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Osteoblast differentiation and survival: A role for A_{2B} adenosine receptor allosteric modulators

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ABSTRACT

The A_{2B} adenosine receptor (A_{2B} AR), activated in response to high levels of endogenous adenosine, is the major AR subtype involved in mesenchymal stem cell (MSC) differentiation to osteoblasts and bone formation. For this reason, targeting of A_{2B} AR with selective allosteric modulators may represent a promising pharmacological approach to the treatment of bone diseases.

Herein, we report the characterization of a 3-keto-indole derivative, 2-(1-benzyl-1*H*-indol-3-yl)-2-oxo-N-phenylacetamide (KI-7), as A_{2B} AR positive allosteric modulator in MSCs, demonstrating that this compound is able to potentiate the effects of either adenosine and synthetic orthosteric A_{2B} AR agonists in mediating osteoblast differentiation in vitro. In detail, we observed that MSC treatment with KI-7 determined an increase in the expression of osteoblast-related genes (Runx2 and osterix) and osteoblast marker proteins (phosphatase alkaline and osteocalcin), associated with a stimulation of osteoblast mineralization.

In the early phase of differentiation programme, KI-7 significantly potentiated physiological and A_{2B} AR agonist-mediated down-regulation of IL-6 release. Conversely, during the late stage of differentiation, when most of the cells have an osteoblast phenotype, KI-7 caused a sustained raise in IL-6 levels and an improvement in osteoblast viability. These data suggest that a positive allosteric modulation of A_{2B} AR not only favours MSC commitment to osteoblasts, but also ensures a greater survival of mature osteoblasts. Our study paves the way for a therapeutic use of selective positive allosteric modulators of A_{2B} AR in the control of osteoblast differentiation, bone formation and fracture repair.

1. Introduction

Bone health is a chief medical concern for the ageing population, because deterioration of the bone is significantly correlated with fracture, disease, and a diminished quality of life [1]. With ageing, bone mass tends to decrease, resulting in the onset of many diseases associated with bone loss. For example, osteoporosis, characterized by low bone mass and structural deterioration of bone tissue with an increased susceptibility to fractures, is a major public health threat to the elderly [2,3]. A deeper understanding of the mechanisms by which bone formation is physiologically regulated is the goal of many studies aimed at finding adequate treatments of all the bone loss diseases. The majority of therapies for osteoporosis are largely based on preventing bone loss by inhibiting osteoclast-mediated bone resorption [4,5]. As regeneration of trabecular architecture by bone-forming osteoblasts (OBs) is required to fully restore the mechanical integrity of bone, there is much interest in developing anabolic agents to stimulate de novo bone formation [6].

Recently, an emerging role for purine receptors, and in particular for the A_{2B} adenosine receptor (AR) subtype, in controlling osteoblast differentiation has been outlined [7–11]. A_{2B} AR is expressed in MSCs and/or osteoblast progenitors and is activated when adenosine levels raise up to micromolar concentrations resulting from ATP degradation during bone injury [12,13]. A_{2B} AR activation promotes the expression of the osteogenic factor Runx2 and phosphatase alkaline (ALP), favouring mesenchymal stem cell (MSC) differentiation to osteoblasts [8]. These data suggest that A_{2B} AR may represent an interesting new target in the treatment of osteoporosis.

Therapies using receptor agonists are often prone to side effects, owing to the widespread distribution of the target receptor in the body. Allosteric modulation of membrane receptors has been intensively studied in the past three decades and is now considered an important mechanism for the control of receptor functions. A significant advantage of a therapy based on a positive allosteric modulator of a membrane receptor over its native, orthosteric activator is that, in principle, a greater tolerability can be achieved [14,15]. Particularly, a positive allosteric

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modulator is expected to enhance the action of the endogenous agonist without effects of its own on the unoccupied receptor. As a consequence, the effects of the endogenous agonist, which might be therapeutically insufficient in a particular disease state, could be magnified through allosteric modulation. An increasing percentage of G Protein-Coupled Receptors (GPCRs), including ARs, has been found to be modulated allosterically by various compounds [16–20].

Recently, we have developed and characterized a new series of 1-benzyl-3-ketoindoles as the first class of positive/negative allosteric modulators of the human A_{2B} AR [21,22].

Subsequently, we investigated whether one of these positive A_{2B} AR allosteric modulators, namely 2-(1-benzyl-1*H*-indol-3-yl)-2-oxo-*N*-phenylacetamide (KI-7) could markedly potentiate orthosteric agonist efficacy, so as to accelerate agonist-mediated osteoblast differentiation by MSC precursors. The results of our study suggest that this compound may represent a prototype of an innovative class of pharmacological agents useful to control disorders where bone destruction exceeds bone formation (e.g. osteoporosis, rheumatoid arthritis, osteogenesis imperfecta, multiple myeloma, fracture mal-union).

2. Materials and methods

2.1. Materials

Human bone marrow MSCs and cell culture medium were purchased by Lonza (Milan, Italy). Adenosine receptor ligands, 5′-*N*-ethylcarboxamidoadenosine (NECA), 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide (BAY 60-6583), and *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS1706) were purchased by Tocris Bioscience (Bristol, UK). RNeasy® Mini Kit was purchased from Qiagen S.p.A. The Script cDNA synthesis kit was furnished by Bio-rad s.r.l. Fluocycle® II SYBR® was obtained from Euroclone s.p.a. (Milan, Italy). KI-7 was synthesized as previously reported [21].

2.2. Cell cultures

MSCs were cultured in normal growth medium (MSCGM, Lonza), plated (5×10^3 cells/cm^2) in 75-cm^2 flasks and incubated at 37 °C in 5% CO_2 and 95% air. The medium was changed to remove nonadherent cells every 3 to 4 days, and the cells were used at passages 0 to 3.

For osteoblast differentiation studies, cells were seeded (3×10^3 cells/cm^2) in normal growth medium and, 24 h later, the medium was changed with osteogenic induction medium [8] containing dexamethasone (10^{-8} M), L-glutamine, ascorbate (50 μg/mL), penicillin/streptomycin (2 mM) and β-glycerophosphate (2 mM).

2.3. Measurement of cAMP levels in MSCs

MSCs were plated in 24-well plates in 0.5 mL of growth medium. After 24 h, the medium was changed with osteogenic induction medium, and the cells were differentiated for different days. At each time of differentiation, cells were incubated at 37 °C for 15 min with noncomplete medium containing 20 μM of the phosphodiesterase inhibitor 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone (Ro 20-1724), and adenosine deaminase (ADA, 1 U/mL). Cells were then treated for 15 min with different AR orthosteric agonists (BAY 60-6583, selective for the A_{2B} AR, NECA or adenosine) and the allosteric modulator KI-7 (1 μM) alone or in combination. The specificity of agonist-mediated responses was evaluated by cell pre-incubation with the selective A_{2B} AR antagonist MRS1706 (15 nM). When adenosine was used, the pre-treatment medium consisted in a noncomplete medium containing 20 μM of Ro 20-1724 without ADA. Intracellular cyclic AMP (cAMP) levels were measured using a competitive protein binding method [23].

2.4. Osteogenic marker expression during MSC differentiation to osteoblasts: RT-PCR analysis

MSCs were cultured in normal growth medium and, 24 h later, were treated with osteogenic medium alone (control), BAY 60-6583 (5 nM), NECA (100 nM) or KI-7 (1 μM), alone or in combination. Treatments were repeated every two days, and the expression levels of osteogenic markers (Runx2, Osterix, ALP and osteocalcin) were quantified after 5, 15 or 21 days of treatment.

