Novel positive allosteric modulators of A2B adenosine receptor acting as bone mineralisation promoters

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ABSTRACT

Small-molecules acting as positive allosteric modulators (PAMs) of the A2B adenosine receptor (A2B AR) could potentially represent a novel therapeutic strategy for pathological conditions characterised by altered bone homeostasis, including osteoporosis. We investigated a library of compounds (4-13) exhibiting different degrees of chemical similarity with three indole derivatives (1-3), which have been recently identified by us as PAMs of the A2B AR able to promote mesenchymal stem cell differentiation and bone formation. Evaluation of mineralisation activity of 4-13 in the presence and in the absence of the agonist BAY60-6583 allowed the identification of lead compounds with therapeutic potential as anti-osteoporosis agents. Further biological characterisation of one of the most performing compounds, the benzofurane derivative 9, confirmed that such a molecule behaves as PAM of the A2B AR.

Introduction

Osteoporosis is a widespread systemic and chronic disorder characterised by decreased bone mass and micro-architectural deterioration, which predisposes to low-energy fractures. It affects the health of many adults, with an incidence that increases with age and varies among genders, being the postmenopausal women at particularly high risk.

Several approaches have been proposed for the management of osteoporosis, in terms of prevention and/or treatment, all finalised to the achievement of normal peak bone mass, including an adequate dietary regimen, calcium and vitamin D supplementation. Among the pharmacological agents, bisphosphonates, calcitonin, oestrogens, inhibitors of receptor activator of nuclear factor κB ligand, parathyroid hormone and parathyroid hormone-related peptide should be mentioned, all possessing one or more drawbacks in terms of efficacy, safety, bioavailability, drug–drug interactions and costs.

The increasing knowledge about the numerous signalling pathways involved in bone and osteocytes, as well as osteoblasts and osteoclasts turnover, has encouraged research projects aimed to investigate innovative therapeutic options for the treatment of osteoporosis.

In this line, evidence emerging supports the role of extracellular adenosine in bone homeostasis. Adenosine is ubiquitous and regulates several physiological and pathological conditions through the activation of specific G-protein coupled receptors, namely the A1, A2A, A2B and A3 adenosine receptors (ARs). The binding of adenosine to its receptors causes the activation of different intracellular signalling pathways such as those involving cAMP, calcium or potassium channels, phospholipase C, phospholipase D and the mitogen-activated protein kinase. The modulation of these pathways significantly affects the mesenchymal stem/stromal cell (MSC) biology by controlling their fates.

MSCs are multipotent cells able to differentiate into osteoblasts or adipocytes. MSCs express various AR subtypes based on the differentiation lineage and stages. Human bone marrow-derived MSCs (BM-MSCs) are the main sources for newly osteoblasts in bone. The extracellular adenosine acts as an autocrine/paracrine hormone by promoting the differentiation of MSCs to mature osteoblasts which results from activation of the A2B AR. The role of A2B AR in osteoblast differentiation has been confirmed by experiments demonstrating that the deletion of this receptor in a mouse model produces a defective bone formation. In addition, the A2B AR negatively controls the MSCs differentiation to adipocytes.

In 2013 and 2014, we disclosed three indole derivatives (1, 2 and 3, Table 1) acting as positive allosteric modulators (PAMs) of the A2B AR. None of these compounds bind with appreciable affinity to the orthosteric site of each of the ARs. Moreover, they increase efficacy of adenosine or synthetic agonists without affecting their potency. To our knowledge, compounds 1, 2 and 3 are still the only PAMs of A2B AR reported in literature.

Subsequent studies on 2, chosen as the most promising of the three, according to in vitro experiments, revealed that this compound promotes MSC differentiation and bone formation by inducing the differentiation of BM-MSCs to osteoblasts.

