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Local delivery of adenosine receptor agonists to promote bone regeneration and defect healing

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ABSTRACT

Adenosine receptor activation has been investigated as a potential therapeutic approach to heal bone. Bone has enhanced regenerative potential when influenced by either direct or indirect adenosine receptor agonism. As investigators continue to elucidate how adenosine influences bone cell homeostasis at the cellular and molecular levels, a small but growing body of literature has reported successful *in vivo* applications of adenosine delivery. This review summarizes the role adenosine receptor ligation plays in osteoblast and osteoclast biology and remodeling/regeneration. It also reports on all the modalities described in the literature at this point for delivery of adenosine through *in vivo* models for bone healing and regeneration.

Contents

1. Introduction

Clinical applications of bone tissue engineering have yet to be realized. Globally, almost nine million fractures occur annually consequent to osteoporosis [1]. Orofacial clefts affect approximately 1 of every 700

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live births, many of which include alveolar cleft defects of the midface [2]. Databases in the U.S. and Europe suggest that bone tumors affect 0.9–1.3 of 100,000 persons per year, but given the improved therapeutic options for these diseases over the past three decades, many of these patients survive with significant bony defects [3, 4]. Dental pathology such as periodontal disease and periapical lesions result in bone remodeling and loss of the bone that structurally supports dentition/teeth [5]. In the United States alone, the treatment of facial fractures is estimated to cost over 1 billion dollars annually [6], and the cost per patient doubles when bony non-union occurs [7]. Consequently, the impetus for advances in bone regeneration remains significant.

For surgical treatment of large bony defects, autologous bone transfer, or harvesting bone from a donor site and replacing a defect within the same patient, is a preferred reconstructive option. However, this type of bone tissue transfer is limited by complications such as donor site morbidity and infection [8, 9]. Many bioactive molecules that can potentially enhance bone regeneration have been investigated, the most well-known of which is recombinant human bone morphogenetic protein 2 (rhBMP-2). BMP-2 has shown significant regenerative potential and is indicated for spinal fusion procedures in adults, but it has also demonstrated concerning side effects, such as ectopic bone formation/exuberant bone growth [10–12]. Recently, adenosine receptor ligation has been gaining attention as another bone-forming pathway that is as effective as BMP-2, but with a safer side effect profile [13].

2. The adenosine receptors

There are four adenosine receptor subtypes: A1, A2A, A2B, and A3. Three of the four subtypes (A1, A2A and A2B) are highly conserved throughout evolution, as they have 80–95% sequence homology whereas A3 receptors vary significantly amongst different species [14]. All four receptors are found in humans and belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors. A1 and A3 receptors are coupled to pertussis toxin-inhibited Gi coupled signal transduction proteins or directly to ion channels, whereas A2 receptors are G-alpha-s-linked receptors and stimulate adenylate cyclase and cAMP accumulation [14]. The cellular function of adenosine appears to be preserved across phyla neutroas in the bacterium *Myxococcus xanthus*—both the mechanism of adenosine formation (product of ATP dephosphorylation) and its physiologic role are similar to mammalian function; adenosine is secreted by a mechanism that does not involve cell lysis and is secreted in response to substrate depletion [5].

A wide array of selected adenosine receptor agonists and antagonists has previously been reported [15]. Of note, these selected agonists and antagonists are limited to compounds that activate or inhibit adenosine receptors directly.

2.1. The role of adenosine

Adenosine is a purine nucleoside found in low concentrations under normal physiological conditions. It is both soluble and stable in solutions with a pH of 6.8 to 7.4, and in the body, it has an extremely short half-life of less than 10 s [16, 17]. Adenosine concentration in the extracellular space, specifically plasma, increases with metabolic stress or significant energy demand (*e.g.* ~1 μM in unstressed tissue, ~4–10 μM in septic patients) [18]. Bioavailability is determined by production, release, cellular uptake, and metabolism: processes that are both interdependent and highly regulated [19]. Such interplay is highlighted during tissue hypoxia or ischemia: intracellular purinergic metabolic pathways increase dephosphorylation of ATP to adenosine by the enzyme 5′ nucleotidase and suppresses adenosine kinase activity, thus preventing rephosphorylation of adenosine [19].