Gene expression was assessed by RT-PCR, as previously described [24]. In brief, total RNA was extracted from 0, 5, 15 and 21 days of osteoblast differentiation using the RNeasy® Mini Kit, according to the manufacturer's instructions. The purity of the RNA samples was determined by measuring the absorbance at 260/280 nm. Reverse-transcription was performed with 500 ng of total RNA using i-Script cDNA synthesis kit. The primers used for the RT-PCR were designed to span intron/exon boundaries (Table 1); the annealing temperature used was 55 °C for all the genes. PCR specificity was determined using both a melting curve analysis and gel electrophoresis, and the data were analysed by the standard curve method. mRNA levels for each sample were normalised against β-actin mRNA levels, and relative expression was calculated using the Ct value.

2.5. Mineralization assay

MSCs were seeded (3×10^3 cells/cm^2) in normal growth medium and, 24 h later were treated for different days with osteogenic medium in the absence (control) or in the presence of NECA (100 nM), BAY 60-6583 (5 nM), KI-7 (500 nM–5 μM), alone or in combination. The specificity of agonist-mediated responses was evaluated by pre-incubation of the cells with selective A_{2B} AR antagonist MRS1706 (15 nM). In addition, the effects of 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, 100 nM–10 μM) and forskolin (1 μM), as activators of cAMP pathway, were evaluated. Treatments were repeated every two days, and the mineralization was quantified after 9, 15 or 21 days of treatment. The mineralization degree was quantified using OsteoImage™ Staining Reagent (Lonza, Milan, Italy), which specifically binds to the hydroxyapatite portion of the bone-like nodules deposited by cells [25,26].

Qualitative measure of the mineralization was also obtained by alizarin red staining [8]. After treatment with AR agonists, as above reported, fixed cells were washed once with distilled water and subsequently stained with 2% alizarin red S dissolved in distilled water for 5 min. The remaining dye was washed out, and the cells were washed once more. Finally, the cells were air-dried and images of the stained cells were captured using a light microscope.

2.6. Cell viability assay (MTS)

MSCs were seeded in 96-well microplates (5000 cells/well) and cultured in osteogenic medium for 21 days in the absence (control) or in the presence of NECA (100 nM), KI-7 (1 μM) and the selective A_{2B} AR antagonist MRS1706 (15 nM), alone or in combination. To evaluate cell viability, MTS assay was used following the manufacturer's instruction (Promega, Milan, Italy).

2.7. IL-6 production

MSCs were cultured in osteogenic medium for 5–21 days in the absence (control) or in the presence of NECA (100 nM) or BAY 60-6583 (5 nM) alone or in the presence of KI-7 (1 μM). The specificity of agonist-mediated responses was evaluated by pre-incubation of the cells with the selective A_{2B} AR antagonist MRS1706 (15 nM). The levels of secreted cytokine IL-6 were assayed in the conditioned medium of cells treated as described above, using ELISA detection kit (R&D Systems DuoSet, Minneapolis, MN).

Table 1
Primers used for RT-PCR.

Gene	Primer nucleotide sequences	Product size (base pairs)
Runx2	FOR: 5′-GGCCCTGGTGTTTAAATGGT-3′ REV: 5′-AGGCTGTTTGACGCCATAGT-3′	178 bp
ALP	FOR: 5′-CTGCAAGGACATCGCCTATC-3′ REV: 5′-CATCAGTTCTGTTCTTGGGGTA-3′	101 bp
Osterix	FOR: 5′-TCCCTGCTTGAGGAGGAAG-3′ REV: 5′-AAAGGTCACTGCCCACAGAG-3′	153 bp
Osteocalcin	FOR: 5′-CCTTTGTGTCCAAGCAGGAG-3′ REV: 5′-GTCAGCCAACTCGTCACAGT-3′	151 bp
β-actin	FOR: 5′-GCACTCTTCCAGCCTTCCTTCC-3′ REV-5′-GAGCCGCCGATCCACACG-3′	254 bp

2.8. Statistical analysis

A non-linear multipurpose curve-fitting programme, Graph-Pad Prism (Version 5.00), was used for data analysis and graphic presentation. Data are reported as the mean ± SEM of 3–4 different experiments. Statistical analyses were performed using a one-way ANOVA study followed by the Bonferroni test for repeated measurements. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Characterization of KI-7 as A_{2B} AR allosteric modulator in MSC: cAMP assay

In previous works we identified and characterized a new class of *N*-(indol-3-ylglyoxyl)amide derivatives as the first allosteric modulators of A_{2B} ARs [21,22]. Among these derivatives, KI-7 was found to act as a positive modulator of A_{2B} AR response provided with a high selectivity towards the other AR subtypes.

Herein, the activity of KI-7 as positive allosteric modulator of A_{2B} AR was evaluated in MSCs during their spontaneous differentiation to osteoblasts.

Functional activity of cAMP pathway in MSCs was demonstrated by the ability of forskolin and cAMP analogue, 8-Br-cAMP, to stimulate cAMP intracellular accumulation (Fig. 2C and D).

The selective A_{2B} AR agonist BAY 60-6583 (5 nM) stimulated cAMP accumulation at all differentiation time points, indicating the presence of functional A_{2B} AR at all stages of osteoblastogenesis (Fig. 1A). When compared with undifferentiated MSC (day 0), agonist-mediated cAMP production increased during the time of osteoblastic induction, reaching the highest levels on days 5 and 9, and then fell back to basal levels on days 15–21. The drop in agonist-mediated effects at the late stage of differentiation process may be most likely ascribed to the reduction in A_{2B} AR protein expression levels in the terminal phase of osteoblast differentiation, as previously reported [8].

The potency of BAY 60-6583, as derived by agonist concentration–response curves performed at 5 day differentiation stage, was in the nanomolar range ($EC_{50} = 6.0 \pm 0.5$ nM), a value comparable to that described for the same agonist in transfected cells (Fig. 1B) [22].

Furthermore, BAY 60-6583-mediated cAMP accumulation appeared to be completely abrogated by the selective A_{2B} AR antagonist MRS1706, confirming that the effect of this ligand was specifically mediated by A_{2B} AR subtype (Fig. 1C).

Thereafter, the effects of KI-7 on the responses evoked by the A_{2B} AR agonist BAY 60-6583 were investigated. Both in undifferentiated (Fig. 1C) and differentiated (5 days, Fig. 1D) MSCs, KI-7, "per se", did not significantly affect cAMP levels, suggesting that this compound is not provided with any intrinsic agonist efficacy. Conversely, the same compound was able to potentiate cAMP accumulation evoked by two different BAY 60-6583 concentrations (5 and 50 nM) and these effects were completely counteracted by MRS1706. These data suggest that KI-7 behaves as positive allosteric modulator of A_{2B} ARs in MSCs, as well as in transfected cell lines [22].

Interestingly, the shape of time course of cAMP production, obtained in the presence of KI-7, appeared to be quite different with respect to that observed with the agonist alone. Indeed, a trend towards a reduction in cAMP production was observed in the late phase of differentiation, but the second messenger's levels still remained high all the time. These results suggest that the positive allosteric modulation of A_{2B} AR allows the agonist to significantly activate the cAMP pathway even when the receptor expression is reduced.

It is well known that the nature of the agonist can make a huge difference in the response to an allosteric compound. Each combination of orthosteric probe and allosteric ligand could be characterized by unique properties. Thus, the "probe-dependence" effects of KI-7 were evaluated during the MSC differentiation. As shown in Fig. 2A and B, NECA and adenosine induced a significant increase in cAMP levels that reached a maximum after 9 days of differentiation and remained unchanged up to 21 days. The differences in the time course of cAMP production obtained using the nonselective A_{2B} AR agonists NECA and adenosine vs the selective A_{2B} AR agonist BAY-606583 may be due to the contribution of other AR subtypes. In particular, a great contribution in the cAMP intracellular accumulation may be ascribed to the A_{2A} AR activity, especially considering that this AR subtype is up-regulated in the terminal phase of MSC differentiation.

