BJP British Journal of Pharmacology

DOI:10.1111/bph.12342
www.brjpharmacol.org

RESEARCH PAPER

Rolofylline, an adenosine A_1 receptor antagonist, inhibits osteoclast differentiation as an inverse agonist

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Keywords
adenosine A_1 receptor; osteoclastogenesis; inverse agonist

Received
10 January 2013
Revised
7 June 2013
Accepted
19 June 2013

BACKGROUND AND PURPOSE

Adenosine may be generated by hydrolysis of extracellular nucleotides by ectonucleotidases, including ectonucleoside triphosphate diphosphohydrolase 1 (CD39), ecto-5′-nucleotidase (CD73), nucleotide pyrophosphatase phosphodiesterase 1 (NPP-1) and tissue non-specific alkaline phosphatase (TNAP). Previous work from our laboratory has uncovered a critical role for adenosine A_1 receptors (A_1R) in osteoclastogenesis; blockade or deletion of these receptors diminishes osteoclast differentiation. Interestingly, selective A_1R agonists neither affect basal osteoclastogenesis nor do they reverse A_1R antagonist-mediated inhibition of osteoclastogenesis. In this study, we determined whether ectonucleotidase-mediated adenosine production was required for A_1R antagonist-mediated inhibition, and, when we saw no effect, determined whether A_1R was constitutively activated and the antagonist was acting as an inverse agonist to diminish osteoclast differentiation.

EXPERIMENTAL APPROACH

Osteoclast formation derived from wild-type, CD39 knockout (KO), CD73 KO, NPP-1 KO and TNAP KO mice was examined by tartrate-resistant acid phosphatase staining of receptor activator of NF-κB ligand–macrophage colony-stimulating factor-stimulated osteoclasts and osteoclast gene expression (*Ctsk*, *Acp5*, *MMP-9* and *NFATc1*). Intracellular cAMP concentration was determined by ELISA.

KEY RESULTS

Rolofylline inhibited osteoclast formation in a dose-dependent manner (IC_{50} = 20–70 nM) in mice lacking all four of these phosphatases, although baseline osteoclast formation was significantly less in precursors from CD73 KO mice. Rolofylline (1 μM) stimulates cAMP production in bone marrow macrophages by 10.23 ± 0.89-fold.

CONCLUSIONS AND IMPLICATIONS

Based on these findings, we hypothesize that the A_1R is constitutively activated in osteoclast precursors, thereby diminishing basal AC activity, and that A_1R antagonists act as inverse agonists to release A_1R-mediated inhibition of basal AC activity and permit osteoclast differentiation. The constitutive activity of A_1R promotes osteoclast formation and down-regulation of this activity blocks osteoclast formation.

Abbreviations

A_1R, adenosine A_1 receptors; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1, 3-dipropylxanthine; PP_i, inorganic pyrophosphate

Introduction

There is growing evidence that adenosine, which acts on its four GPCRs (A_1, A_{2a}, A_{2b} and A_3), plays an important role in bone metabolism (Evans *et al.*, 2006; Russell *et al.*, 2007; Kara *et al.*, 2010; He and Cronstein, 2011; 2012; Carroll *et al.*, 2012; Mediero *et al.*, 2012b). Previous work from our laboratory (Kara *et al.*, 2010; He and Cronstein, 2011; 2012) has

shown that blockade or deletion of the adenosine A_1 receptor (A_1R), the adenosine receptor with the highest ligand affinity, inhibits osteoclast differentiation and function both *in vivo* and *in vitro*. The effects of A_1R blockade are not reversed by A_1 agonists, raising questions about the mechanism by which A_1Rs regulate osteoclast function. One possibility by which A_1R antagonists could regulate osteoclast differentiation is that adenosine generated on the surface of osteoclasts is sufficient to constitutively activate A_1Rs, a conclusion suggested by the work of Chen *et al*. (2006) demonstrating that adenosine released from neutrophils at low levels (<0.15 μM) induces the localization and activation of A_3 receptors on the cell surface. Alternatively, A_1R antagonists could be acting as inverse agonists to progressively diminish the signalling activity of A_1Rs (de Ligt and Ap, 2002; Nobles *et al*., 2005).

Adenosine synthesis is controlled by the action of a series of ectonucleotidases located on the plasma membrane, including ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD-1) (CD39), ecto-5′-nucleotidase (CD73, EC 3.1.3.5), nucleotide pyrophosphatase phosphodiesterase 1 (NPP-1, EC 3.1.4.1) and tissue non-specific alkaline phosphatase (TNAP). These enzymes act in concert to sequentially hydrolyse extracellular ATP to ADP, AMP and adenosine. Whereas adenosine is a ubiquitous signalling molecule, ectonucleotidases are widely distributed in mammals. In particular, CD39 and CD73 are expressed on primitive haematopoietic stem cells and whole bone marrow cells (Barbosa *et al*., 2011) and monocyte/macrophages (Dwyer *et al*., 2007), which can be differentiated into osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). We have detected CD39 and CD73 mRNA in murine osteoclast precursors and mature osteoclasts as well (Supporting Information Figure S1). A recent study further supports the importance of CD73 in bone remodelling, demonstrating that CD73 KO mice exhibit osteopenia due to diminished osteoblast function (Takedachi *et al*., 2012). TNAP is abundantly expressed in bone, including osteoblast precursors and mature osteoblasts, osteoclast basolateral membrane and bone lining cells (Bernard, 1978; Hoshi *et al*., 1997; Miao and Scutt, 2002; Nakano *et al*., 2007). Besides hydrolysing ATP directly to AMP, NPP-1 also modulates bone remodelling as an important inorganic pyrophosphate (PP_i)-generating enzyme on osteoblasts and chondrocytes, which controls bone mineralization. NPP-1 is expressed in a large variety of tissues, including skeletal muscle and cartilage (Bollen *et al*., 2000).