Adenosine accumulates to high extracellular levels by likely one of two mechanisms. During metabolic stress, precursor adenine nucleotides like ATP and ADP/AMP are released from the cell and catabolized into adenosine by a cascade of ectonucleosidases, such as nucleoside triphosphate dephosphorylase (CD39) (to AMP) and 5′ ectonucleotidase (CD73) (to adenosine). The second mechanism involves the accumulation of high concentrations of adenosine intracellularly, followed by shunting of adenosine into interstitial space *via* specialized nucleoside transporters such as equilibrative nucleoside transpoter-1 [19] (Fig. 1). Equilibrative nucleoside transporters also exist on endothelial cells, which release adenosine into plasma and result in adenosine uptake by erythrocytes, which likely contribute to the salvage of these molecules for ATP synthesis *via* orthophosphate [20].

Adenosine accumulation is limited in humans: adenosine deaminase catabolizes hydrolysis of adenosine to inosine, and inosine is degraded to uric acid by xanthine oxidase [14]. Adenosine is a 'retaliatory metabolite' and autacoid with a mechanism of formation fundamentally different from any known hormone: rather than being secreted in response to an initiating signal, adenosine is formed consequent to metabolic status within the cell—analogous to regulatory metabolites citrate and AMP [21]. As early as 1929, adenosine was reported to exert a protective effect on cardiac myocytes: Drury and Szent-Gyorgi demonstrated adenosine to be a potent negative inotropic and coronary

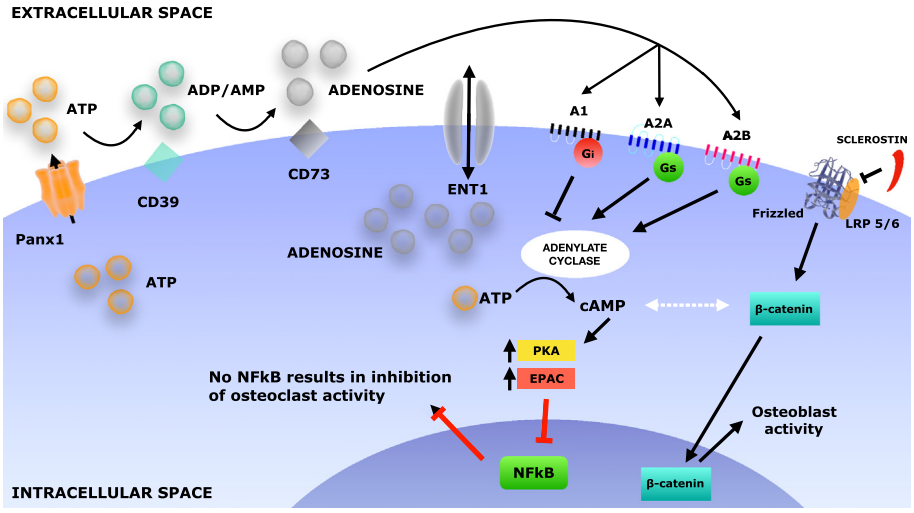

Fig. 1. Schematic depicting how adenosine receptor activation alters osteoclast and osteoblast activity.

vasodilator, and thereby postulated that adenosine was protective in settings of significant metabolic demand [1], highlighting its unique and direct link to metabolism and regional tissue signaling.

All cells synthesize or release adenosine. Adenosine's effects have been observed across nearly every organ system, ranging from the inhibition of platelet aggregation to attenuation of nerve firing to modification of glomerular filtration rate *via* macula densa cell release. Key contributors to extracellular adenosine include endothelial cells and neutrophils, both of which have consistently been reported to release high levels of adenosine at sites of inflammation/infection or increased metabolic need [19]. When released, adenosine binds to receptors on immune cells and induces a potent immunosuppressive pathway characteristic of the regulation of an excessive immune response. Indeed, stimulation of adenosine receptors on neutrophils suppresses the stimulated generation of the superoxide anion in neutrophils [2].