However, in the early phase of differentiation (up to 5 days of differentiation), the effects elicited by both NECA and adenosine were completely counteracted by the A_{2B} AR antagonist, MRS1706, thus demonstrating that these agonists mainly label A_{2B} ARs in differentiating MSCs (Fig. 2C and D).

KI-7 caused a significant increase in NECA and adenosine mediated cAMP production at all the differentiation stages (Fig. 2A and B) and these effects were completely counteracted by the A_{2B} AR antagonist MRS1706.

In summary, these results demonstrate that KI-7 allosterically modulates, in a probe-independent manner, the effects elicited by different classes of A_{2B} AR ligands on cAMP pathway, as demonstrated in transfected cell lines [22].

3.2. Effect of KI-7 on osteogenic marker expression during MSC differentiation

The effects of the A_{2B} AR orthosteric agonists, NECA and BAY 60-6583, and of the positive allosteric modulator, KI-7, on the regulation of different osteogenic marker expression were evaluated. In detail, the expression of two pivotal transcription factors that lead cells towards osteoblast phenotype, Runx2 and Osterix, was first evaluated. In parallel, the transcriptions of ALP, that is an early marker of osteoblast differentiation, and of osteocalcin, that appears later concomitantly with mineralization, were evaluated as markers of later osteoblastogenesis stage [27–29].

During MSC differentiation in osteogenic medium, the expression of the transcription factors Osterix and Runx2 followed the typical time course of these gene targets. Runx2 mRNA peaked after 15 days of treatment and tended to decrease in the late stage of differentiation (Fig. 3A). Osterix, a downstream gene of Runx2, was indeed expressed in a later stage during differentiation and its levels were maintained up to 21 days of the differentiation process (Fig. 3B). Treatment of the cells with NECA, BAY 60-6583 or KI-7 alone induced a significant increase in mRNA expression of Runx2 and Osterix, at all the differentiation stages. Moreover, KI-7 was able to significantly increase the effects of both NECA and of the selective A_{2B} AR agonist BAY 60-6583, demonstrating its positive modulator activity on A_{2B} AR (Fig. 3A and B).

NECA and BAY 60-6583 were also able to potentiate the expression of both ALP and osteocalcin proteins, and these effects were strongly increased by KI-7 (Fig. 3C and D). These results demonstrate that KI-7 facilitates the A_{2B} AR agonist-mediated signalling, so as to accelerate and strength the osteoblast differentiation process.

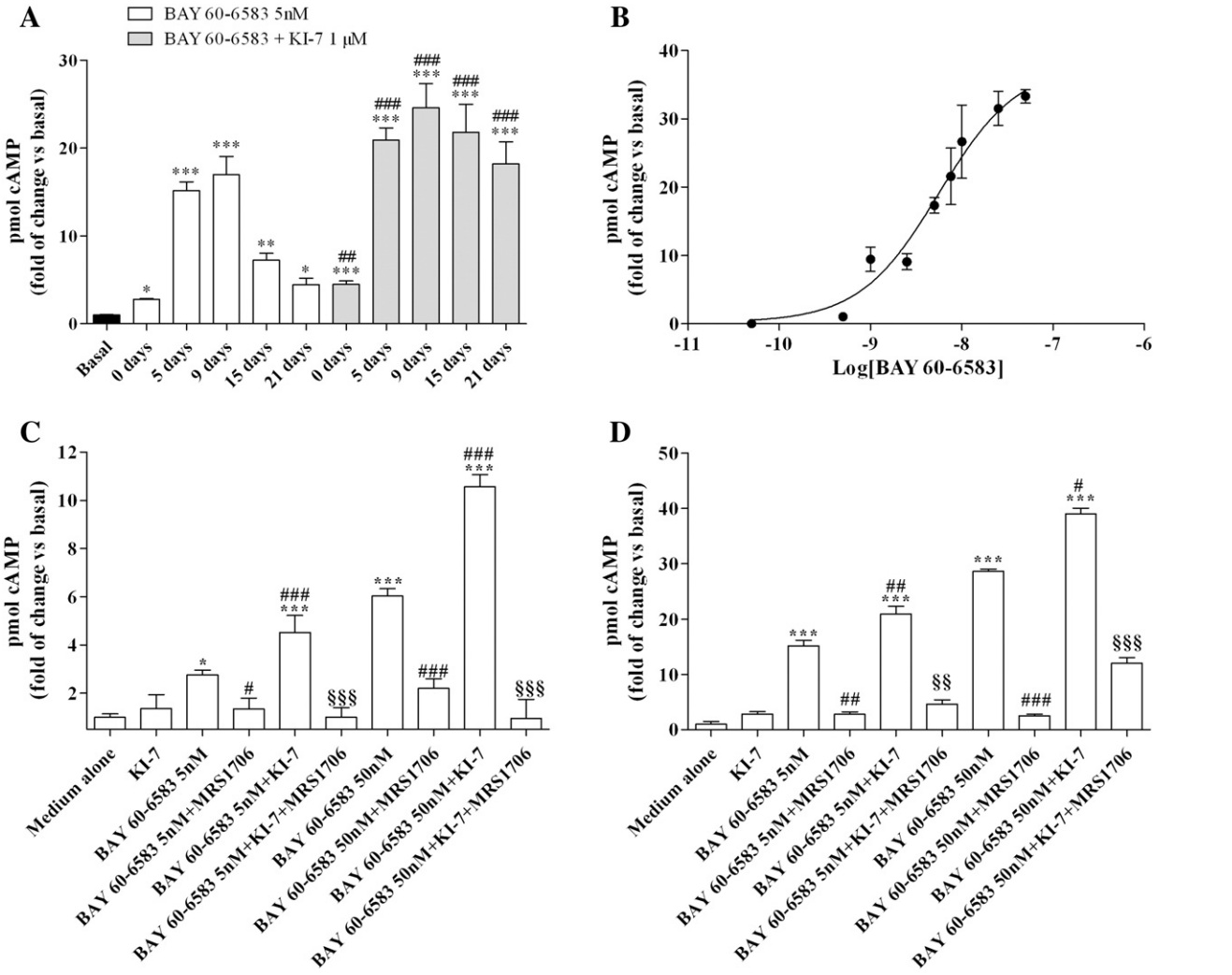

Fig. 1. Effect of BAY 60-6583 and KI-7 on cAMP accumulation in MSC during differentiation process. (A) Undifferentiated MSCs were cultured under osteogenic medium for different times (0–21 days). At each time, cells were treated for 15 min with 5 nM BAY 60-6583 in the absence or presence of 1 μM KI-7 and then, cAMP production was evaluated. (B) Dose-response curve of BAY 60-6583 in MSCs, cultured under osteogenic medium for 5 days. (C, D) undifferentiated (C) and 5 day differentiated cells (D) were treated for 15 min with 5–50 nM BAY 60-6583 or with 1 μM KI-7, alone or in combination. Aliquots of cells were treated with 15 nM A_{2B} AR antagonist MRS1706, alone or in combination with the agonists. After ligand treatments, intracellular cAMP levels were quantified. The data are expressed as fold of cAMP change versus basal value, which was set to 1, and represent the mean ± SEM of three different experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs respective basal, set to 1; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs BAY 60-6583; $^{\S\S}P < 0.01$, and $^{\S\S\S}P < 0.001$ vs BAY 60-6583 + KI-7.