To determine how adenosine A_1R blockade or deletion regulates osteoclast differentiation where A_1 agonists have no effect alone and do not reverse the effect of antagonists on osteoclast differentiation, we examined the effect of the highly selective A_1R antagonist rolofylline (K_i = 0.72 nM for human and 0.19 nM for rat, respectively; Nonaka *et al*., 1996; Pfister *et al*., 1997) on M-CSF/RANKL-induced differentiation of osteoclast precursors derived from bone marrow of wild-type, CD39-, CD73-, NPP-1- or TNAP-deficient primary bone marrow-derived myeloid precursors. We also tested the alternative hypothesis that A_1Rs are coupled endogenously to signalling mechanisms that regulate osteoclast differentiation in myeloid precursors.

Methods

Mice and reagents

C57BL/6 wild-type mice and TNAP KO inbred onto C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD39 KO mice were kindly provided by Dr. Simon Robson (Beth Israel Deaconess Medical Center, Boston, MA, USA). CD73 KO mice were provided as a gift by Dr. Linda Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) (Thompson *et al*., 2004). NPP-1 KO mice were kindly provided by Robert Terkeltaub (Veterans Affairs Medical Center, San Diego, CA, USA) (Johnson *et al*., 2005). CD39, CD73 and NPP-1 knockout mice were all bred onto C57BL/6 background (>10 backcrosses) in the New York University School of Medicine Animal Facility. All experimental mice were 6- to 8-week-old female mice. All experimental procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the School of Medicine of New York University. Rolofylline (K3769) and forskolin (F6886) were purchased from Sigma-Aldrich (St Louis, MO, USA). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al*., 2010; McGrath *et al*., 2010).

Osteoclast formation

For generation of bone marrow-derived osteoclasts, primary bone marrow cells from 6- to 8-week-old female mice were cultured as described previously (Kara *et al*., 2010; He and Cronstein, 2012). Briefly, bone marrow was extracted from femora and tibia of mice. Cells were grown in complete α-Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS for 24 h. Then the non-adherent bone marrow macrophages (BMMs) were collected and replated in culture dishes at 1×10^5 cells·cm^{-2} density with murine M-CSF (30 ng·mL^{-1}) for 2 days. Cells at this stage were considered M-CSF-dependent BMMs and used as osteoclast precursors. Induction of differentiation to osteoclasts was achieved by culturing the BMM cells with the osteoclastogenic medium containing M-CSF (30 ng·mL^{-1}) and recombinant murine RANKL (30 ng·mL^{-1}), with or without A_1R antagonist, rolofylline, for 5 days. The culture medium was replaced with fresh medium containing these reagents every 2 days.

Tartrate-resistant acid phosphatase (TRAP) staining

Osteoclast differentiation was evaluated by staining for TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich) as previously described (He and Cronstein, 2012). TRAP-positive multinucleated cells (≥3 nuclei) were counted as osteoclasts.

Real-time reverse transcription-PCR

Total RNA was isolated from culture cells using RNeasy Kit (Qiagen, Valencia, CA, USA) as previously described (He and Cronstein, 2012). Briefly, cDNA was synthesized from 1 μg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen) in a volume of 20 μL. Real-time PCR was performed using Master SYBR Green Kit (Stratagene, La Jolla, CA,

Table 1
Oligonucleotides used for quantitative real-time PCR

Target mouse gene	Sequence	Gene bank reference
β-Actin	(F) 5′-ACTATTGGCAACGAGCGGTT-3′ (R) 5′-CAGGATTCCATACCCAAGAAGGA-3′	NM_007393.3
Acp5	(F) 5′- CGTCTCTGCACAGATTGCAT-3′ (R) 5′-TGAAGCGCAAACGGTAGTA-3′	NM_007388
Ctsk	(F) 5′- GGAGGCGGCTATATGACCA-3′ (R) 5′-ACAACTTTCATCCTGGGCCCA-3′	NM_007802
MMP-9	(F) 5′- CCTGTGTGTTCCCGTTCATCT-3′ (R) 5′- GCCATACAGTTTATCCTGGTCA-3′	NM_013599.2
NFATc1	(F) 5′-CTCGAAAGACAGCACTGGA-3′ (R) 5′-AGGTGCTGGAAGGTGTACT-3′	AF309389.1
c-fos	(F) 5′-GAACAACACACTCCATGCGGG-3′ (R) 5′-GGAGGACCTTACCTGTTCGTGA-3′	NM_010234.2
CD39	(F) 5′-AGCTGCCCCTTATGGAAGAT-3′ (R) 5′-ATCACAGCCAAGATAGAGGT-3′	NM_009848.3
CD73	(F) 5′-CAAATCCCACACAACCACTG-3′ (R) 5′-TGCTCACTTGGTCACAGGAC-3′	NM_011851.4

USA). Primers are listed in Table 1. PCR conditions were 95°C for 5 min followed by 38 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Each experiment was carried out in triplicate and results were standardized against the message level of β-actin. The comparative CT method was used to calculate the expression levels of RNA transcripts.