It has also been shown that removal of endogenous adenosine signaling increases immune activation and aggravates tissue dysfunction following acute injurious stimuli [3]. There have recently been attempts under way to make use of adenosine receptor antagonism to increase immunologic reactions to tumors as a novel approach to cancer therapy [22]. This is consistent with the "retaliatory" description of adenosine—while inflammation and immune reactions protect the host by fighting invading microorganisms/eliminating debris at sites of tissue injury, when inflammatory mechanisms are unchecked, they may injure surrounding tissue through exuberant responses, and it is this exuberance that adenosine has been discovered to protect against [19].

It is because of the widespread, systemic effects of adenosine that localized delivery for bone healing/formation is critical. Local delivery prevents unintended activation of other systems affected by adenosine—which likely includes every organ system. Local implementation also allows for prolonged adenosine receptor activation through greater control of release kinetics, without a need to regularly ingest medication. Furthermore, the concentrations of adenosine receptor agonists such as Dipyridamole needed to induce bone regeneration are much lower than already established safety profiles for the same drugs at systemic doses [23, 24], thereby promoting a bioactive molecule that can potentially be rapidly deployed for clinical investigations.

3. Stimulation of bone formation by targeting adenosine pathways

In bone homeostasis, extracellular adenine nucleotide accrues in response to bone loading, fracture, and repair [25]. While physiologic concentrations of extracellular adenosine have not been reported to affect bone healing, pharmacologic activation of adenosine receptors has demonstrated significant regenerative potential by affecting the differentiation and activity of osteoblasts and osteoclasts in several *in vitro* and *in vivo* reports.

3.1. Cellular players in bone mass homeostasis

3.1.1. Osteoblasts

Bone homeostasis requires the linked functioning of osteoblasts and osteoclasts. Progenitor mesenchymal stem cells differentiate into osteoblasts, which produce matrix proteins such as type 1 collagen, osteocalcin, osteopontin, and sialoprotein [26]. Osteoblasts migrate to lacunar spaces and produce non-mineralized bone matrix [26, 27]. Osteoblast formation requires several transcription factors, including core-binding factor alpha-1 (Cbfa-1) also called RUNX-2, a *runt*-domain gene family factor that, when deficient in murine models, results in an absence of bone formation [28]. When Cbfa-1 is over-expressed, osteoblast-related genes are expressed in non-osteogenic cells [29]. Cbfa-1 contributes to bone matrix protein production [30] and it upregulates the expression of genes of several key bone matrix proteins, including osteopontin, type 1 collagen, and osteocalcin [31–33]. Other transcription factors that have been suggested to play a role in the regulation, maturation, and proliferation of osteoblasts include proto-oncogenes (c-myc, c-jum, c-fos), zinc-finger proteins, and Osterix (Osx) [26, 31, 34].

The Wnt pathway also plays a role in regulating osteoblast activity by signaling the canonical pathway mediated by β-catenin [35]. Wnt proteins co-activate a frizzled family receptor and a low-density lipoprotein receptor-related protein (Lrp5 or Lrp6) [26, 36], which together form a complex and stabilize β-catenin, permitting its translocation to the nucleus. This complex targets several transcription factors, the best characterized of which are Lef1/Tcf [37]. B-catenin then displaces co-repressors from Lef1/Tcf, recruits co-activators, and induces expression of genes related to osteoblast activity [37]. This pathway was realized when loss-of-function mutations in Lrp5 resulted in decreased bone mass, and gain-of-function mutations resulted in an increase in bone mass [26, 38, 39]. Additionally, sclerostin inhibits osteoblast activity by binding to Lrp5 and 6, thereby inhibiting both the canonical pathway and mature osteoblast activity [40].

3.1.2. Osteoclasts

Osteoclasts are multinucleated bone-resorbing cells that differentiate from myeloid progenitors. Hydrochloric acid and proteases released from these cells resorb the matrix and mineral components of bone [41–43]. How osteoclasts recognize bone and subsequently bind to its surface is not well understood, but it is known that integrins play a key role [41]. αvβ3 is the dominant osteoclast integrin and its expression is induced by the receptor activator of nuclear factor kappa-B ligand (RANKL).