These data suggest that the use of small molecules able to produce a positive A2B AR allosteric modulation could potentially represent a novel therapeutic strategy not only for osteoporosis.
Table 1. Structures of the reference indoles 1-3\textsuperscript{20,21} and novel compounds 4–13.
but also for all the pathological conditions characterised by an excessive bone destruction and/or a decrease in bone formation (e.g. fracture mal-union, osteogenesis imperfecta, rheumatoid arthritis and multiple myeloma), as well as in pathology characterised by a high bone fragility (e.g. congenital insensitivity to pain, CIP)²³. These results prompted a deep investigation to identify new lead compounds, structurally related to 1, 2 and 3, endowed with the ability to allosterically enhance A2B AR agonists and to stimulate matrix mineralisation when used alone or in the presence of the orthosteric agonist BAY60-6583. In this respect, the present study was aimed at gaining information about the pharmacophoric requirements of this novel class of mineralisation agents and hopefully identifying new compounds with improved activity as compared to 2. Thus, we selected ten compounds, whose structures are reported in Table 1, exhibiting different degrees of chemical similarity with the reference indole derivatives 1, 2 and 3.

The library includes novel indole-based derivatives featuring modified substitution patterns at 1- and/or 3-positions, as well as compounds in which the indole scaffold was replaced by different aromatic heterocycles. Specifically, compounds 4-7 feature side chains at 3-position of the indole nucleus corresponding to those of 2 (N-phenylglyoxylylamide) or 1 (N-benzylglyoxylylamide), whereas compounds 8 and 10 feature the phenylglyoxylyl side chain present in 3. In compounds 4-7 and 12-13 the indole nucleus was maintained and was decorated with a phenylethyl (4, 5 and 12) or a methyl (6, 7 and 13) moiety at 1-position in place of the benzyl group of 1-3; instead, in derivatives 8-11 the indole was replaced by different heterocycles such as benzothiophene (8), benzofurane (9), thiophene (10) and N-methylpyrrole (11). With the exception of the commercially available 11, the above compounds were easily prepared by means of experimental protocols modified according to reported procedures or set up ex novo in order to obtain the desired products with high yield and purity degree.

The present paper describes the evaluation of compounds 4-13 as novel bone mineralisation agents and the resulting structure-activity relationships.

### Materials and methods

#### Chemistry

Uncorrected melting points were determined using a Reichert Kofler hot-stage apparatus. NMR spectra were obtained on a Bruker AVANCE 400. The coupling constants (J) are given in Hertz. Magnesium sulphate was used as the drying agent. Evaporations were made in vacuo (rotating evaporator). Analytical TLC have been carried out on Merck 0.2 mm precoated silica gel aluminium sheets (60F-254). Silica gel 60 (230–400 mesh) was used for column chromatography. Purity of the target inhibitors 4-13 was determined, using a Shimadzu LC-20AD SP liquid chromatograph equipped with a DDA Detector (λ = 254 nm) using a column C18 (250 mm × 4.6 mm, 5 µm, Shim-pack); the mobile phase, delivered at isocratic flow, consisted of acetonitrile (60-70%) and water (40-30%); flow rate 1 ml/min. All the compounds showed percent purity ≥ 95%. Reagents, starting materials and solvents were purchased from commercial suppliers and used as received. The N-phenyl-indol-3-ylglyoxylylamide 16, N-benzyl-indol-3-ylglyoxylylamide 17 were prepared according to a reported procedure²⁴. Compound 11 is commercially available (Ambinter); its structure was confirmed by ¹H-NMR and ¹³C-NMR.

#### N-Benzyl-2-{(1-methyl-1H-pyrrrol-2-yl)-glyoxylyamide (11)}

¹H-NMR (DMSO-d₆ ppm): 3.92 (s, 3H); 4.39 (s, 2H); 6.19-6.21 (m, 1H); 7.26-7.36 (m, 7H); 9.22 (bs, 1H). ¹³C-NMR (DMSO-d₆ ppm): 37.60; 42.41; 109.54; 124.75; 127.39; 127.68; 128.80; 134.77; 139.34; 164.74; 178.74.

#### General procedure for the synthesis of N-(indol-3-yl)glyoxylylamides 4-7 and 3-(phenylglyoxylyl)indoles 12-13

Sodium hydride (60% dispersion in mineral oil, 44 mg, 1.10 mmol) was portionwise added to an ice-cooled solution of 16,17 or 26 (1.10 mmol) in 5 ml of anhydrous DMF. The resulting mixture was stirred at room temperature for 1 h. Then, the appropriate halide ((2-bromoethyl)benzene for 4, 5 and 12 or methyl iodide for 6, 7 and 13, 1.10 mmol) was added and stirring was continued overnight. The reaction mixture was diluted with water and ice and the formed precipitate was collected by filtration and purified by recrystallization from ethanol (for 4-7) or by flash chromatography (for 12-13, petroleum ether 60–80°C/ethyl acetate in ratio 8:2 as eluting system).