Measurement of cAMP accumulation

BMMs were plated at a density of 5×10^5 cells·cm^{-2} in 6-well plates. To determine the intracellular content of cAMP, cells were starved in serum-free medium containing M-CSF (30 ng·mL^{-1}) for 1 h to inhibit cAMP phosphodiesterase activity. After pre-incubation, cells were treated with 100 ng·mL^{-1} RANKL with or without different doses of rolofylline for 30 min. Forskolin treatment serves as a positive control. Acetylated intracellular cAMP concentrations were measured using a Direct cAMP ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's protocol. cAMP results were expressed in pmol·mL^{-1}.

Statistical analysis

Data are shown as the means ± SD from at least three independent experiments using cells from different mice. Statistical analysis was performed using Prism 4.02 software (GraphPad Software Inc., San Diego, CA, USA). All data were evaluated by ANOVA followed by Bonferroni *post hoc* testing. $P < 0.05$ was considered to be significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Results

Rolofylline inhibits osteoclast formation by bone marrow precursors derived from wild-type mice

Consistent with our previous observation (He and Cronstein, 2012), blockade of adenosine A_1R with the A_1R-selective antagonist, rolofylline, suppresses M-CSF/RANKL-induced osteoclast differentiation of murine bone marrow cells (TRAP$^+$ cells with more than three nuclei) in a dose-dependent manner (IC_{50} = 12.8 nM; Figure 1A,B). Although not tested directly, it is interesting to note that rolofylline appears to be more potent than 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) as an inhibitor of osteoclast differentiation consistent with prior reports that rolofylline has higher binding affinity for the A_1R (Nonaka *et al.*, 1996; He and Cronstein, 2012).

Rolofylline inhibits expression of mRNA for osteoclast markers during osteoclast differentiation by bone marrow precursors derived from wild-type mice

To confirm the inhibitory effects of rolofylline on osteoclast formation, the expression of osteoclast-specific genes, such as cathepsin K (*Ctsk*), TRAP (*Acp5*), *MMP-9* and the master transcription factor, nuclear factor of activated T-cells (*NFATc1*), were analysed using real-time PCR. Rolofylline at concentration of 100 nM or more significantly reduced the transcription of these genes at day 5 for *Ctsk*, *Acp5* and *MMP-9*; day 4 for *NFATc1*; and day 2 for *c-fos* respectively (Figure 2A–E). The concentrations of rolofylline required to inhibit expression of these genes were similar to those required for inhibition of osteoclast differentiation. These results further demonstrate that rolofylline inhibits osteoclast formation derived from normal mice bone marrow.

Rolofylline inhibits osteoclast formation derived from nucleotidase-deficient mice

To identify the biological source of adenosine in modulating osteoclast formation, *ex vivo* experiments to examine osteoclast differentiation of cultured ectonucleotidase-deficient bone marrow precursors into osteoclasts were next performed. As shown in Figure 1B, cells from mice lacking TNAP, CD39, CD73 and NPP-1 differentiated into osteoclasts and