Macrophage colony-stimulating factor (M-CSF) and RANKL are the key cytokine drivers of macrophage differentiation and osteoclast formation, respectively. M-CSF promotes osteoclast precursor proliferation and survival by binding to its receptor c-Fms and activating its tyrosine kinase domain [44]. RANKL is expressed on the surface of osteoblasts and a soluble form is released by osteoblasts and osteoblast precursors, which is upregulated when stimulated by indirect osteoclast-stimulating agents such as PTH and TNF-α [41]. These agents initially bind to osteoblasts and their precursors, which in turn secrete RANKL. RANKL then interacts with RANK (its receptor) expressed on osteoclast progenitors. RANKL activates various downstream mediators, including NFkB, which contributes to both osteoclast activation and differentiation [45]. This is balanced by osteoblast secretion of osteoprotegerin, a decoy receptor that binds to RANKL. Mature osteoclasts participate in resorption of bone *via* podosome-mediated osteoclast attachment to the extracellular matrix, and this is characterized by fusion of intracellular vesicles and the plasma membrane facing bone within an actin ring. Apatite crystal dissolution results from the low pH in the sealed resorption area. Afterwards, cells either relocate to a new site of resorption or undergo apoptosis [46].

3.2. Role of adenosine receptors in bone homeostasis

Adenosine modulates both osteoblastogenesis and osteoclastogenesis [47]. The effects of adenosine on osteoblasts have been described for over two decades. Caplan reported that adenosine had mitogenic effects on a murine model osteoblastic cell line [27]. Several groups have reported that adenosine signaling diminishes osteoprotegerin and increases IL-6 levels, which are important contributors to both normal osteoblast function and Ca^{2+} regulation [48]. Interestingly, Evans et al. suggested that sufficient adenosine release from osteoblasts can stimulate osteoclastogenesis [47].

Costa et al. indicated that while adenosine receptor-mediated mechanisms were capable of altering osteoblast differentiation and proliferation, this was not the case at non-stressed physiologic levels [49]. Non-stressed cell cultures did not accumulate sufficient extracellular

adenosine to activate any of its receptors, even when treated with continuous adenosine deaminase inhibition.

3.2.1. Adenosine receptors effects on osteoblasts

Costa and colleagues also reported that continuous application of 0.5 μM of Dipyridamole, a pharmacological agent that blocks adenosine uptake, resulted in increased osteogenic differentiation in human cells [49]. The activity of the ectonucleotidase pathway in human primary osteoblast-like cells (HPOC) was mapped to better define patterns of extracellular adenosine formation: extracellular ATP and AMP degradation were higher as HPOC became more differentiated and proliferated less [49]. Adenosine receptor stimulation of osteoblast proliferation and differentiation from human bone marrow stromal cells suggest that the A2B receptor plays the most prominent role for HPOC cell osteogenicity [49]. Osteogenic differentiation was inferred by the activity of alkaline phosphatase (ALP), which was greatest when cells were subjected to A2B agonism [49]. The effect of A1 agonism was less than A2B but still upregulatory, and A2A receptor agonism actually resulted in a concentration-dependent decrease in ALP activity [49]. These agonists were then tested in the presence of their respective antagonists to determine if these effects were receptor specific, and A1 and A2B antagonists both inhibited their respective agonist counterparts from inducing osteogenic differentiation, while the A2A antagonist reversed the inhibitory agonist effect towards osteogenic differentiation [49].

Indeed, Gharibi et al. suggested that A2BRs played a more significant role in commitment and differentiation of rat stem cells towards osteoblast lineage, and that A2ARs played a larger role in osteoblast maturation and osteoblast phenotype maintenance [50]. This was based on observations that A2BRs were the dominant adenosine receptor subtype expressed in rat undifferentiated mesenchymal stem cells (MSCs). A2BR activation in these cells promoted osteoblast lineage markers, osteoblastogenesis, and osteoblast mineralization, but gene and protein expression occurred only transiently. In contrast, they reported that A2AR gene and protein expression was upregulated at later stages of differentiation relative to A2BRs [50]. The A2BR was the dominant receptor throughout osteoblast differentiation, a finding that was supported by Larsen et al. who also reported A2BR upregulation in a human bone marrow stromal cell population that induced heterotopic bone formation [51].