3.3. Effect of A_{2B} AR orthosteric ligands and KI-7 on mineralization of MSC-derived osteoblasts

As A_{2B} AR plays a role in osteoblastogenesis, it was investigated whether the activation of these receptors by KI-7 and orthosteric agonists could stimulate matrix mineralization. MSCs, cultured under osteogenic medium, underwent a spontaneous, time-dependent, mineralization. Cell treatment with either NECA or BAY 60-6583 induced a significant increase, to the same extent and in all differentiation stages, in the mineralization process of MSCs. The presence in the incubation medium of ADA, which removes endogenous adenosine, did not significantly affect neither spontaneous nor A_{2B} AR agonist-induced MSC differentiation, suggesting endogenous adenosine "per se" is not enough to regulate the differentiation process. The A_{2B} AR agonist effects were most completely counteracted by cell pre-incubation with the A_{2B} AR antagonist MRS1706, suggesting that A_{2B} AR is the major subtype responsible for the induction of mineralization (Fig. 4A, B and C).

Since the A_{2B} AR mainly signals through cAMP/PKA pathway, the effects of adenylate cyclase activator, forskolin, and of the cAMP analogue, 8-Br-cAMP on the induction of mineralization were evaluated (Fig. 4A,B and C). The results demonstrate that both forskolin and 8-Br-cAMP were able to regulate MSC differentiation, with peculiar effects, dependent on both drug concentration and time of cell exposure. MSC short-time exposure to a low concentration of a cAMP activator induced a significant and marked increase in the mineralization process. These effects gradually decreased when a high compound concentration and/or a long time cell exposure conditions were used. By the comparison between the data obtained in mineralization studies, using cAMP modulators and A_{2B} AR ligands, we can speculate that A_{2B} AR-mediated effects on osteogenesis involve, at least partially, the cAMP pathway, but this mechanism may not be primary responsible for the terminal phase of osteoblast maturation.

Treatment of MSCs with KI-7 alone induced a concentration-dependent increase in osteoblast mineralization at all differentiation stages (Fig. 5A, B and C), and this effect appeared to be completely abrogated by cell pre-incubation with the A_{2B} AR antagonist MRS1706. Interestingly, the effect of KI-7 on the mineralization process was likewise almost completely abrogated when cells were treated with ADA, which removes endogenous adenosine (Fig. 5C). These results suggest that KI-7 potentiates adenosine activity in favouring osteoblast mineralization trough activation of A_{2B} AR.

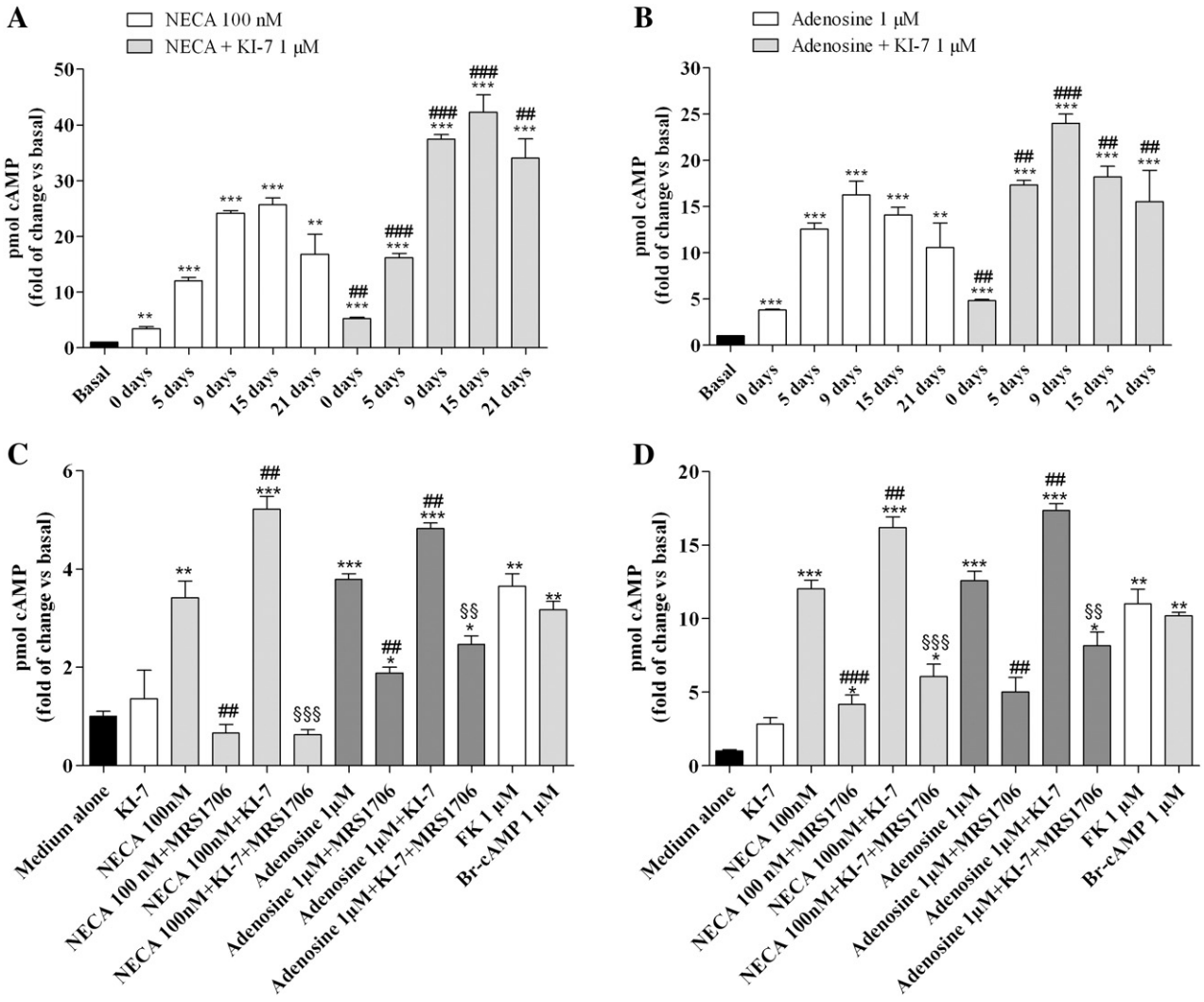

Fig. 2. Effect of KI-7 on NECA or adenosine-mediated cAMP accumulation in MSC during differentiation process. (A, B) Undifferentiated MSCs were cultured under osteogenic medium for different times (0–21 days). At each time, cells were treated with 100 nM NECA (A) or 1 μM Adenosine (B) alone or in combination with 1 μM KI-7 and then, cAMP levels were quantified. (C, D) undifferentiated (C) and 5 day differentiated cells (D) were treated for 15 min with 100 nM NECA or 1 μM Adenosine alone or in combination with 1 μM KI-7. Aliquots of cells were treated with 15 nM A_{2B} AR antagonist MRS1706, alone or in combination with the agonists. Aliquots of cells were treated for 15 min with 1 μM Forskolin (FK) or 1 μM 8-Br-cAMP. After ligand treatments, intracellular cAMP levels were quantified. The data are expressed as fold of cAMP change versus basal value, which was set to 1, and represent the mean ± SEM of three different experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs respective basal, set to 1; $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs BAY 60-6583; and $^{\S\S}P < 0.01$, and $^{\S\S\S}P < 0.001$ vs BAY 60-6583 + KI-7.

The ability of KI-7 to modulate the effects of A_{2B} AR orthosteric agonists on mineralization was then investigated. KI-7 potentiated osteoblast mineralization evoked by both NECA and BAY 60-6583 in a concentration-dependent manner at all differentiation stages (Fig. 6A, B and C), and these effects appeared to be completely counteracted by the A_{2B} AR antagonist MRS1706. These data confirm that KI-7, acting as a positive allosteric modulator of A_{2B} AR, favours cell mineralization resulting from the A_{2B} AR activation. These results were qualitatively confirmed by alizarin-Red staining of differentiated cells (Fig. 6D and E).