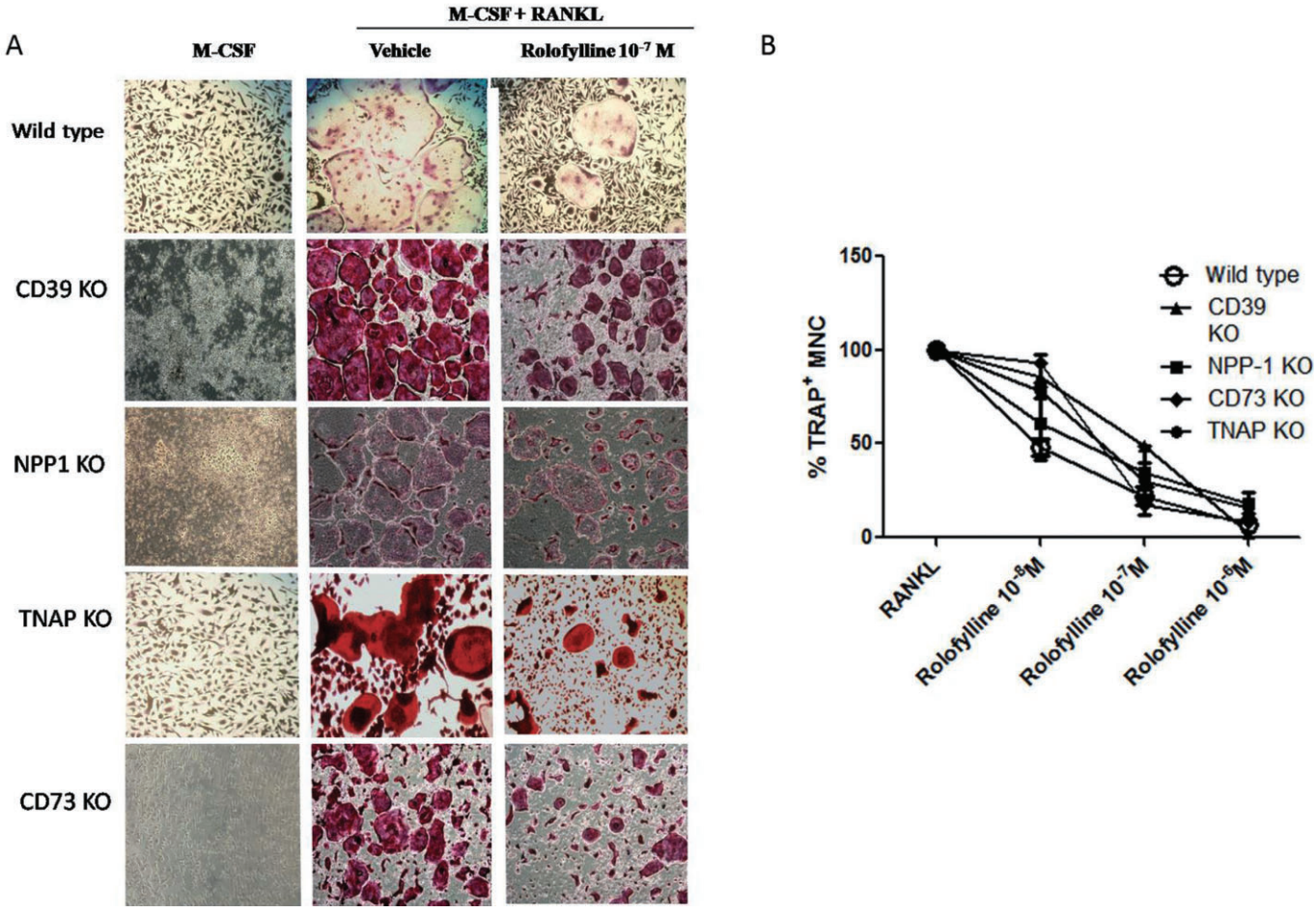

Figure 1

Suppression of osteoclast formation by A_1R-selective antagonist, rolofylline. Murine BMMs derived from wild-type, CD39 KO, NPP-1 KO, CD73 KO or TNAP KO (1×10^5 cells·cm^{-2}) were cultured with M-CSF and RANKL (30 ng·mL^{-1} each), with or without various concentrations of rolofylline for 5 days in 48-well plates for TRAP staining (A). (B) Cells that are TRAP-positive multinuclear containing more than three nuclei (TRAP$^+$ MNC) were counted and plotted against the RANKL control (set as 100%). Data are expressed as the mean ± SEM of four independent experiments.

rolofylline inhibited osteoclast differentiation similarly in cells from all four different nucleotidase-deficient strains. The concentration of rolofylline required to inhibit osteoclast differentiation was similar in all four ectonucleotidase-deficient strains except CD39 KO mice (Table 2). The osteoclast formation derived from CD39 KO mice significantly increased by 21.6 ± 2.5% compared with those isolated from wild-type mice (402 ± 19 vs. 324 ± 15 multinuclear cells per well; $P < 0.01$). In contrast, CD73 KO mice had much less osteoclast formation than that from wild-type mice (162 ± 22 vs. 324 ± 15 multinuclear cells per well; $P < 0.001$). Mice deficient in TNAP or NPP-1 had similar osteoclast formation with wild-type (TNAP KO: 289 ± 25 and NPP-1 KO: 285 ± 31 multinuclear cells per well). Similarly, rolofylline inhibited M-CSF/RANKL-induced expression of mRNA for osteoclast markers in osteoclasts differentiated from cells lacking these ectonucleotidases (Figure 2). Thus, these results indicate that it is unlikely that adenosine A_1R blockade or deletion inhibits the action of locally generated adenosine on osteoclast differentiation.

Table 2

Calculated IC_{50} values for rolofylline-mediated inhibition on osteoclast formation derived from wild-type, CD39 KO, NPP-1 KO, CD73 KO and TNAP mice

	IC_{50} (nM)
Wild type	12.8 ± 1.44
CD39 KO	77.1 ± 15.99*
NPP-1 KO	20.4 ± 12.09
CD73 KO	45.7 ± 0.74
TNAP KO	19.8 ± 7.32

Data are expressed as the mean ± SEM of four independent experiments.
*$P < 0.01$, compared with wild type.