3.2.2. Adenosine receptor effects on osteoclasts

The A1AR was initially suggested as a useful target for diseases characterized by excessive bone resorption (*e.g.* osteoporosis, prosthetic joint loosening) in mouse models [52]. Once A1 receptors were elucidated to play an important role in promoting human monocyte fusion into giant cells *in vitro* [53], this was explored in osteoclast fusion but resulted in mixed results: while multinucleation was inhibited *in vitro*, this was not in the case *in vivo* in a murine model [52]. Furthermore, osteoclast number was not affected as measured by TRAP staining [52]. However, osteoclasts that were differentiated from bone marrow cells of A1 knockout mice did have alterations compared to the age and sex matched control group. A1R deficiency was associated with a defect in M-CSF and RANKL induced osteoclastogenesis *in vitro* (*e.g.* significantly fewer TRAP positive cells in A1 knockout bone marrow macrophages than wild type counterparts, smaller and atypically smaller spread of TRAP positive multinucleated cells). The same observations were made in murine bone marrow cells when subjected to a DPCPX, a chemical compound that is a selective and potent A1 receptor antagonist [52].

In a murine model of post-menopausal osteoporosis, A1R-KO mice subjected to ovariectomy demonstrated increased bone density, and the pharmacological blockage of A1 receptors in WT mice prevented bone loss after ovariectomy [52]. A1R blockade diminished osteoclast function *via* degradation of TRAF6, a RANK signaling protein essential for osteoclast differentiation and function [52].

Merrill et al. expanded on reports that A1R occupancy promoted human monocyte fusion/giant cell formation *in vitro* [54] and reported that adenosine plays a key role in monocyte-progenitor osteoclast cells [55]. Their group reported A1R deletion or blockade resulted in prevention of bone loss in a murine model [55]. In both deletion or blockade of A1R, osteoclast differentiation from bone marrow precursor cells was inhibited, suggesting a critical role for A1Rs in osteoclast differentiation. They also reported that A1R blockade inhibited early stage RANKL-induced osteoclast formation [55]. A1RKO osteoclasts were demonstrated to form ineffective actin rings and consequently demonstrate less capacity for bone resorption [55].

A2AR agonists such as CGS21680 have been observed to inhibit osteoclastogenesis in a dose-dependent manner in murine models as well. Furthermore, the A2AR antagonist ZM241385 was found to have dose-dependent activation of osteoclastogenesis in the same model [56]. Together, these findings suggest that adenosine inhibits osteoclast formation through an autocrine mechanism of action [56]. This was supported through work demonstrating that neither agonist nor antagonist affected osteoclast proliferation in A2AKO mice [56]. When assessing for which stage of osteoclast formation/function was most affected by A2AR mediated effects, it was determined that the most significant effects occurred at the beginning of differentiation (0–4 days) while no effect was noted after that timeframe [56]. In groups of maturing osteoclasts, A2AR agonists significantly increased the percentage of least-differentiated osteoclasts [56]. These findings provide the impetus for investigating local adenosine delivery: by introducing a localized source of adenosine receptor activation, inhibition of osteoclastogenesis can be leveraged to facilitate desirable shifts in bone homeostasis.