3.4. Effects of A_{2B} AR orthosteric ligands and of KI-7 on osteoblast survival

The effects of the A_{2B} AR orthosteric ligand NECA and of allosteric modulator KI-7 on survival of MSC cultures, differentiated in osteogenic medium for 15 or 21 days, were then evaluated. Cell treatment with NECA or KI-7 induced a significant increase in cell viability in both differentiation stages (Fig. 7A and B). The effects evoked by the two compounds were almost completely reversed by the A_{2B} AR antagonist MRS1706, demonstrating the specific involvement of the A_{2B} AR subtype. Moreover, KI-7 induced a significant increase in the pro-survival effects of the orthosteric agonist. These data suggest that orthosteric agonists and positive allosteric modulators of A_{2B} AR, in addition to favouring osteoblast differentiation, play a crucial role in regulating the viability of differentiated cells.

3.5. Effects of A_{2B} AR orthosteric ligands and of KI-7 on IL-6 release

It is know that activation of A_{2B} AR promotes IL-6 expression in different cell lines [30–32] and that this cytokine has a role, even if quite controversial, in the control of osteoblast differentiation [33, 34]. On this basis, we investigated the effects of A_{2B} AR orthosteric ligands and KI-7 on IL-6 production during all the stages of MSC differentiation to osteoblasts. In control cells, cultured under osteogenic medium, IL-6 release significantly decreased during the differentiation process (Fig. 8).

Treatment of the cells for 5 or 9 days with NECA or BAY 60-6583 or KI-7 favoured the spontaneous decrease in IL-6 production. This effect was strongly potentiated when NECA or BAY 60-6583 were used in combination with KI-7. These data suggest that agonist-mediated A_{2B} AR activation reduces IL-6 production, thus favouring differentiation of

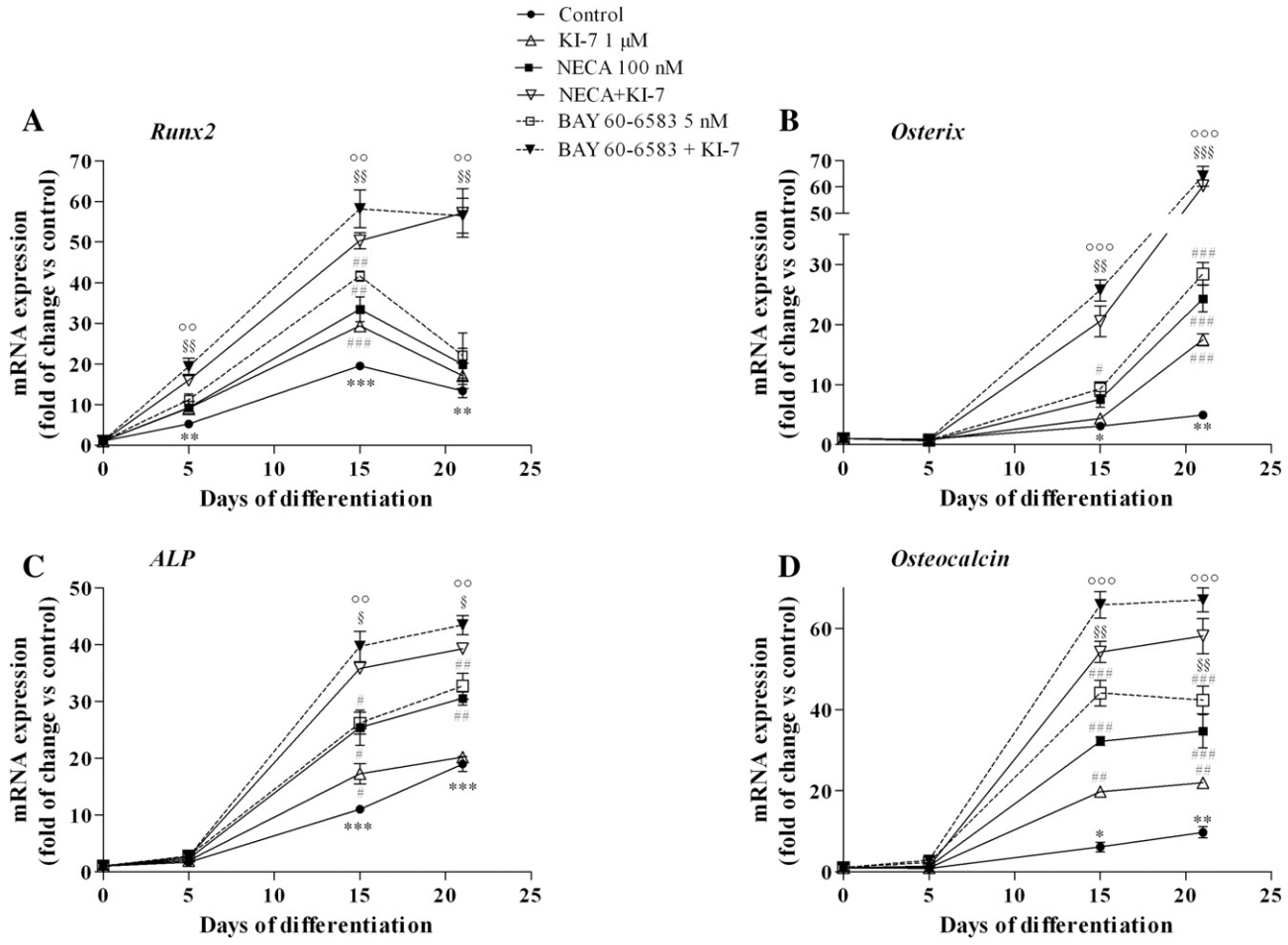

Fig. 3. Effect of NECA and KI-7 on osteogenic marker expression. MSCs were cultured in osteogenic medium for different time (0–21 days), in the absence (control) or in the presence of 100 nM NECA, 5 nM BAY 60-6583 or 1 μM KI-7, alone or in combination. At each time, mRNA expression levels of transcription factors Runx2 (A) and Osterix (B), and of osteogenic protein markers, ALP (C) and osteocalcin (D) were quantified by real time PCR. Data are expressed as fold of changes versus basal value (set to 1) and represent the mean ± SEM of three different experiments. *P < 0.05; **P < 0.01, and ***P < 0.001 vs basal; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs untreated cells; §P < 0.05, §§P < 0.01, and §§§P < 0.001 vs NECA alone; °°P < 0.01, and °°°P < 0.001 vs BAY 60-6583 alone.

MSCs, and that this effect is potentiated by positive allosteric modulation of A_{2B} AR.

Surprisingly, when the MSC differentiation process was prolonged to 15 or 21 days, a time corresponding to the late stage of differentiation, NECA, BAY 60-6583 and KI-7 induced a strong increase in IL-6 production. In addition, KI-7 was able to potentiate the effects of orthosteric agonists in both differentiation stages, even if the effect became significant only at 21 days.

