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Human stem cell osteoblastogenesis mediated by novel glycogen synthase kinase 3 inhibitors induces bone formation and a unique bone turnover biomarker profile in rats☆,☆☆

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ABSTRACT

Wnt activation by inhibiting glycogen synthase kinase 3 (GSK-3) causes bone anabolism in rodents making GSK-3 a potential therapeutic target for osteoporotic and osteolytic metastatic bone disease. To understand the wnt pathway related to human disease translation, the ability of 3 potent inhibitors of GSK-3 (AZD2858, AR79, AZ13282107) to 1) drive osteoblast differentiation and mineralisation using human adipose-derived stem cells (hADSC) *in vitro*; and 2) stimulate rat bone formation *in vivo* was investigated. Bone anabolism/resorption was determined using clinically relevant serum biomarkers as indicators of bone turnover and bone formation assessed in femurs by histopathology and pQCT/μCT imaging.

GSK-3 inhibitors caused β-catenin stabilisation in human and rat mesenchymal stem cells, stimulated hADSC commitment towards osteoblasts and osteogenic mineralisation *in vitro*. AZD2858 produced time-dependent changes in serum bone turnover biomarkers and increased bone mass over 28 days exposure in rats. After 7 days, AZD2858, AR79 or AZ13282107 exposure increased the bone formation biomarker P1NP, and reduced the resorption biomarker TRAcP-5b, indicating increased bone anabolism and reduced resorption in rats. This biomarker profile was differentiated from anabolic agent PTH_{1-34} or the anti-resorptive Alendronate-induced changes. Increased bone formation in cortical and cancellous bone as assessed by femur histopathology supported biomarker changes. 14 day AR79 treatment increased bone mineral density and trabecular thickness, and decreased trabecular number and connectivity assessed by pQCT/μCT.

GSK-3 inhibition caused hADSC osteoblastogenesis and mineralisation *in vitro*. Increased femur bone mass associated with changes in bone turnover biomarkers confirmed *in vivo* bone formation and indicated uncoupling of bone formation and resorption.

Introduction

The process of bone remodelling involves tightly coupled bone resorption and formation activities and dysregulation of bone turnover can lead to a number of bone disorders such as osteopetrosis and osteoporosis. In malignant diseases, bone destruction through increased bone resorption produces osteolytic lesions and is a characteristic feature of multiple myeloma and breast cancer metastatic disease (Theriault, 2012). Current therapies aimed for osteoporotic and osteolytic bone diseases are primarily aimed at reducing bone resorption. However, stimulating anabolic activity is an alternative and complementary therapeutic strategy. Therefore, understanding of the biochemical pathways governing osteoblast differentiation and function has aided the search for anabolic therapies and recent research has highlighted the importance of Wnt signalling in bone formation. Canonical Wnt signalling is critical for osteoblast maturation and function and activation of Wnt signalling through inhibition of constitutively active GSK-3 results in induction of β-catenin and Runx2 transcriptional activities (Hartmann, 2006; Kugimiya et al., 2007; Monroe et al., 2012; Reinhold and Naski, 2007). *In vitro*, GSK-3 inhibition leads to osteoblast differentiation in a C3H10T1/2 murine mesenchymal stem cell (MSC)

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population (Kulkarni et al., 2006). Accordingly *in vivo*, haploinsufficient GSK-3β mice display increased bone density in comparison to wildtype mice (Kugimiya et al., 2007) and small molecule inhibitors of GSK-3α/β have demonstrated increased bone mass in normal and ovariectomized rodent models (Clement-Lacroix et al., 2005; Gambardella et al., 2011; Kulkarni et al., 2006; Marsell et al., 2012).

In order to determine the role of GSK-3 in human osteoblastogenesis, three structurally diverse, potent GSK-3 inhibitors were tested in hADSC assays *in vitro*. We have assessed the ability of these GSK-3 inhibitors to stimulate canonical Wnt/β-catenin signalling through β-catenin stabilisation and osteogenic mineralisation. In addition, we have studied the commitment of hADSC towards osteoblastogenesis through the assessment of protein transcriptional co-activator with PDZ-binding motif (TAZ), and the osteoblast-specific marker Osterix. TAZ modulates the activity of the critical osteoblast transcription factor runt-related transcription factor 2 (RUNX2) and PPARγ during MSC lineage commitment (Hong et al., 2005) and has recently been reported as a downstream component of the Wnt/β-catenin signalling cascade (Azzolin et al., 2012). Osterix has been reported to be essential for osteoblast differentiation and bone formation as exemplified by the complete lack of osteoblast differentiation and maturation in osterix-null mice (Nakashima et al., 2002). In order to demonstate translation of these *in vitro* findings to bone anabolism in an intact system, each GSK-3 inhibitor was dosed daily to rats, and bone formation *in vivo* was investigated by quantifying changes in serum biomarkers of bone turnover and femur bone changes. One challenge for pre-clinical assessment of potential bone anabolic agents is the ability to measure changes over a short period of time in *in vivo* models as many agents that modulate bone turnover have been tested in lengthy disease models such as the ovariectomized rat model. Therefore, serum bone turnover markers, which are used in the clinical setting, were used in these studies to evaluate treatment effectiveness before mature bone formation could be observed in healthy rats. Initially, a time course of changes in serum bone turnover biomarkers was established following daily dosing of AZD2858 which has known anabolic activity in rats (Marsell et al., 2012). Then, the effect of three different GSK-3 inhibitors (AZD2858, AR79 and AZ13282107), PTH_{1-34} and Alendronate on serum biomarkers in short term rat studies (7 days) were assessed by quantifying N-terminal propeptide of type I procollagen (P1NP), a marker of bone anabolism (Hale et al., 2007) and osteoblast activity and Tartrate-resistant acid phosphatase (TRAcP-5b) a marker of bone metabolism (Halleen et al., 2006). In addition, confirmation of bone formation was assessed using histology and imaging (pQCT, μCT) of femurs.