In addition to these reports, in human cells, A2AR activation has been demonstrated to inhibit osteoclast differentiation from bone marrow derived macrophages from both healthy and multiple myeloma models [57]. A closer look at the pathway by which this occurs in murine cells has suggested that this is consequent to A2AR stimulation of cAMP, and subsequently both protein kinase A (PKA) and exchange protein activated by cAMP (EPAC) [58]. Furthermore, the cAMP-PKA-ERK1/2 pathway has been shown to inhibit the translocation of NFkB to the nucleus, thereby inhibiting osteoclast differentiation [58]. Other work investigating where extracellular adenosine originates led to the finding that Type 1 equilibrative nucleoside transporter (ENT1) is a key contributor, and it plays an essential role in maintaining bone density [59]. Hinton and colleagues demonstrated that ENT1 deletion in a murine model has important consequences for bone homeostasis: osteoclast activity and bone formation activity are upregulated, but reduced bone mineral density is also observed. This suggests that resorption is disproportionately favored in the absence of ENT1 [59]. From a functional standpoint, ENT1-deficient mice had notable deficits in motor coordination and locomotor activity, which was reported to be consequent to unfavorable alterations in bone density [59].

Teramachi et al. demonstrated that methotrexate induced suppression of bone destruction in rats was reversed through an A2BR mechanism when adenosine levels rose to significant at high levels (>40 μM) [60]. Their findings revealed that adenosine inhibited the expression of osteoprotegerin mRNA and subsequent osteoclastogenesis. While seemingly inconsistent with findings from others, Teramachi et al. suggest that differences in effect on osteoclastogenesis can be attributed to receptor subtype affinity.

IB-MECA is a chemical compound that has selective A3R agonism properties, and when investigated it attenuated bone resorption and osteoclast number in a murine arthritis model [61]. This effect was postulated to occur *via* A3R-mediated downregulation of inflammation through modulation of apoptosis (down-regulation of PI3K, PKB/Akt, IKK, NF-kB, TNF-alpha and RANKL were all observed) [61]. No direct

effects of A3AR stimulation/blockade have been shown in either osteoclastogenesis or osteoblast bone production.

3.2.3. Wnt signaling & adenosine-mediated osteoblast/osteoclast activity

The role of Wnt signaling may also be influenced by the A1 receptor. D'Alimonte and colleagues observed that in dental pulp-derived stem cells (DPSCs), A1 receptor agonists induced osteoblastic differentiation and activity as measured by cell culture mineralization, RUNX-2 expression and ALP activity. They also observed that differentiation was accompanied by an increase in canonical wingless signaling proteins Dishevelled (Dvl) as well as phosphorylated glycogen synthase kinase-3β (pSGSK-3β) [62]. Dvl is the most proximal intracellular protein in the Wnt signaling pathway, and pSGSK-3β is the inactive form of SGSK-3β, which upon phosphorylation cannot phosphorylate and ubiquitinate β-catenin, and consequently facilitates β-catenin nuclear translocation. Additionally, Dvl-2 knockdown model DPSCs failed to demonstrate the same ALP activity as control cells.

Wnt signaling has been shown to inhibit osteoclast differentiation as well, but the relationship between this mechanism and adenosine-mediated pathway remains unclear. A potential site of crosstalk has been described by Weivoda and colleagues who reported that Wnt3a activates the cAMP/protein kinase A (PKA) pathway [63], which is also downstream of A2A and A2B receptor activation.

3.3. Adenosine delivery in bone defects

Matrices or scaffolds capable of supporting the delivery of bioactive molecules to facilitate tissue regeneration are one of the three main principles of tissue engineering [64]. Matrix designs include ceramics, bioactive glass, metallic alloys, and polymers, or composite materials [65]. Each material has unique limitations pertaining to manipulation of their shape design, rigidity, degradation kinetics, and mechanical stability [65]. Approaches to drug delivery have explored molecules such as growth factors (*e.g.* bone morphogenetic proteins), chemotherapeutics, and antimicrobial agents, *via* mechanisms ranging from diffusion to microcapsule delivery, over various time points. For example, calcium-phosphate and polymer-based scaffolds have demonstrated local antibiotic and growth factor release kinetics extending different periods of time [66, 67].

To date, there are few reports on use of matrices or scaffolds to deliver agents targeting adenosine receptors for promotion of bone growth (Table 1). The first report on use of a matrix (collagen sponge) to deliver adenosine receptor agonists or antagonists to bone defects

Table 1
Review of studies investigating the role of adenosine receptor activation in bone defect regeneration.