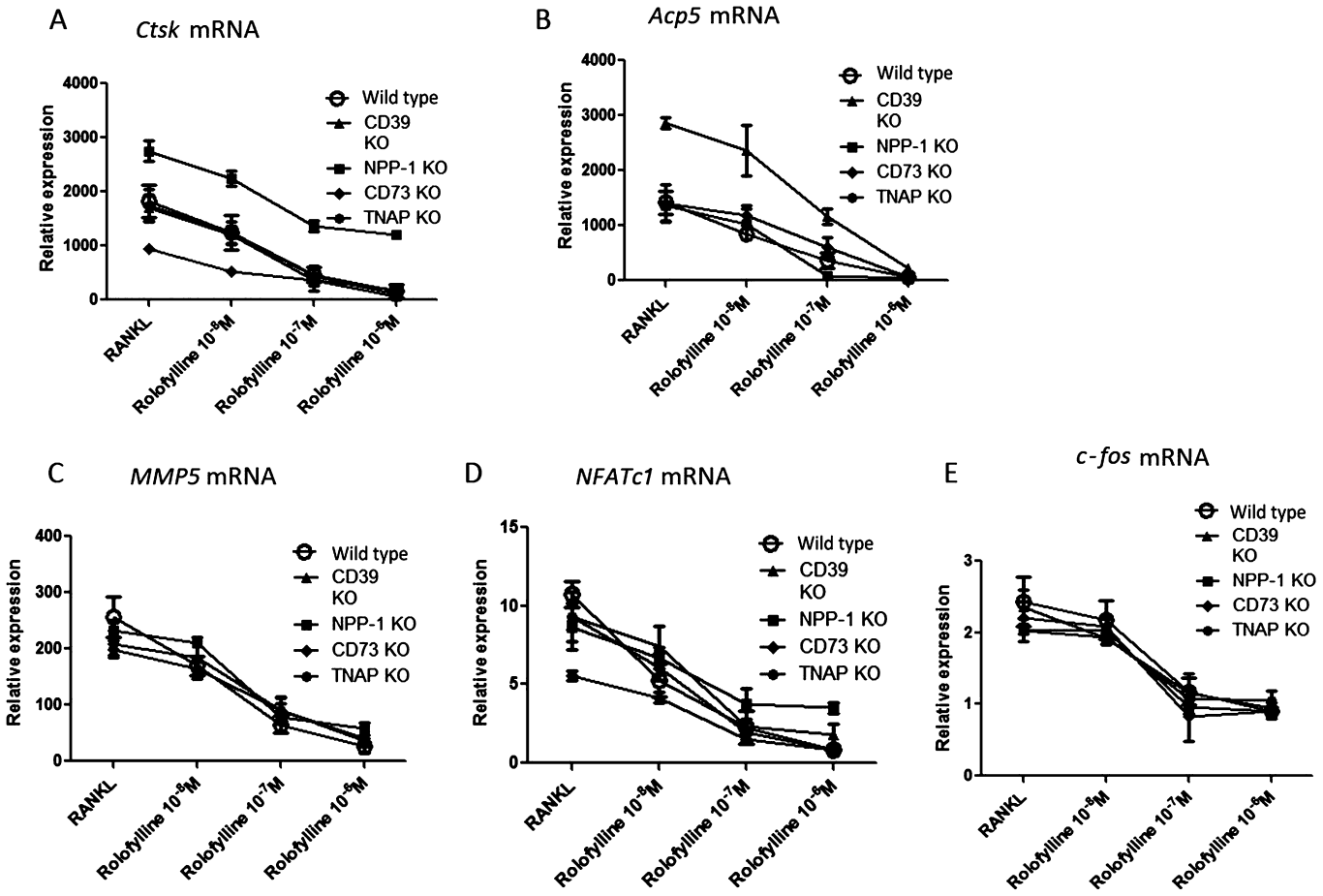

Figure 2

Expression of the osteoclast-specific genes was inhibited by A_1R-selective antagonist, rolofylline. BMMs were cultured with M-CSF and RANKL (30 ng·mL^{-1} each), with or without various concentrations of rolofylline in 6-well plates for 5 days prior to RNA extraction and real-time PCR for *Ctsk*, *MMP9*, *Acp5* 4 days prior to RNA extraction, and real-time PCR for *NFATc1* and 2 days prior to RNA extraction and real-time PCR for *c-fos* respectively. β-Actin served as PCR control. Relative expression was calculated relative to M-CSF only cells (fold value 1). Data are expressed as the mean ± SEM of four independent experiments. (A) *Ctsk* mRNA; (B) *Acp5* mRNA; (C) *MMP5* mRNA; (D) *NFATc1* mRNA; (E) *c-fos* mRNA.

Rolofylline increases the intracellular level of cAMP in BMMs

Previous studies have documented that in some cell types and under certain circumstances, adenosine A_1R are constitutively active and A_1R antagonists, like DPCPX and rolofylline, have negative intrinsic activity (act as inverse agonists). Because A_1R are G_i-linked receptors, they inhibit stimulated cAMP generation and inverse agonists at A_1R would block this intrinsic inhibition of cAMP generation and would increase cellular cAMP content in the absence of agonists (Ma and Green, 1992; Shryock *et al.*, 1998; Nobles *et al.*, 2005). Because our previous observations had demonstrated that the adenosine A_1R agonist, N^6-cyclopentyladenosine (CPA), neither affected basal osteoclast formation nor reversed A_1R antagonist-mediated inhibition of osteoclast formation, we tested the hypothesis that adenosine A_1R antagonists could act as inverse agonists for cAMP generation in osteoclast precursors. To test this hypothesis, osteoclast precursors (BMMs treated with M-CSF) were incubated with RANKL with or without the presence of different doses of rolofylline for 30 min, and the amount of intracellular cAMP in osteoclast precursors was determined by ELISA. Whereas RANKL alone barely elevated the cAMP level, rolofylline strikingly increased the intracellular content of cAMP by 2.68 ± 0.32-fold at 10 nM, 4.09 ± 0.55-fold at 100 nM and 10.23 ± 0.89-fold at 1 μM respectively (Figure 3). As a positive control, forskolin increased the cAMP level by 20.25 ± 1.1-fold (Figure 4).

Rolofylline inhibits osteoclast differentiation and increases cAMP generation in normal human bone marrow-derived osteoclast precursors

Rolofylline inhibited osteoclast differentiation by M-CSF/RANKL-stimulated human bone marrow-derived myeloid cells in a dose-dependent fashion, although the extent of inhibition observed was not as great as that observed in cells of murine origin (Figure 5). Similarly, rolofylline increased