#### N-Phenyl-2-{(1-phenylethyl-1H-indol-3-yl)glyoxylyamide (4)}

Yield: 61%; mp 139–140°C; ¹H-NMR (DMSO-d₆ ppm): 3.14 (t, 2H, J = 7.4 Hz); 4.59 (t, 2H, J = 7.4 Hz); 17.9-7.21 (m, 2H); 7.26-7.32 (m, 4H); 7.34-7.41 (m, 4H); 7.71-7.74 (m, 1H); 7.87 (d, 2H, J = 8.0 Hz); 8.31-8.33 (m, 1H); 8.75 (s, 1H); 10.67 (s, 1H). ¹³C-NMR (DMSO-d₆ ppm): 36.04; 48.28; 111.43; 111.84; 120.69; 121.95; 123.53; 124.11; 124.71; 127.00; 127.20; 128.84; 129.20; 129.33; 136.74; 138.40; 138.53; 141.59; 162.74; 182.05.

#### N-Benzyl-2-{(1-phenylethyl-1H-indol-3-yl)glyoxylyamide (5)}

Yield: 67%; mp 83–85°C; ¹H-NMR (DMSO-d₆ ppm): 3.12 (t, 2H, J = 7.4 Hz); 4.42 (d, 2H, J = 6.4 Hz); 4.56 (t, 2H, J = 7.4 Hz); 7.18-7.37 (m, 12H); 7.69-7.71 (m, 1H); 8.26-8.28 (m, 1H); 8.75 (s, 1H); 9.29 (t, 1H, J = 6.4 Hz). ¹³C-NMR (DMSO-d₆ ppm): 36.01; 42.53; 48.23; 111.64; 111.72; 122.01; 123.37; 123.97; 126.99; 127.26; 127.35; 127.82; 128.78; 128.83; 129.31; 136.60; 138.41; 139.48; 141.54; 163.92; 182.06.

#### N-Phenyl-2-{(1-phenylethyl-1H-indol-3-yl)glyoxylyamide (6)}

Yield: 82%; mp 167–169°C, lit ref²³ mp 175–177°C.

#### N-Benzyl-2-{(1-phenylethyl-1H-indol-3-yl)glyoxylyamide (7)}

Yield: 71%; mp 147–149°C, lit ref²³ mp 147–148°C.

#### 1-{(1-Phenylethyl-1H-indol-3-yl)-2-phenyl-1,2-ethanedione (12)}

Yield: 71%; mp 101–103°C; ¹H-NMR (DMSO-d₆ ppm): 3.08 (t, 2H, J = 7.2 Hz); 4.52 (t, 2H, J = 7.2 Hz); 7.10-7.22 (m, 5H); 7.34-7.40 (m, 2H); 7.60–7.64 (m, 2H); 7.74–7.88 (m, 2H); 7.93 (d, 2H, J = 7.2 Hz); 8.07 (s, 1H); 8.24 (d, 1H, J = 6.8 Hz). ¹³C-NMR (DMSO-d₆ ppm): 35.82; 48.27; 111.94; 111.99; 121.88; 123.65; 124.38; 125.94; 126.94; 128.75; 129.25; 129.63; 130.15; 133.31; 135.19; 137.26; 138.30; 140.85; 188.58; 194.36.
1-(1-Methyl-1H-indol-3-yl)-2-phenyl-1,2-ethanediene (13)
Yield: 72%; mp 88–90 °C; lit ref\textsuperscript{26} mp 91–92 °C.

**General procedure for the synthesis of 2-(benzothen-3-yl)glyoxylamide 8**
An excess of thiophenyl chloride (0.28 ml, 3.86 mmol) was added at 0 °C to a suspension of the acids \textbf{18-20} (1.93 mmol) in dry toluene (2 ml). The mixture was refluxed for 3 h and then the excess of thiophenyl chloride was distilled off under reduced pressure and the residue was washed three times with dry toluene. The oily residue obtained was dissolved in 10 ml of anhydrous THF, cooled at 0 °C and added with diethylamine (0.29 ml, 2.12 mmol). Then, a solution of the appropriate amine (benzylamine for 8 and 10) and thionyl chloride was added to the cooled mixture. The reaction mixture was kept at room temperature for 24–48 h (TLC analysis; petroleum ether 60°C/ethyl acetate in ratio 8:2 as eluting system). Yield: 65%; mp 93–94 °C (petroleum ether 60–80 °C).