Authors	Year	Molecule(s) investigated	Receptor(s) of interest	Model used	Anatomic site	Delivery method	Duration	Outcomes
Mediero et al.	2015	CGS21680 (1 μM), Dipyridamole (1 μM)	A2A	Murine	Calvaria	Collagen scaffold, daily injection	8 weeks	Direct A2AR stimulation *via* CGS and indirect stimulation *via* Dipyridamole enhances bone regeneration significantly more than control group. Both regenerated bone as well as BMP-2.
Mediero et al.	2016	Ticagrelor (1 μM, 10 μM for sponge, 1 mM for scaffold) Clopidogrel (CAM) (1 μM, 10 μM for sponge, 1 mM for scaffold) Dipyridamole (*in vitro only*)	A2A, A2B	Murine	Calvaria	Collagen sponge, HA/β-TCP scaffold	4 weeks	Ticagrelor and CAM treated sponges had significantly greater bone formation than control at 10 μM but not at 1 μM. Ticagrelor and CAM treated scaffolds regenerated significantly more bone than scaffold alone, as much as BMP-2.
Ischack et al.	2017	Dipyridamole (100 μM)	A2A	Murine	Calvaria	HA/β-TCP scaffold	2, 4, 8 weeks	Ticagrelor, CAM, and Dipyridamole all inhibited osteoclast differentiation and promoted osteoblast differentiation *in vitro via* A2AR (Ticagrelor & Dipyridamole) or A2BR (CAM). At 4 weeks, 2AKO mice failed to regenerate significantly greater bone regeneration than control when treated with Dipyridamole. A2AKO group treated with BMP-2 did have significantly greater bone regeneration. At 2, 4, and 8 weeks, scaffolds coated in Dipyridamole regenerated bone significantly greater than control group and statistically equal to BMP-2 group in normal mice.
Zhou et al.	2017	Calcium phosphate-phosphorylated adenosine (CPPA) paired with doxorubicin	N/A	Murine	Subcutaneous osteosarcoma at armpit	Intratumor injection	4 weeks	Biocompatible microspheres facilitated stable doxorubicin treatment for longer than direct intratumor doxorubicin injection, with safer systemic outcomes than intravenous doxorubicin treatment or control group.
Bekisz et al.	2017	Dipyridamole	A2A	Ovine	Calvaria	β-TCP scaffold	3 & 6 weeks	CCPA microspheres promoted osteogenic differentiation of human bone mesenchymal stem cells through AMPK pathway Dipyridamole coated scaffolds had significantly greater bone formation than control group at 3 weeks, marginally significantly greater bone formation at 6 weeks, and significant amounts of bone formation localized to the interface between calvaria and dura mater.

was reported by Mediero and colleagues. This group utilized a collagen sponge implanted in a calvarial defect in mice to deliver an A1R antagonist, a direct A2AR agonist CGS21680, or Dipyridamole, an indirect agonist, to regenerate bone in a 3 mm trephine defect in a murine model [13] and found that all of these agents promoted bone growth as well as BMP-2. Both demonstrated an *in vivo* increase in osteoblast number and decrease in osteoclast number, and A2AKO murine models failed to demonstrate these alterations [8].

The same group also reported that Ticagrelor, an agent that like Dipyridamole also upregulates extracellular adenosine by blocking ENT-1, also promoted bone regeneration capacity equivalent to BMP-2 [68]. However, the efficacy of collagen sponge delivery in these studies was dependent on daily dosed molecule administration, which significantly limits translational model applications. To address this limitation, the same group tested the delivery of Ticagrelor by coating it on to 3D printed bioactive ceramic scaffolds. By adding this adenosine receptor agonist to 3D printed ceramic structures of porous design through a collagen coat, bone regeneration comparable to BMP-2 was reported without the need for daily adenosine receptor activator administration [68]. Regenerative capacity of Ticagrelor was abrogated in A2AKO mice, supporting that the bone regenerative mechanism of Ticagrelor is mediated by adenosine receptor ligation. Ishack et al. utilized constructs made of bioactive ceramics coated with collagen that were immersed in Dipyridamole prior to implantation, to regenerate critical-size cranial defects in a murine model. They tested this method of delivery in both control mice and A2AKO mice, with results indicating that A2A receptors were a promising target for bone regeneration [69].

