associated bone loss and co-administration of a $P2Y_{12}$ inhibitor with cisplatin led to enhanced cytotoxic response in breast cancer cells [19,50]. These findings also present the $P2Y_{12}$ receptor as a potential target to treat CIBD in combination with chemotherapeutic agents. Furthermore, ADPase, which hydrolyses ADP, has been reported to significantly inhibit bone tumours in

combination with aspirin, while bisphosphonates can promote cancer cell apoptosis due to the formation of an ATP analogue (Apppl) interacting with P2X7 receptors [47]. These findings provide the foundation for further understanding of P2 receptor signalling in CIBD [47]. Finally, blockade of P2X3 and P2X2/X3 receptors in murine models has been shown to attenuate cancer-induced bone pain [51,52], whilst P2X7 receptor-deficient mice were still susceptible to bone cancer pain and showed earlier onset of pain related behaviours [53]. Although this latter observation may suggest that P2X7 receptors are not involved in cancer induced bone pain, many researchers believe them to still be viable targets with significant medicinal chemistry advances being made recently in using selective P2X7 receptor antagonists for chronic pain [53-55]. These findings represent new targets for pharmacotherapy in cancer-induced bone pain.

Summary

Significant progress has been made in the field of puringeric signalling recently, furthering our understanding of the role of P2 receptors in the musculoskeletal system. In particular studies of P2X7, $P2Y_6$, $P2Y_{12}$, and $P2Y_{13}$ receptors present potential novel pharmacological targets to treat series musculoskeletal diseases such as osteoporosis, arthritis, and CIBD. Drug design based on agonists or antagonists of these P2 receptors should be the next focus point, in addition to the further elucidation of other P2 receptor subtypes' role in musculoskeletal system including P2X4, $P2Y_1$ and $P2Y_2$ receptors.

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