**N-Benzyl-2-(benzothen-3-yl)glyoxylamide (8)**
Yield: 75%; mp 87–89 °C (petroleum ether 60–80 °C); \textsuperscript{1}H-NMR (DMSO-\textit{d}_{6}, ppm): 4.49 (d, 2H, J = 6.0 Hz); 7.27–7.30 (m, 1H); 7.33–7.37 (m, 4H); 7.51 (t, 1H, J = 7.2 Hz); 7.57 (t, 1H, J = 7.2 Hz); 8.15 (d, 1H, J = 8.0 Hz); 8.63 (d, 1H, J = 8.0 Hz); 9.34 (s, 1H); 9.52 (bs, 1H). \textsuperscript{13}C-NMR (DMSO-\textit{d}_{6}, ppm): 42.65; 123.59; 124.76; 126.19; 126.63; 127.49; 127.89; 128.86; 130.21; 136.93; 139.06; 139.63; 146.24; 164.14; 183.92.

**N-(4-Chlorobenzyl)-2-(benzofuran-2-yl)glyoxylamide (9)**
Yield: 64%; mp 137–139 °C (ethyl acetate); \textsuperscript{1}H-NMR (DMSO-\textit{d}_{6}, ppm): 4.45 (d, 2H, J = 6.4 Hz); 7.36-7.42 (m, 5H); 7.59-7.63 (m, 1H); 7.76 (d, 1H, J = 8.4 Hz); 7.93 (d, 1H, J = 7.6 Hz); 8.35 (s, 1H); 9.61 (t, 1H, J = 6.4 Hz). \textsuperscript{13}C-NMR (DMSO-\textit{d}_{6}, ppm): 42.05; 112.75; 121.32; 124.81; 125.07; 127.25; 128.78; 129.79; 130.21; 132.09; 137.96; 149.64; 155.98; 161.83; 177.48.

**N-Benzyl-2-(thien-2-yl)glyoxylamide (10)**
Yield: 65%; mp 95.6–95.8 °C, lit ref\textsuperscript{27} mp 93–94 °C (petroleum ether 60–80 °C).

**Adenosine receptor binding assay**
\textsuperscript{[3H]}DPCPX and \textsuperscript{[3H]}NECA were obtained from DuPont-NEN (Boston, MA). ADA (Cat. N. 10102105001) was from Sigma Aldrich (Italy). All other reagents were from commercial sources and of the highest commercially available grade. CHO cells stably expressing human A1, A2A, A3 and A2B ARs were kindly supplied by Prof. K. N. Klotz, Wurzburg University, Germany. MSCs (Cat. N. SCC034) were purchased by Sigma Aldrich (Milan, Italy).

**Human A1 adenosine receptors**
Competition binding experiments were performed as previously reported\textsuperscript{29}. Briefly, membranes of A1 CHO cells were incubated at 25 °C for 180 min in 500 μL of buffer containing \textsuperscript{[3H]}DPCPX (3 nM) and different concentrations of the compounds. R-PIA (50 μM) was used for the determination of non-specific binding. The dissociation constant (Kd) of \textsuperscript{[3H]}DPCPX in A1 CHO cell membranes was 3 nM.

**Human A2A adenosine receptors**
Competition binding experiments were performed as previously reported\textsuperscript{29}. Briefly, membranes of A2A CHO cells were incubated at 25 °C for 90 min in 500 μL of buffer in the presence of \textsuperscript{[3H]}NECA (30 nM) and six different concentrations of the newly synthesised compounds. NECA (100 μM) was used for the determination of non-specific binding. The dissociation constant (Kd) of \textsuperscript{[3H]}NECA in A2A CHO cell membranes was 30 nM.