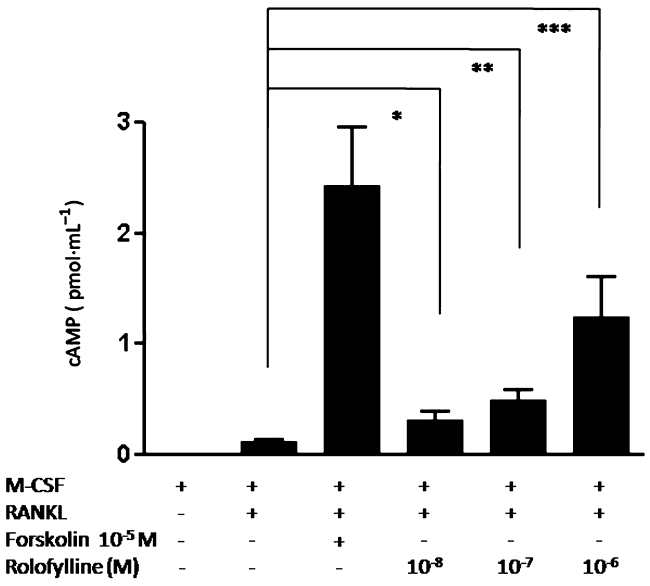

Figure 3

Induction of intracellular cAMP level by A_1R-selective antagonist, rolofylline, in murine BMMs. BMMs were starved in serum-free media supplemented with M-CSF (30 ng·mL^{-1}) for 1 h before the addition of 100 ng·mL^{-1} RANKL with or without various concentration of rolofylline for 30 min. Forskolin treatment serves as a positive control. Acetylated intracellular cAMP concentrations were measured using a Direct cAMP ELISA kit (Enzo Life Sciences) following the manufacturer's protocol. Data are expressed as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

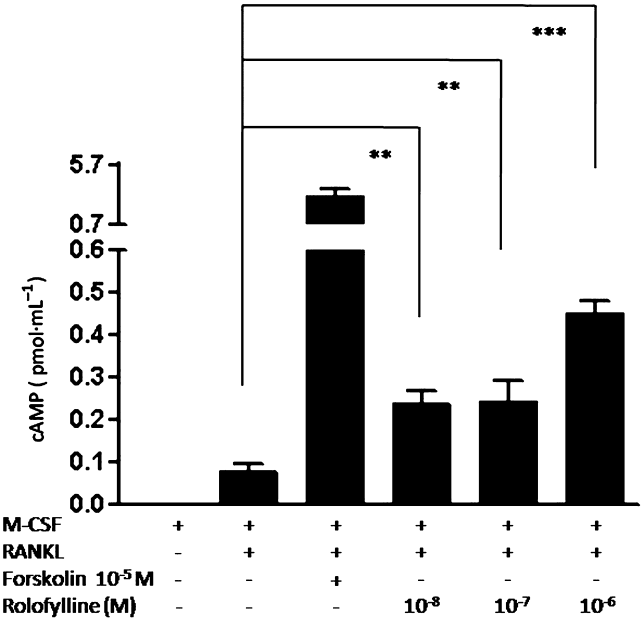

Figure 4

Induction of intracellular cAMP level by A_1R-selective antagonist, rolofylline, in human bone marrow-derived osteoclast precursors. Monocytes isolated from fresh human bone marrow were starved in serum-free media supplemented with M-CSF (30 ng·mL^{-1}) for 1 h before the addition of 100 ng·mL^{-1} RANKL with or without various concentration of rolofylline for 30 min. Forskolin treatment serves as a positive control. Acetylated intracellular cAMP concentrations were measured using a Direct cAMP ELISA kit (Enzo Life Sciences) following the manufacturer's protocol. Data are expressed as the mean ± SEM of three independent experiments. **P < 0.01, ***P < 0.001.

the intracellular cAMP level in normal human bone marrow-derived osteoclast precursors by 3.09 ± 0.23-fold at 10 nM, 3.28 ± 0.15-fold at 100 nM and 5.89 ± 0.67-fold at 1 μM respectively. These results are consistent with the hypothesis that rolofylline, an A_1R antagonist, acts as an inverse agonist at A_1Rs in primary human osteoclast precursors as well.

Discussion

Over the past 10 years, the discovery that receptors are constitutively active and antagonists act as inverse agonists has been made for almost every known GPCR, including adenosine A_1 and A_{2b} receptors (Shryock *et al.*, 1998; de Ligt and Ap, 2002; Nobles *et al.*, 2005; Li *et al.*, 2007; Kiesman *et al.*, 2009). Inverse antagonists reverse downstream signalling at the receptor of interest by altering receptor conformation rather than by directly competing with ligand at the binding site, as shown for A_1R. Here, we report that rolofylline treatment reverses downstream signalling at A_1R; rolofylline increases intracellular cAMP levels, a reversal of the effect of constitutively active A_1R on endogenous adenylate cyclase activity and intracellular cAMP levels. In our previous work, we found that blockade or deletion of adenosine A_1R suppresses osteoclast differentiation both *in vivo* and *in vitro* (Kara *et al.*, 2010; Mediero *et al.*, 2012b). Interestingly, selective A_1R agonists neither affect basal osteoclast formation nor reverse A_1R antagonist-mediated inhibition of osteoclast formation (He and Cronstein, 2012). Thus, although we have not formally demonstrated that competitive inhibition of A_1R is not responsible for the effects of rolofylline (and DPCPX) on osteoclast differentiation and the intracellular signalling events observed here, the finding that high affinity ligands for A_1R (e.g. CPA) do not reverse the effects of rolofylline is most consistent with the hypothesis that adenosine A_1R on both primary murine and human osteoclast precursors are constitutively active, and the antagonists studied act as inverse agonists at A_1R. Moreover, we found no evidence to support the notion that adenosine generated at the cell surface by ectonucleotidases activated A_1Rs to stimulate osteoclast differentiation, an effect that could be blocked by A_1R antagonists.