Bekisz and colleagues investigated the regenerative potential of Dipyridamole in a translational large animal model (sheep calvaria) defects [70]. Dipyridamole-coated scaffolds demonstrated significantly greater bone regeneration than control scaffolds. Of note, A2AR ligation significantly augmented bone healing in a pattern that was suggestive of dura-mediated healing, rather than only osseoconduction from defect borders violated by osteotomy.

Rao et al. reported an A2BR driven mechanism of adenosine-induced osteogenesis in human embryonic stem cells (hESCs). Using bio-mineralized calcium-phosphate matrices that directed hESC osteogenic differentiation, osteogenesis was seen. However, without the bio-mineralization of these matrices (composed of poly(ethylene glycol)-diacrylate PEGDA-*co* A6ACA hydrogel matrix) intrinsic osteogenicity was not possible and required the introduction of exogenous adenosine to facilitate osteogenesis, thereby confirming the role of A2BR [71]. Building on the knowledge that adenosine modulates bone metabolism, Zhou and colleagues synthesized an inorganic-organic hybrid, hollow, microsphere structure consisting of calcium phosphate and phosphorylated adenosine (CPPA), with its hollow structure permitting a high degree of doxorubicin [72]. These CPPA microspheres loaded with doxorubicin demonstrated therapeutic efficacy on osteosarcoma both *in vitro* and in mice *in vivo*, while also demonstrating induction of osteogenic differentiation of human bone marrow stem cells, as reported by stage-specific markers (ALP, collagen type 1, *etc.*). The authors suggested that bone regeneration occurred through an AMP kinase-dependent mechanism [72].

3D printed bioactive ceramic scaffolds and collagen sponge delivery of adenosine receptor agonists facilitate bone regenerative capacity similar to BMP-2 in murine and sheep calvarial models, and knockout murine models have confirmed the importance of the A2AR in these processes. Additionally, the A2BR mechanism of action has also been investigated through hydrogel matrix delivery. Finally, the adenosine regenerative mechanism has also been studied with microsphere delivery of chemotherapeutics.

4. Conclusions & future directions

Delivery of adenosine or adenosine-increasing agents on sponges, scaffolds, microspheres, and other modalities may be useful approaches to the regeneration of bone in critical-sized bone defects. The effects of adenosine receptor ligation on bone cell homeostasis are appreciated most at early time points, and thus encourage early delivery of adenosine receptor activators at concentrations high enough to leverage these mechanisms. Given that essentially every organ system has cells with adenosine receptors, local drug delivery will be critical for developing specific and safe bone regenerative therapeutics.

It has only recently been realized that the adenosine pathway can influence bone healing. Consequently, there is a paucity of *in vivo* investigations to date. As translational findings that demonstrate safe yet robust bone formation continue to be reported, clinical interest will rise in the adenosine receptor pathway as a viable alternative to current standards of care for osteogenic agents. Dipyridamole in particular shows significant translational promise due to its robust effects on bone healing and its well-established safety profile. With decades of clinical use, Dipyridamole is indicated in settings requiring antithrombotic therapy or vasodilatory testing, such as cardiac stress testing. Work using human erythroleukemia K562 cells quantified Dipyridamole's role as an ENT1 inhibitor ($Ki = 8.18$ nm and $86.7 \pm 0.1\%$ inhibition at 10 μM) and inducer of adenosine accumulation extracellularly [73]. The concentrations of Dipyridamole used for these therapies range from 75 to 100 mg per dose per 6 h in adults, and 2–5 mg/kg per 8 h in pediatric populations [74–76]. In contrast, Bekisz et al. utilized a one-time coating of approximately 5 mg of Dipyridamole and saw significant calvarial defect healing overall.

Adenosine receptor activation can modulate bone metabolism and potentially promote regeneration, thereby constituting possible novel targets for bone regenerative therapies.

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