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A SUGAR-BASED PHASE-TRANSITIONING DELIVERY SYSTEM FOR BONE TISSUE-ENGINEERING

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Abstract

Bone tissue engineering approaches commonly involve the delivery of recombinant human bone morphogenetic proteins (rhBMPs). However, there are limitations associated with the currently used carriers, including the need for surgical implantation and the associated increase in infection risk. As an alternative to traditional porous collagen sponge, we have adopted a solution of the injectable sucrose acetate isobutyrate (SAIB) as a carrier for rhBMP-2. The ability to deliver rhBMP-2 and other agents by injection reduces the infection risk and lesion size whilst in surgery, with the potential to avoid open surgery altogether in some indications.

The primary methodology used for this *in vivo* study was a C57BL6/J mouse ectopic bone formation model. Specimens were examined by x-ray, microCT, and histology at 3 weeks. SAIB was delivered non-invasively and produced up to 3-fold greater bone volume compared to collagen. To further refine and improve upon the formulation, SAIB containing rhBMP-2 was admixed with candidate compounds including ceramic microparticles, anti-resorptives, and cell signalling inhibitors and further tested *in vivo*. The formulation combining SAIB/rhBMP-2, the bisphosphonate zoledronic acid (ZA), and hydroxyapatite (HA) microparticles yielded a 10-fold greater bone volume than SAIB/rhBMP-2 alone. To investigate the mechanism underlying the synergy between ZA and HA, we used *in vitro* binding assays and *in vivo* fluorescent biodistribution studies to demonstrate that ceramic particles could bind and sequester the bisphosphonate. These data show the utility of SAIB as a non-invasive rhBMP delivery system as well as describing an optimised formulation for bone tissue engineering.

Keywords: Sucrose acetate isobutyrate; injectable scaffold; bisphosphonate; hydroxyapatite; bone tissue engineering.

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Introduction

Current tissue engineering techniques largely rely on surgical implantation of scaffolds. However, the creation of an open wound brings an inherent increase in infection risk. This has been shown by numerous clinical studies. For example, in a prospective randomised trial of closed reduction *versus* open reduction of tibial fractures showed 1 superficial infection in 34 closed fractures (3 %) and 6 superficial infections and 1 deep infection in 30 open fractures (24 %) (Im and Tae, 2005). In another study, the risk of septic knee after retrograde intramedullary femoral nailing was four times greater in open fractures than closed fractures (Halvorson *et al.*, 2012). In a trial comparing recombinant human bone morphogenetic protein-2 (rhBMP-2)/collagen sponge treatment with the standard of care treatment in open tibial fractures, the rhBMP-2 treated group showed a significant increase in infections (Aro *et al.*, 2011). Thus the development of a system able to deliver rhBMPs percutaneously to an orthopaedic site could reduce lesion size and minimise infection risk.

Although various polymeric materials, such as degradable hydrogels, have been developed by many groups to serve as injectable scaffolds (Miyamoto and Takaoka, 1993; Han and Hubbell, 1996; Burdick and Anseth, 2002; Lutolf *et al.*, 2003), progression towards clinical application has been limited. However, we have chosen a material called sucrose acetate isobutyrate (SAIB), a sugar-based ester that is currently used as a food additive (emulsifier E444), as well as in cosmetics and industrial processes. In the United States, SAIB is approved by the Food and Drug Administration as a food additive, and has an allowable daily intake of 20 mg/kg/day (21 CFR 172.833; 64 Fed. Reg. 29949, 4 June 1999). It has been considered safe for use in beverages in at least 28 countries worldwide, including Australia. It has been judged to have a low risk by the World Health Organisation (WHO, 1997). SAIB is manufactured by the esterification of sucrose with acetic and isobutyric anhydrides. SAIB is highly viscous and demonstrates properties of a semi-solid material (Lin *et al.*, 2012). However, the addition of solvents (such as 10-20 % ethanol) leads to a significant decrease in viscosity. When the SAIB/solvent mixture is injected into the muscle, the solvent disperses rapidly, and the SAIB phase transitions to a highly viscous (semi-solid) depot (Lu *et al.*, 2007). The extensive, and safe, use of SAIB in the past few decades in many forms highlights its longstanding excellent biocompatibility.

SAIB has been proposed as a carrier system for systemic drug delivery (anti-psychotics) and as local drug delivery (local anaesthetic) under the brand name SABER™. The phase transitioning properties of SAIB allow it to be locally injected where it forms a depot capable of long term drug release. SAIB is currently being trialled for sustained delivery of Risperidone to schizophrenia patients (clinicaltrials.gov NCT01592110) (Lu *et al.*, 2007). Trials for the treatment of pain (particularly associated with hernia) with bupivacaine *via* SAIB have been recently completed (clinicaltrials.gov NCT01052012, NCT00974350) with no safety issues reported (Hadj *et al.*, 2012). The intramuscular delivery of SAIB has seen to be well tolerated in animal models. The co-delivery of SAIB, ethanol and recombinant human growth hormone, was shown to result in localised inflammation after 7 days in rats, and was considered well tolerated (Okumu *et al.*, 2002). Administration of SAIB, ethanol, and poly(L-lactic acid) showed again a mild localised inflammatory response that had cleared by 28 days in a rat (Lu *et al.*, 2007).