To characterize the impact of the cellular contribution of ectonucleotidases (CD39, NPP-1, CD73 and TNAP) on adenosine-mediated regulation of osteoclast formation, we compared the osteoclast formation derived from these ectonucleotidase knockouts to wild-type animals. It is intriguing to find that myeloid cells from mice that lacked CD73, a key enzyme required for the production of adenosine, have markedly reduced osteoclast formation in *ex vivo* bone marrow-derived culture. In contrast, a recent study by Takedachi *et al.* (2012) showed that there is no bone abnormality in female CD73-deficient mice, and CD73-deficient male mice have normal levels of osteoclastic markers with reduced *in vitro*

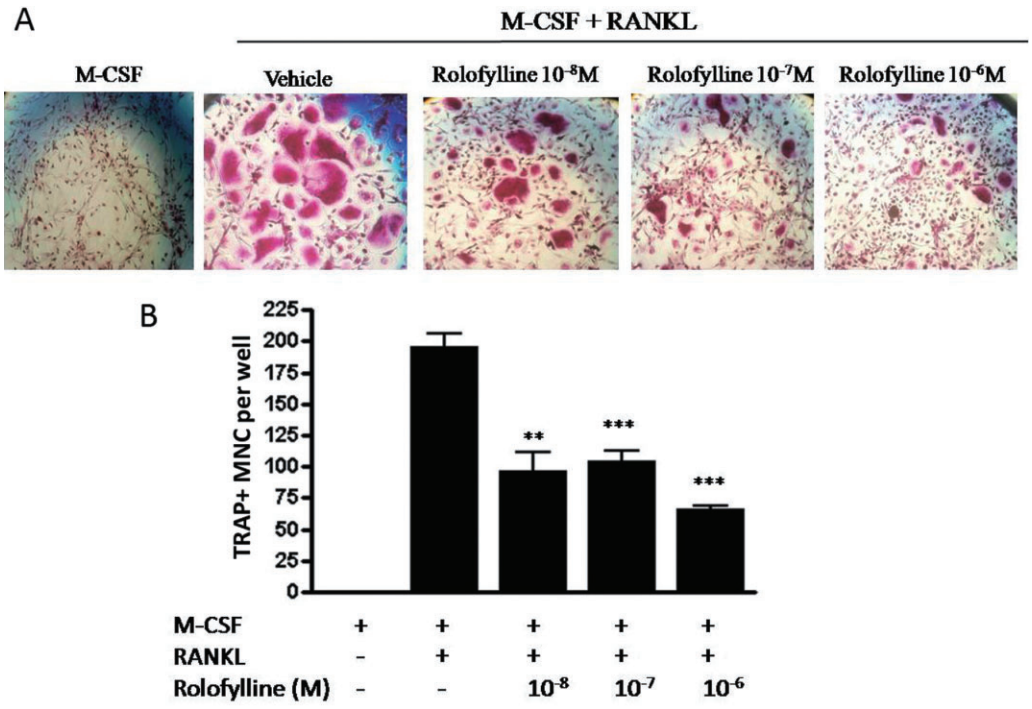

Figure 5

Inhibition of osteoclast formation derived from normal human bone marrow by A_1R-selective antagonist, rolofylline, in a dose-dependent manner. Human haematopoietic stem cells derived from normal human bone marrow (1×10^5 cells·cm^{-2}) were cultured with M-CSF and RANKL (30 ng·mL^{-1} each), with or without various concentrations of rolofylline for 7 days in 48-well plates for TRAP staining (A). (B) Cells that are TRAP-positive multinuclear containing more than three nuclei (TRAP$^+$ MNC) were counted. Data are expressed as the mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

osteoblast differentiation; adenosine generated by the activity of CD73 stimulates A_{2b} receptor on osteoblast precursors to increase osteoblast differentiation. One explanation for the difference in osteoclast generation in culture and whole mice is that the defective ability of these CD73$^{-/-}$ osteoclast precursors to differentiate into osteoclasts *in vitro* can be compensated by an increased number of osteoclast precursors *in vivo*. Another more likely explanation for this apparent discrepancy is the existence of a RANKL-independent compensatory regulatory mechanism for osteoclast formation, such as cytokines TNF-α and IL-6 (Kudo *et al.*, 2003; Hemingway *et al.*, 2011), which are elevated in CD73-deficient mice (Hasko *et al.*, 2011; Blume *et al.*, 2012).

In general, the types and functions of adenosine receptors on specific cell types or organs have been similar in different mammalian species. Nonetheless, we have previously reported that A_{2a} receptor stimulation inhibits mouse and human osteoclast differentiation (Mediero *et al.*, 2012a,b), whereas Pellegatti *et al.* (2011) have observed that adenosine A_{2a} receptor stimulation of human osteoclast precursors increased osteoclast fusion. Similarly, Pellegatti and colleagues have observed that CD39 inhibition markedly inhibits osteoclast differentiation, an observation that is inconsistent with our finding that CD39 deletion increased osteoclast differentiation. It is difficult to account for the differences between Pellegatti's observations and those we have reported. Purinergic signalling is a complex network composed of extracellular nucleotides, including ATP and ADP, and extracellular nucleosides, such as adenosine. CD39/ENTPD-1 is an ectoenzyme that metabolizes ATP to ADP and ADP to 5′-AMP. Blockade of CD39 transiently increases extracellular concentration of ATP and ADP, which are agonists at P2 receptors (Imai *et al.*, 1999). Therefore, CD39 is an important modulator of P2 receptor signalling, including ligand-gated ion channel P2X7 and GPCR P2Y6, which has been implicated in regulation of osteoclast function and formation (Gartland *et al.*, 2003; Korcok *et al.*, 2005; Beldi *et al.*, 2010). In addition, ATP is a potent stimulus of osteoclast formation at 0.2–2.0 μM (Morrison *et al.*, 1998; Hoebertz *et al.*, 2001). Here, we show that haematopoietic stem cells generated from CD39 knockout mice have increased osteoclast formation, which is likely due to an increase in the concentration of ATP in the pericellular space. In contrast, we observed that deletion of CD73, which leads to diminished adenosine generation from 5′-AMP and accumulation of 5′-AMP results in diminished osteoclast formation. One possible explanation for diminished osteoclast differentiation by these cells could be enhanced activation of AMP kinase, which diminishes osteoclast differentiation induced by exposure to RANKL (Lee *et al.*, 2010).