Since almost comparable results were obtained using NECA or the selective A_{2B} AR agonist BAY 60-6583, we can conclude that the increase in

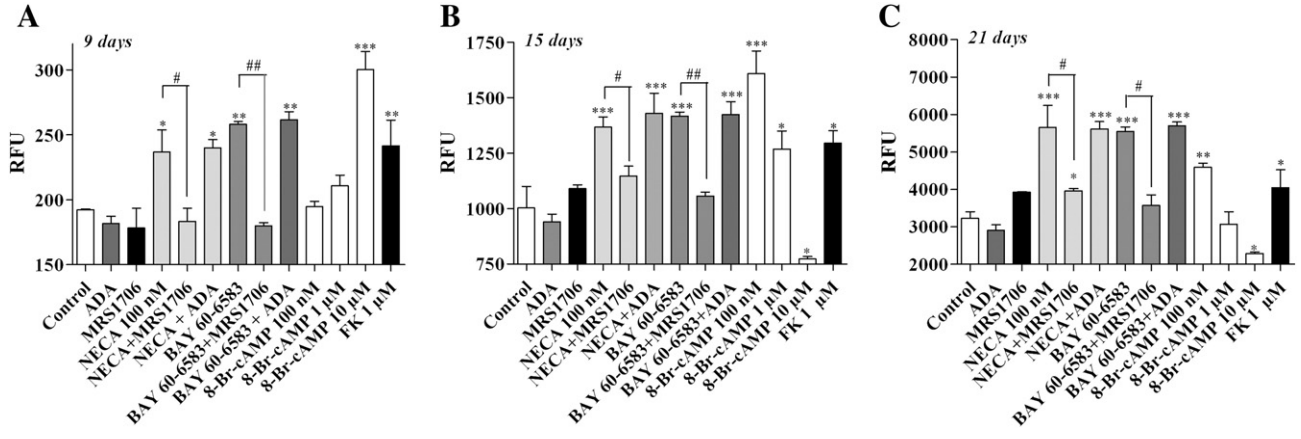

Fig. 4. Effect of A_{2B} AR orthosteric agonists on MSC mineralization. MSCs were cultured for 9 (A), 15 (B) or 21 (C) days with 100 nM NECA or 5 nM BAY 60-6583 or MRS1706 (15 nM) or ADA (0.5 U/mL), alone or in combination. Aliquots of cells were treated with Forskolin (1 μM) or 8-Br-cAMP (100 nM–10 μM). After treatments, cells were stained with fluorescent OsteoImage™ Staining Reagent and florescence was counted using a plate reader (excitation/emission wavelengths 485/535 nm). Data are expressed as relative fluorescence Unit and represent the mean ± SEM of three different experiments performed in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control; and #P < 0.05, and ##P < 0.001 vs agonist alone.

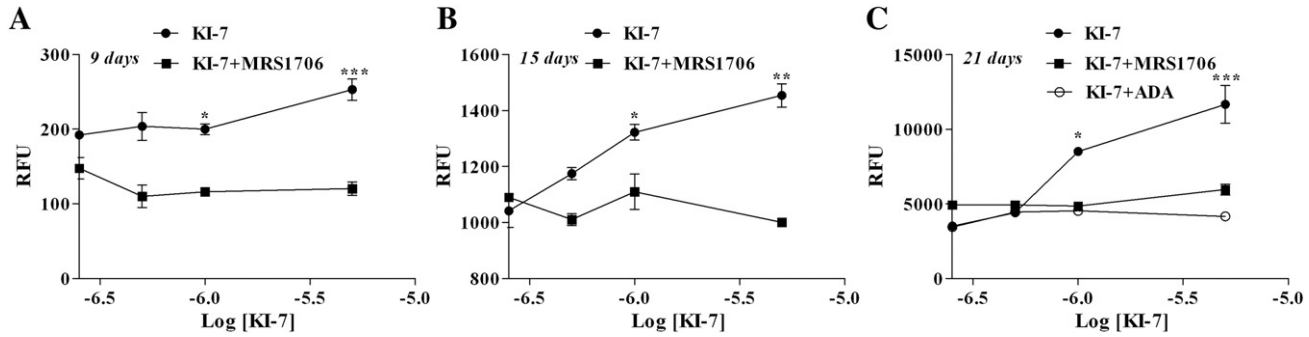

Fig. 5. Effect of KI-7 on MSC mineralization. MSCs were cultured for 9 (A), 15 (B) or 21 (C) days with KI-7 at different concentrations (500 nM–5 μM) in the absence or in the presence of the A_{2B} AR antagonist MRS1706 (15 nM) or ADA (0.5 U/mL). After treatments, cells were stained with fluorescent OsteoImage™ Staining Reagent and florescence was counted using a plate reader (excitation/emission wavelengths 485/535 nm). Data are expressed as relative fluorescence Unit and represent the mean ± SEM of three different experiments performed in triplicate. *P < 0.05, ** P < 0.01, and ***P < 0.001 vs control.

IL-6 release evoked by two ligands is ascribed to the selective recruitment of A_{2B} AR subtype. These data were also confirmed by the demonstration that NECA-mediated IL-6 release was almost completely counteracted by cell pre-incubation with the selective A_{2B} AR antagonist MRS1706.

4. Discussion

The identification of factors that regulate MSC differentiation into osteoblasts may be of great interest to understand both normal bone development and pathological conditions. A deeper knowledge of such phenomena may be also helpful in the search for therapeutic treatments of bone diseases, such as osteoporosis.

Adenosine and its receptors are known to play a fundamental role in bone homeostasis. Under basal conditions, adenosine concentration in cells and tissue fluids are low (30 to 300 nM). When cells become stressed (e.g., during hypoxic events, inflammation, tissue injury), adenosine accumulates at high concentrations (up to 30 μM) in the extracellular space [35–37] and signals through the activation of all AR