**Human A3 adenosine receptors**
Competition binding experiments were performed as previously reported\textsuperscript{29}. Briefly, cell membranes of A3 CHO cells were incubated at 25 °C for 180 min in 500 μL of buffer in the presence of \textsuperscript{[3H]}NECA (30 nM) and six different concentrations of the newly synthesised compounds. NECA (100 μM) was used for the determination of non-specific binding.

**Measurement of cyclic AMP levels in A2B AR CHO**
Intracellular cyclic AMP (cAMP) levels were measured using a competitive protein binding method, as previously reported\textsuperscript{26}. A2B AR CHO cells were seeded at a density of 24 000 cell/cm\textsupersqrm in a 24 multi-well plate. After 48 h, the medium was removed and the cells were incubated at 37 °C for 15 min with 500 μL of Dulbecco’s Modified Eagle Medium (DMEM) in the presence of ADA (1 U/mL) and the phosphodiesterase inhibitor Ro20-1724 (20 μM).

The agonist efficacy profile of the compounds at ARs was evaluated by assessing their ability to modulate intracellular cAMP levels in the absence of standard agonists. The antagonist profile of the new compounds was evaluated by assessing their ability to inhibit 100 nM NECA-mediated accumulation of cAMP. The new compounds were incubated 10 min before the addiction of the agonists. Then, cells were incubated in the reaction medium for 15 min at 37 °C. The reaction was stopped by the removal of the medium and the addition of 0.4 N HCl. After 30 min, lysates were neutralised with 4 N KOH and the suspension was centrifuged at 800 g for 5 min. For the determination of cAMP production, bovine adrenal cAMP-binding protein was incubated with \textsuperscript{[3H]}cAMP (2 nM) at 0 °C for 150 min in a total volume of 300 μL. Bound radioactivity was separated by rapid filtration. The radioactivity was measured by liquid scintillation spectrometry.
Cell cultures

Human bone marrow derived MSCs were cultured in normal growth medium (MSCGM, Sigma Aldrich), plated (5,000 cells/cm²) in 75-cm² flasks and incubated at 37°C in 5% CO₂ and 95% air. The medium was changed to remove non adherent cells every 3 to 4 days and the cells were used at passages 2 to 6.

Quantification of mineralisation

MSCs were seeded at a density of 3300 cells/cm² and maintained in growth medium for 2 days. Then, medium was replaced with Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cat. N. C-28013, Sigma Aldrich) and treated in the absence (control) or in the presence of BAY60-6583 (5 nM) alone or in combination with the new compounds. Treatments were repeated every 3 days and the mineralisation was quantified after 15 days of treatment.

Statistical analysis

GraphPad Prism (version 6.00) was used for data analysis and graphic presentation. Data are reported as the means ± standard errors of the means (SEM). Differences were considered statistically significant for p values of <0.05.

Results and discussion

Chemistry

The synthesis of compounds 4-7 was carried out following our previously procedure reported for 6 and 7, with slight modifications (Scheme 1). Acylation of indole 14 with oxalyl chloride, in anhydrous diethyl ether, at room temperature, yielded the indol-3-ylglyoxyl chloride 15, which was directly allowed to react overnight with the appropriate amine (aniline or benzylamine), in the presence of triethylamine in dry toluene solution at room temperature, to obtain the amides 16, 17 (Scheme 1)24. Treatment of 16 and 17 with sodium hydride and subsequent addition of the appropriate halide (2-bromoethyl)benzene (for 4 and 5) or methyl iodide (for 6 and 7) in dry DMF yielded the target derivatives 4-7, finally purified by recrystallization from ethanol.

Target compounds 8-10 were prepared according to the experimental procedure outlined in Scheme 2. The acid chlorides 21-23 were prepared by treating the commercially available glyoxyllic acids 18-20 with an excess of thionyl chloride in refluxing toluene and used in the following reaction without further purification. The condensation of 21-23 with the appropriate amine (benzylamine for 8 and 10, 4-chlorobenzylamine for 9) in dry THF solution, in the presence of triethylamine, yielded the target derivatives 8-10, finally purified by recrystallization from the appropriate solvent.