There is redundancy in the roles of the different ectonucleotidases in the catabolism of nucleotides to adenosine. For example, CD39 can hydrolyse both nucleoside triphosphates (ATP) and diphosphates (ADP) to generate AMP, whereas NPP-1 breaks down ATP directly to AMP. Therefore, although our results (Figures 1 and 2) indicate that adenosine A_1R

blockade or deletion does not inhibit the action of locally generated adenosine on osteoclast differentiation, it remains possible that more than one ectonucleotidase deletion may be required to see any effects.

Another example is that TNAP competes with CD73 for the conversion of AMP into adenosine. However, whereas TNAP has a comparatively lower affinity for AMP (K_m = 0.5–10.0 mM) compared with CD73 (K_m = 3–19 μM), it only accounts for 15% of the overall hydrolytic activity at physiological pH (Ikehara *et al.*, 1978; Picher *et al.*, 2003; Hunsucker *et al.*, 2005). And the fact that TNAP generally co-localizes with other ectonucleotidases suggests that TNAP may support CD73 under conditions that require more effective AMP hydrolysis and adenosine production (Langer *et al.*, 2007). Therefore, CD73 has been proposed as the rate-limiting enzyme in the production of adenosine during metabolic stress (Lennon *et al.*, 1998; Eckle *et al.*, 2007). This role for CD73 is consistent with our findings that only osteoclast precursors derived from CD73-deficient mice have impaired osteoclast formation among all four ectonucleotidase knockouts. Observations made in TNAP knockout mice, which shows that the functional status of osteoclasts are normal in TNAP –/– mice (Wennberg *et al.*, 2000), are in line with the observations we made here that TNAP knockout mice derived osteoclasts differentiate normally (Figure 5).

In conclusion, here we have examined the biological basis for adenosine A_1R-mediated regulation of osteoclast differentiation using *ex vivo* bone marrow cell cultures. Our data support the hypothesis that A_1R antagonists act as inverse agonists on osteoclast precursors to diminish osteoclast differentiation. In contrast, our results indicate that generation of adenosine at the surface of the osteoclast by most ectonucleotidases plays little role in osteoclast differentiation.

Acknowledgements

This work was supported by grants from the National Institutes of Health (AR56672, AR56672S1 and AR54897), the New York University–Health and Hospitals Corporation Clinical and Translational Science Institute (UL1RR029893) and the Vilcek Foundation.

Conflict of interest

Bruce N. Cronstein holds patents on use of adenosine A_{2a} receptor agonists to promote wound healing and use of A_{2a} receptor antagonists to inhibit fibrosis, use of adenosine A_1 receptor (A_1R) antagonists to treat osteoporosis and other diseases of bone, use of adenosine A_1 and A_{2b} receptor antagonists to treat fatty liver and use of adenosine A_{2a} receptor agonists to prevent prosthesis loosening. Bruce N. Cronstein is consultant for Bristol-Myers Squibb; Novartis; Can-Fite Biopharmaceuticals; Cypress Laboratories; Regeneron (Westat, DSMB); Endocyte; Protalex; Allos, Inc.; Savient; Gismo Therapeutics; Antares Pharmaceutical and MediVector. Dr. He and Ms. Wilder have no conflicts. Bruce Cronstein, MD, Consultant (within the past 2 years), all <$10 000: Bristol-Myers Squibb; Novartis; Can-Fite Biopharmaceuticals; Cypress Laboratories; Regeneron (Westat, DSMB); Endocyte; Protalex; Allos, Inc.; Savient. Equity: Can-Fite Biopharmaceuticals received for membership in Scientific Advisory Board. Grants: King Pharmaceuticals, National Institutes of Health, Vilcek Foundation, OSI Pharmaceuticals, URL Pharmaceuticals, Inc., Board Member: Vilcek Foundation. Intellectual Property: Patents on use of adenosine A_{2a} receptor agonists to promote wound healing and use of A_{2a} receptor antagonists to inhibit fibrosis. Patent on use of adenosine A_1R antagonists to treat osteoporosis and other diseases of bone. Patent on the use of adenosine A_1 and A_{2b} receptor antagonists to treat fatty liver. Patent on the use of adenosine A_{2a} receptor agonists to prevent prosthesis loosening.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12342

Figure S1 (a) mRNA expression of CD39 and CD73 by mouse BMMs and mature osteoclasts. Mouse bone marrow cells were extracted and BMMs were stimulated with M-CSF/RANKL for 7 days and total mRNA was extracted. Expression of mRNA was detected by RT-PCR. (b) Quantification of CD39 and CD73 mRNA expression by real-time RT-PCR.