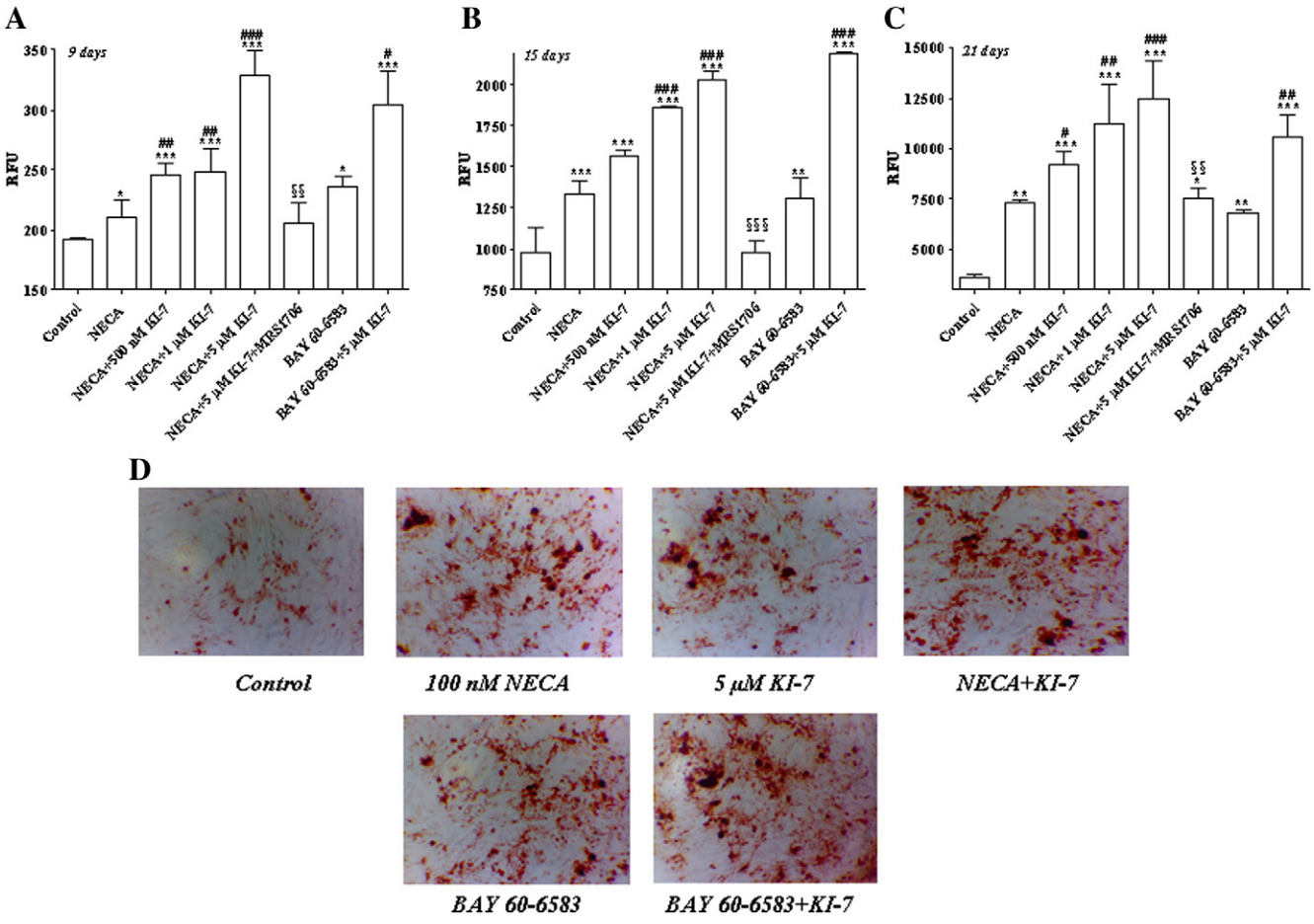

Fig. 6. Effect of KI-7 on osteoblast mineralization induced by orthosteric A_{2B} AR agonists. MSCs were cultured for 9 (A), 15 (B) or 21 (C) days with different KI-7 concentrations (500 nM–5 μM) in the presence of 100 nM NECA or 5 nM BAY 60-6583. In a set of cells, the A_{2B} AR antagonist MRS1706 (15 nM) was also included in the incubation medium. After treatments, cells were stained with fluorescent OsteoImage™ Staining Reagent and florescence was counted using a plate reader (excitation/emission wavelengths 485/535 nm). Data are expressed as relative fluorescence Unit and represent the mean ± SEM of three different experiments performed in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs agonist alone; and §§P < 0.01, and §§§P < 0.001 vs agonist + KI-7. D) Representative photograph (10× magnified images) of cultures stained with alizarin red S to visualize mineral nodules, after osteo-induction for 21 days with different ligands, alone and in combination.

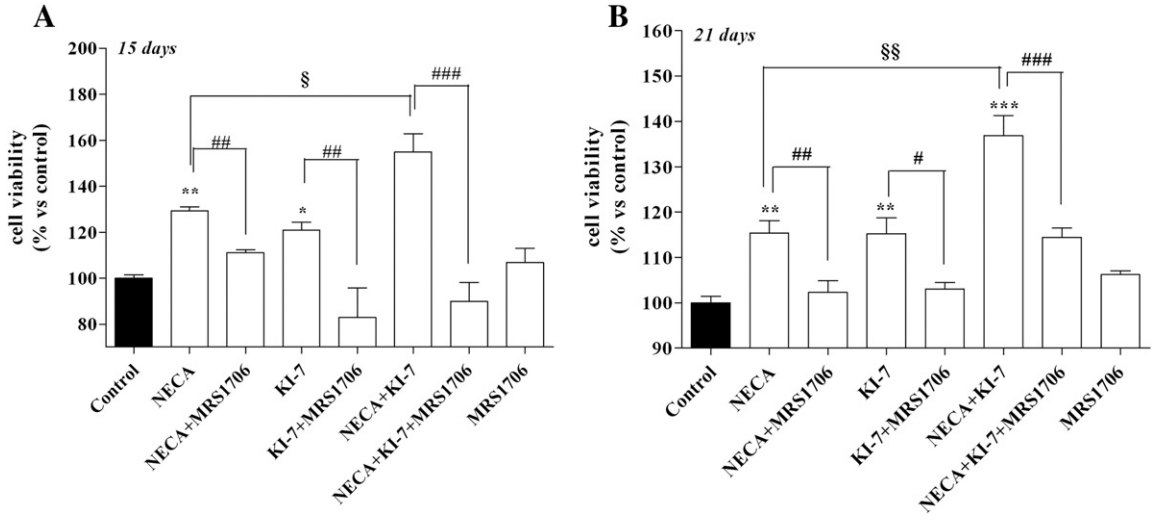

Fig. 7. Effect of KI-7 and NECA on differentiated osteoblast viability. MSCs, cultured under osteogenic medium, were treated for 15 (A) or 21 (B) days with 100 nM NECA or with 1 μM KI-7, alone or in combination. Aliquots of cells were treated with 15 nM A_{2B} AR antagonist MRS1706, alone or in combination with NECA or KI-7. After treatments, cell viability was detected by MTS assay. The data are expressed as percentage of cell viability versus control, set to 100%, and represent the mean ± SEM of three different experiments performed in duplicate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs agonist alone; and §P < 0.05, and §§P < 0.01 vs NECA.

subtypes, which are expressed in MSCs [38–41]. Among the different AR subtypes, the A_{2B} AR is a prime player in the commitment and differentiation of MSCs to osteoblasts: it enhances the three stages of initiation, maturation, and mineralization, and its activation or over-expression in undifferentiated cells is sufficient to promote the expression of osteoblast lineage markers [7–11].

The above findings suggest that targeting A_{2B} AR with selective compounds may represent an innovative therapeutic approach in bone diseases. In the perspective of a systemic use of an orthosteric agonist of A_{2B} AR, the wide distribution of ARs throughout the body would increase the risk of adverse effects. For this reason, in the last years the development of allosteric modulators of ARs has represented an area of active research [14,15]. Indeed, these compounds may represent a more "physiologic" alternative to orthosteric ligands thanks to their capability of modulating the interaction between the receptor and its endogenous ligands. Particularly, positive allosteric modulators of ARs (compounds potentiating the effects of endogenous adenosine) can lead to site-specific and event-specific responses mainly in damaged tissues, where adenosine is massively released. Such a pharmacological activity should be in principle characterized by less side effects compared with those potentially caused by orthosteric agonists.

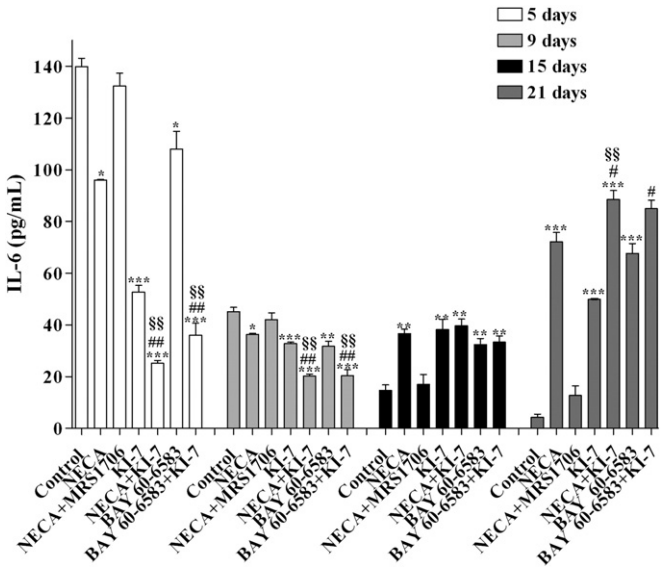

Fig. 8. Effect of A_{2B} AR activation on the release of IL-6 by MSC, during osteogenic differentiation. MSCs, cultured under osteogenic medium, were treated for different days with 100 nM NECA or 5 nM BAY 60-6583, alone or in combination with 1 μM KI-7. Aliquots of cells were pre-incubated with 15 nM MRS 1706 before NECA treatment. At each time, IL-6 protein levels in medium were quantified using an ELISA kit. Data are expressed as pg/mL and represent the mean ± SEM of two different experiments performed in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control; #P < 0.05, and ##P < 0.01 vs NECA or BAY 60-6583; and §§P < 0.01 vs KI-7.