Scheme 3 reports the synthesis of compounds 12 and 13. Phenylglyoxylic acid 24 was reacted with thionyl chloride in dry dichloromethane, in the presence of DMAP, for 3 h at room temperature to provide the 2-oxo-2-phenylacetyl chloride 25 that was

Reagents and conditions: i) anhydrous Et₂O, oxalyl chloride, room temperature, 5 h; ii) aniline (for 4 and 6) or benzylamine (for 5 and 7), NEt₃, anhydrous toluene, room temperature, overnight; iii) NaH, (2-bromoethyl)benzene (for 4 and 5) or methyl iodide (for 6 and 7), anhydrous DMF, room temperature, overnight.

Scheme 1. Synthesis of target compounds 4–7. Scheme 2. Synthesis of target compounds 8–10. Scheme 3. Synthesis of target compounds 12–13.
then treated with 14 at 50°C for 2 h, using DMAP as a base, to yield the desired α-diketo derivative 26. Treatment of 26 with sodium hydride and subsequent addition of the appropriate halide ((2-bromoethyl)benzene for 12 or methyl iodide for 13) in dry DMF yielded the target derivatives 12 and 13, finally purified by flash chromatography.

Reagents and conditions: i) SOCl₂, DMAP, anhydrous DCM, room temperature, 3 h; ii) benzyamine (for 8 and 10) or 4-chlorobenzyamine (for 9), NEt₃, anhydrous THF, room temperature, 24–48 h.

Effects of 4-13 on MSCs differentiation to osteoblasts

The A₂B AR plays a pivotal role in MSCs differentiation to osteoblasts leading to the increase of mineralisation process. Herein, we investigated whether a series of newly synthetised compounds (4-13), potentially able to allosterically modulate the A₂B AR, could stimulate matrix mineralisation when used in the presence of the orthosteric agonist BAY60-6583 or alone (Figure 1).

To evaluate the mineralisation activity of compounds 4-13, they were first assayed in the presence of BAY60-6583 at 5 nM concentration (Figure 1(A)). Such a concentration was selected based on the reported potency of BAY60-6583 on MSCs (EC₅₀ = 6.0 nM)⁰⁶. Under these experimental conditions it can be safely assumed that the orthosteric site is mainly occupied by BAY60-6583 rather than adenosine, owing to the much higher potency and concentration of the synthetic agonist compared with the natural transmitter present in the culture medium at physiological concentrations³⁳. The mineralisation activity was expressed as percentage with respect to 100% assigned to the control.
Among these, only derivatives 4 and 9 significantly ameliorated BAY60-6583 activity, while for compounds 5, 8 and 10 the increase is not statistically significant. It is worth noting that 4 is more active than the reference compound 2.

The activity of compound 9 suggests that the indole scaffold together with a N-arylyl substituent (benzyl or 2-phenylethyl) at 1-position are probably not key pharmacophore elements to display positive allosteric modulation of the A2B AR. Of course, such a conclusion should be supported by further experiments demonstrating the precise nature of the events occurring at the molecular levels. The activity measured in the mineralisation assay reflects, in fact, not only pharmacodynamic factors but also pharmacokinetic phenomena, such as stability to metabolism, which cannot be taken into account separately.

The compounds able to modify the mineralisation induced by BAY60-6583 in the above described assay (4, 5, 8, 9 and 10) were subsequently evaluated for their mineralisation activity in absence of BAY60-6583 in order to assess their therapeutic potential (Figure 1(B)). It has been demonstrated that the presence of endogenous adenosine in the medium affects MSCs differentiation; thus, under these experimental conditions, the tested compounds alone could potentiate the adenosine activity mediated by the A2B AR activation, acting as PAMs. In this assay, those compounds displaying a mineralisation activity higher than that of the control (set to 100%) were considered active. Three compounds revealed to be more active than the control, specifically 4 significantly enhanced the mineralisation with respect to the control (130.0 ± 6.8%, p < 0.01), while compounds 5 (128.7 ± 3.6%) and 9 (111.5 ± 4.6%) displayed a mineralisation activity higher compared with the control although not significant. These products might be considered potential anti-osteoporosis agents as they are able to increase the bone mineralisation in experimental conditions close to the physio-pathological ones. The inactivity of 8 and 10 might be ascribed to their low affinity for the allosteric site of the A2B AR. However, as mentioned before, we cannot rule out that factors others than the pharmacodynamic ones can interfere with the ability of 8 and 10 to act as A2B AR allosteric enhancers of adenosine.