Materials and methods

Small molecule compounds AR79, AZD2858 and AZ13282107 were synthesised at AstraZeneca R&D, Loughborough UK (structures in Fig. 1).

GSK-3 and kinase selectivity assays. The potency of compounds at GSK-3β and cyclin-dependent protein kinase 2 (CDK2, kinase with closest homology to GSK-3β) was assessed using Z-LYTE™ Kinase assay kit (Invitrogen, UK) in the presence of 7 and 80 μM ATP respectively. A ratiometric method was used to calculate the ratio of donor emission (445 nm) to acceptor emission (520 nm) after excitation of the donor fluorophore at 400 nm to quantitate the reaction progress. Kinase selectivity with AR79, AZD2858 and AZ13282107 were determined using the KinaseProfiler Service (Millipore, Watford, UK) or University of Dundee Kinase (MRC Protein Phosphorylation Unit, UK). Over 80 different kinases were assessed at a single concentration of 1 or 10 μM of AR79, AZD2858 and AZ13282107. Concentration-inhibition 10-point curves to compounds that showed activity were constructed to determine pIC_{50} estimations. Also, in some kinase assays these pIC_{50} estimations were converted to binding affinity values (pK_i) using the Cheng–Prussoff equation to correct for the concentration of ATP used.

β-catenin stabilisation in mesenchymal stem cells. Human adipose-derived stem cells (hADSC; Invitrogen, UK) and rat MSCs (isolated from bone marrow of Sprague Dawley rats at ≤8 weeks after gestation; Invitrogen, UK) were cultured in a basal media of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, UK) containing 5% (v/v) foetal bovine serum (FBS, Gibco, UK) and 2 mM GlutaMax (Invitrogen, UK). Cells were seeded in basal media into 96-well plates (3–5000 cells/well) for 18 h before treatment with AZD2858, AR79 or AZ13282107 (0.3 nM to 20 mM). After 24 h, β-catenin stabilisation was measured as previously described (Gambardella et al., 2011).

Osteoblast-specific marker expression. hADSC were seeded at a density of 5000 cells/well into black 96-well Viewplates (Perkin-Elmer Inc., UK) in basal media (as detailed above). All cells were incubated at 37 °C in 5% CO_2/95% air humidified atmosphere. After overnight incubation, basal media was replaced and compounds, at concentrations of 6 nM to 20 μM, or 0.2% DMSO vehicle were added. After 18 h, cells were fixed in 4% paraformaldehyde (PFA) for 20 min followed by washing in PBS. The cells were blocked in PBS supplemented with 1.1% BSA (Sigma-Aldrich) and 0.2% Triton X-100 for 30 min at room temperature. Anti-osterix (1:50 dilution, ab22552, Abcam, UK) and TAZ antibody (1:500 dilution, #2149, Cell Signalling Technology) were added overnight at 4 °C in blocking buffer, followed by addition of Alexa Fluor AF647 donkey anti-rabbit IgG (Invitrogen, UK) and 1 μM Hoechst 33342 in blocking buffer for 1 h at room temperature. Cells were washed and stored in PBS at 4 °C prior to image acquisition and analysis, using an in-built object intensity algorithm, on an IN Cell Analyzer 3000 platform (Amersham Biosciences, UK).

Osteogenic differentiation studies — Alizarin Red staining and automated image analysis. hADSC were seeded at a density of 5000 cells/cm^2 and were cultured in basal media (as above). After overnight incubation, basal media was replaced and compounds were added. In osteogenic positive control wells, basal media was replaced with an osteogenic differentiation media: phenol red-free DMEM supplemented with 5% FBS, 2 mM GlutaMax, 50 μg/ml L-ascorbic acid (Sigma-Aldrich, UK), 5 mM β-glycerophosphate (Calbiochem, UK), and 10 nM dexamethasone (Sigma-Aldrich, UK). Cells were incubated at 37 °C in 5% CO_2 with media and compounds being replaced every 3–4 days over a 25–28 day time-course. Osteogenic mineralisation was visualised using Alizarin Red staining. Cells were washed with PBS followed by

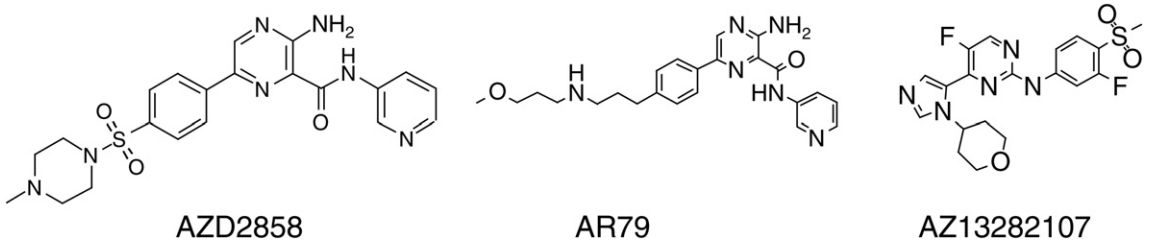

Fig. 1. Small molecule GSK-3 inhibitors from 3 chemical series.