Recently, we have developed and characterized a new class of 1-benzyl-3-ketoindoles as the first allosteric modulators of A_{2B} AR, provided with high efficacy and selectivity towards the other ARs [21,22]. Particularly, we have shown that these compounds are able to increase efficacy of A_{2B} AR orthosteric agonists in a transfected cell line, by facilitating A_{2B} AR–Gs protein coupling, and thus increasing the functional response of A_{2B} AR agonists on the cAMP signalling pathway. On the contrary, the same compounds were not able to affect A_1, A_{2A} and A_3 AR responses, suggesting they may represent useful tools to selectively target A_{2B} AR.

Herein, we demonstrate that one of these compounds, namely KI-7, acts as allosteric modulator of A_{2B} AR signalling in MSCs, and exerts a potent effect in favouring differentiation to osteoblast phenotype of MSCs in response to A_{2B} AR agonists.

By cAMP functional assays we demonstrate that KI-7, in a probe-independent manner, potentiated the stimulation of cAMP accumulation evoked by selective A_{2B} AR agonists, through a selective allosteric modulation of A_{2B} AR subtype, as described in transfected cells [22]. Furthermore, KI-7 induced a significant change in the time course of cAMP production in response to A_{2B} AR agonists: while the functional response of A_{2B} ARs appeared to gradually decrease in the terminal phase of MSC differentiation process, in the presence of KI-7 the second messenger's levels still remained high all the times. From these data we can speculate that KI-7, through an allosteric modulation of A_{2B} ARs, is able to increase A_{2B} AR coupling to effector system over all the differentiation time. The allosteric modulator may thus slow down the receptor switch-off, that is due to the physiological decrease in receptor protein expression in the late phase of the differentiation process [8].

Moreover, KI-7 showed a great facilitatory effect on the ability of A_{2B} AR orthosteric agonists in promoting MSC differentiation to osteoblasts by i) increasing the expression of osteoblast related genes and ii) accelerating/increasing osteoblast mineralization. Intriguingly, KI-7 was also able to increase cell mineralization in the

absence of a synthetic A_{2B} AR agonist by modulating and potentiating the effects of endogenous adenosine. It has been demonstrated that extracellular accumulation of adenosine in non-stressed cell cultures is not enough to activate any of its receptors and to regulate cell proliferation and osteogenic differentiation [13]. Accordingly, we demonstrated that cell treatment with ADA, to remove endogenous adenosine, did not alter differentiation programme. In the presence of KI-7, the pro-osteogenic effects of endogenous nucleoside became detectable, suggesting that this compound potentiates adenosine physiological responses, without the need of an exogenous agonist. Furthermore, the selectivity of KI-7 towards A_{2B} AR may ensure that signals evoked by adenosine through A_{2B} AR activation could get the upper hand on the effects mediated by the other AR subtypes, causing in turn a forced commitment of MSC towards osteoblast lineage.

Then, we investigated the mechanism by which A_{2B} ARs regulate MSC differentiation. The cAMP/PKA pathway has been demonstrated to play a crucial role in controlling osteogenic process [42,43], also in response to different Gs-coupled receptors including A_{2B} ARs and receptors for melatonin and parathyroid hormone (PTH) [11,44–47]. Actually, how Gs-mediated cAMP signalling regulates MSC development remains controversial. It has been demonstrated that cAMP stimulation in MSCs enhances the osteogenic response, while continuous activation of cAMP in differentiating osteoblasts negatively affects mineralization. These different effects could be attributed to the cell types which express Gs-coupled receptors to receive the signal. These data perfectly agree with our results demonstrating that i) cAMP activators induced a significant and marked increase in the mineralization process within the first differentiation days and ii) these effects gradually decreased during differentiation time. From these data we can speculate that cAMP signalling is crucial to prime lineage commitment of MSC towards osteoblast phenotype but is not involved in the terminal phase of osteoblast maturation, as previously suggested [8,11,47,48].

Bone turnover processes are regulated by several hormones, growth factors and cytokines [49–51]. Among cytokines, it is worth outlining the role of IL-6 in activating target genes involved in proliferation, differentiation, survival and apoptosis in a variety of cells [52]. Several findings indicate that IL-6, even if it has been reported to have contradictory effects on bone resorption and osteoblast function [34,53,54], is a key regulator in bone remodelling and is essential for bone homeostasis. The maintenance of low levels of this cytokine is required to allow MSC to complete differentiation process [33,55], while the sustained increase in its levels in the terminal phase ensures a pro-survival effect on differentiated osteoblasts [56]. On the other hand, several data demonstrate the cross-talk between purinergic receptors and cytokines in the regulation of cellular homeostasis. In particular, A_{2B} AR acts as an eternal clue to modulate the release of IL-6 in several cells [30–32,57] and cytokine themselves play a role in the control of AR responses [58–60]. Concerning bone homeostasis, A_{2B} AR has been identified as a key regulator of osteoblast differentiation and as local contributor to regulate bone formation, also through stimulation of IL-6 release [38].

Based on this evidence, suggesting a functional interplay between A_{2B} AR and IL-6 in the regulation of bone homeostasis, we investigated the effects of A_{2B} AR agonists on IL-6 release, and in parallel on the viability of MSCs during differentiation program. We demonstrate that IL-6 levels are down-regulated under spontaneous osteoblast differentiation of MSCs. The A_{2B} AR activation by orthosteric agonists and by the positive allosteric modulator KI-7, either each alone and much more in combination, potentiated the physiological decrease of IL-6 release during the differentiation program. Based on these data, we speculate that the reduction of IL-6 levels, induced by the activation of A_{2B} AR, may represent one of the mechanisms by which adenosine accelerates MSC differentiation.

Intriguingly, at the terminal phase of differentiation process, A_{2B} AR agonists caused a sustained increase in IL-6 release and this effect was raised by KI-7. In parallel, a significant increase in cell viability was observed. Since the effects elicited by A_{2B} AR agonists were completely abrogated by the selective A_{2B} AR antagonist MRS1706, we can most likely ascertain that they are specifically mediated by A_{2B} AR and do not involve the other AR subtypes.

Noticeably, the increase in IL-6 levels did not correlate with the production of intracellular cAMP. Based on these evidences, we can speculate that A_{2B} AR mediates IL-6 release through the activation of different intracellular signalling pathways, depending on the stage of cell differentiation (i.e., undifferentiated MSCs or differentiating osteoblasts). In this respect, in cardiac fibroblasts and in pituitary folliculostellate cells it has been demonstrated that the A_{2B} AR induce IL-6 release via PKC-p38 signalling pathway [61,62]. Studies are in progress to investigate the different signal pathways involved in A_{2B} AR-mediated control of IL-6 release, especially in the late phase of differentiation process when cAMP levels in response to A_{2B} AR agonists fall-down.

These results suggest that, depending on cell phenotype, A_{2B} ARs have distinct effects on IL-6 release, with different physiological implications: in the first stage of differentiation, it would reduce IL-6 levels and favour MSC commitment to osteoblasts; in terminal phases, it would promote IL-6 release and rescue differentiated cells from physiological death. IL-6 restoration induced by A_{2B} AR agonists in differentiated MSCs should have a great therapeutic impact, by enhancing long-term ability of MSC for tissue repair and regeneration. In this context, the development of positive allosteric modulators of A_{2B} AR, capable to differentiate MSC and ensure expression of soluble immunosuppressive factors, may represent a goal for regenerative medicine in bone disease.

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