The obtained results prompted us to focalise our attention on the most promising compounds able to enhance osteoblast differentiation. However, unlike 4 and 5 that are structurally analogues of our already reported PAMs, 9 features a peculiar chemical structure that could make it a potential lead compound for the development of novel PAMs. In this respect, a more accurate characterisation of 9 was carried out.

When assayed in radioligand binding assays, 9 displayed no appreciable binding affinity for A1, A2A and A3 ARs (Ki > 10 000 nM). Compound 9 was then evaluated for its ability to modulate the increase in cAMP levels, either alone or in the presence of an EC50 concentration of the agonist NECA in human A2B AR transfected CHO cells (Table 2). When tested alone at a 10 µM concentration, 9 did not significantly increase cAMP levels, demonstrating a lack of A2B AR agonist activity. Interestingly, 9 potentiated the effects of NECA, to a higher extent with respect to the...
parent compound 2, suggesting that this compound may behave as PAM of the A2B AR.

Then, the potency of 9 in modulating the activity of A2B AR agonists was determined by assessing the effects of different concentrations (ranging from 1 nM to 10 μM) on cAMP accumulation induced by an EC50 concentration of the agonist NECA (100 nM).

The resulting concentration–response curves (Figure 2(A)) indicated that 9 exhibited a positive allosteric modulation of A2B AR with an EC50 636.2 ± 65.3 nM. Subsequently, the effects of 9 on the potency and efficacy of NECA were evaluated by assessing agonist concentration–response curves in the absence or presence of two different concentrations of 9 (650 nM and 6.5 μM). Compound 9 significantly enhanced the efficacy of NECA in stimulating cAMP accumulation without affecting agonist potency (Figure 2(B)).

Conclusions

A small library of molecules (4-13) exhibiting different degrees of chemical similarity with the recently identified indole-based positive allosteric modulators (PAMs) of the A2B AR 1-3 was investigated for their potential as bone mineralisation agents. Compounds 4 and 9 turned out to stimulate matrix mineralisation in MSCs either in the presence or in the absence of the agonist BAY60-6583. The structure-activity relationships of 4-13 suggest that the indole scaffold and a 1-arylalkyl substituent (benzyl or 2-phenylethyl) at 1-position are probably not key pharmacophore elements to display positive allosteric modulation of the A2B AR. The biological properties of the benzofurane derivative 9, featuring a peculiar chemical structure with respect to the reference indole 1-3, were more accurately characterised. The obtained results confirmed that such a molecule behaves as PAM of the A2B AR, making it a lead structure for the development of novel compounds potentially acting as anti-osteoporosis agents.

Author contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

1. Lane JM, Russell L, Khan SN. Osteoporosis. Clin Orthop Relat Res 2000;372:139–50.
2. Lamichhane AP. Osteoporosis - an update. J Nepal Med Assoc 2005;44:60–6.
3. Roush K. Prevention and treatment of osteoporosis in postmenopausal women: a review. Am J Nurs 2011;111:26–35.
4. Francis RM. Non-response to osteoporosis treatment. J Br Menopause Soc 2004;10:76–80.
5. Salarí SP, Abdollahi M, Larijani B. Current, new and future treatments of osteoporosis. Rheumatol Int 2011;31:289–300.
6. Strazzulla LC, Cronstein BN. Regulation of bone and cartilage by adenosine signaling. Purinergic Signal 2016;12:583–93.
7. Cheng X, Cheng G, Xing X, et al. Controlled release of adenosine from core-shell nanofibers to promote bone regeneration through STAT3 signaling pathway. J Control Release 2020;319:234–45.

8. Shih YR, Liu M, Kwon SK, et al. Dysregulation of ectonucleotide- mediated extracellular adenosine during postmenopausal bone loss. Sci Adv 2019;5:eaaax1387

9. Fredholm BB, Uzerman AP, Jacobson KA, et al. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine receptors—an update. Pharmacol Rev 2011;63:1–34.

10. Langemeijer EV, Verzijl D, Dekker SJ, Uzerman AP. Functional selectivity of adenosine A1 receptor ligands? Purinergic Signal 2013;9:91–100.

11. Giacomelli C, Daniele S, Romei C, et al. The A2B adenosine receptor modulates the epithelial-mesenchymal transition through the balance of CAMP/PKA and MAPK/ERK pathway activation in human epithelial lung cells. Front Pharmacol 2018; 9:54.

12. Zezula J, Freimuth M. The A 2A-adenosine receptor: A GPCR with unique features? Br J Pharmacol 2008;153: S184–S190.

13. Gharibi B, Abraham AA, Ham J, Evans BAJ. Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes. J Bone Miner Res 2011;26:2112–24.

14. Costa MA, Barbosa A, Neto E, et al. On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. J Cell Physiol 2011;226:1353–66.

15. Shih YR, Hwang Y, Phadke A, et al. Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. Proc Natl Acad Sci U S A 2014;111:990–5.

16. Trincavelli ML, Daniele S, Giacomelli C, et al. Osteoblast differentiation and survival: a role for A2B adenosine receptor allosteric modulators. Biochim Biophys Acta - Mol Cell Res 2014;1843:2957–66.

17. He W, Mazumder A, Wilder T, Cronstein BN. Adenosine regulates bone metabolism via A1, A2A, and A2B receptors in bone marrow cells from normal humans and patients with multiple myeloma. Faseb J 2013;27:3446–54.

18. Carroll SH, Wigner NA, Kulkarni N, et al. A2B Adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation in vivo. J Biol Chem 2012; 287:15718–27.

19. Eisenstein A, Carroll SH, Johnston-Cox H, et al. An adenosine receptor-Krüppel-like factor 4 protein axis inhibits adipogenesis. J Biol Chem 2014;289:21071–81.

20. Taliani S, Trincavelli ML, Cosimelli B, et al. Modulation of A2B adenosine receptor by 1-benzyl-3-ketoindole derivatives. Eur J Med Chem 2013;69:331–7.

21. Trincavelli ML, Giacomelli C, Daniele S, et al. Allosteric modulators of human A2B adenosine receptor. Biochim Biophys Acta - Gen Subj 2014;1840:1194–203.

22. Taliani SD, Settimo F, Martini C, et al. Exploiting the indole scaffold to design compounds binding to different pharmacological targets. Molecules 2020;25:2331.

23. Zhang Y, Haga N. Skeletal complications in congenital insensitivity to pain with anhidrosis: a case series of 14 patients and review of articles published in Japanese. J Orthop Sci 2014;19:827–31.

24. Da Settimo A, Primofiore G, Da Settimo F, et al. Structure-activity relationships, and molecular modeling studies of N-(indol-3-ylglyoxylyl)benzylamine derivatives acting at the benzodiazepine receptor. J Med Chem 1996;39:5083–91.

25. Thompson MJ, Borsenberger V, Louth JC, et al. Design, synthesis, and structure – activity relationship of indole-3-glyoxylamide libraries possessing highly potent activity in a cell line model of prion disease. J Med Chem 2009;52:7503–11.

26. Katritzky AR, Zhang D, Kirichenko K. Synthesis of heteroaryl 1,2-diketones. J Org Chem 2005;70:3271–4.

27. Zhang YK, Wang B. Synthesis of z-ketoamides from β-ketoamides and primary amines: a catalyst-free oxidative decyanation-amidation reaction. Eur J Org Chem 2019;2019:5732–5.

28. Ivinin SP, Lapandin AV. Conversions of unsymmetrical benzoins in basic media. ARKIVOC 2004; 2005:4–9.

29. Graziano A, Giovannoni MP, Cilibrizzi A, et al. C. New pyrazolo[1,5-a]pyrimidin-4(3H)-ones fluoroderivatives as human A1 adenosine receptor ligands. Acta Chimica Slovenica 2012;59:648–55.

30. Cosimelli B, Greco G, Laneri S, et al. 4-Amino-6-alkoxy-2-alkylthiopyrimidine derivatives as novel non-nucleoside agonists for the adenosine A1 receptor. Chem Biol Drug Des 2016;88:724–9.

31. Nordstedt C, Fredholm BB. A modification of a protein-binding method for rapid quantification of CAMP in cell-culture supernatants and body fluid. Anal Biochem 1990;189:231–4.

32. Giacomelli C, Natale L, Nisi M, et al. Negative effects of a benzodiazepine receptor. J Med Chem 1996;39:5083–91.