SAIB has been previously suggested as a coating system for the delivery of osteoinductive proteins. In a 2005 study, collagen-chondroitin sulphate discs were coated in a mixture of SAIB and poly(L-lactic acid) and loaded with rhBMP-2 (Keskin *et al.*, 2005). However, rhBMP-2 was not directly added to the SAIB and the capacity for injection and subsequent phase transitioning was not employed. The investigator's main purpose in using SAIB was to slow rhBMP-2 release from the collagen-chondroitin sulphate discs, rather than to develop an injectable delivery system.

Under normal homeostatic conditions, bone exists in a state of dynamic equilibrium of anabolism and catabolism (Little *et al.*, 2007). The addition of rhBMP-2 drives a strong anabolic response that induces bone production, but it is also accompanied by a catabolic increase in osteoclast numbers (Giannoudis *et al.*, 2007b). These processes are a part of a normal coupled response to bone injury, and ensure remodelling occurs following bone formation to restore mechanical strength. However, for a majority of bone tissue engineering applications, the new bone is largely unloaded and this lack of mechanical stimulation can lead to premature or excessive resorption (Giannoudis *et al.*, 2007a). There is an increasingly established paradigm that fracture repair outcomes represent a sum of both bone forming and bone resorbing responses (Little *et al.*, 2007). This paradigm is now beginning to be applied to bone tissue engineering strategies with drugs such as bisphosphonates being shown to act synergistically with rhBMPs (Little *et al.*, 2005). Bisphosphonates bind to bone and prevent resorption, although binding affinities can vary between bisphosphonates. *In vitro* assays have shown zoledronic acid (ZA) has a strong affinity for bone mineral (Lawson *et al.*, 2010). The development of strategies that take into account bone resorption and take advantage of the anti-resorptive properties of bisphosphonates as well as their capacity to bind with high affinity to hydroxyapatite (HA) are a natural evolution for bone tissue engineering carriers.

There are many other adjunctive agents alongside bisphosphonates with the potential to affect bone formation. A range of agents was tested in this study, including drugs that modulate cell fate decisions (BADGE, SB216763, PD0325901, and SP600125) and bone anti-resorptives (ZA, HA, PS1145, and AFG495). SB216763 is a Wnt agonist (Adachi *et al.*, 2011), PD0325901 is a MEK inhibitor (Zhang *et al.*, 2012), and SP600125 is a JNK inhibitor (Doan *et al.*, 2012), with all three pathways noted to affect osteogenic differentiation. PS1145 is an IKK inhibitor (Carr *et al.*, 2012), and AFG-496 is an inhibitor for Cathepsin K (rodent).

In this study, we describe the testing of SAIB as a delivery system for rhBMP-2 and as a bone tissue engineering construct. Initially, rhBMP-2 delivered using SAIB was tested in terms of dose response. Subsequently, rhBMP-2/SAIB was compared to the current clinical standard of rhBMP-2/collagen sponge in terms of bone forming capacity. The effects of a range of adjunctive agents were then screened for synergistic effects when delivered alongside rhBMP-2 in SAIB. Based on these studies, we focused on the combination of rhBMP-2/SAIB with anti-catabolic agents to prevent resorption of unloaded bone as well as the synergy seen with the addition of ceramic (hydroxyapatite) microparticles.

Materials and Methods

Chemicals and pharmaceuticals

Food grade (FG) SAIB was purchased from SAFC, a branch of Sigma-Aldrich (St. Louis, MO, USA) and used for the majority of experiments to ensure batch consistency. rhBMP-2 and porous collagen sponge were purchased as a part of the INFUSE™ bone graft kit from Medtronic (Minneapolis, MN, USA). ZA was purchased from AXXORA, LLC (San Diego, CA, USA). Radiolabelled carbon-14 ZA (^{14}C-ZA) was a gift from Dr Jürg Gasser (Novartis AC, Basel, Switzerland). Pre-calcined HA microparticles were from Plasma Biotal Ltd. (Product P149) (www.plasma-biotal.com). Bisphenol A diglycidyl ether (BADGE), SB216763, SP600125 and PS1145 were purchased from Sigma-Aldrich. PD0325901 was purchased from Selleck (Houston, TX, USA). AFG495 was a gift from Novartis (Basel, Switzerland). Pamidronate (Cipla, Bombay, India) was fluorescently labelled with the commercially available AlexaFluor 555 (Life Technologies Australia, Mulgrave, VIC, Australia) according to the protocol provided by Life Technologies. The resultant fluorescently labelled bisphosphonate was termed AlexaPam.

For the study that screened a variety of potential adjunctive agents, the following doses were used. BADGE and SB216763 were used at 15 μg per pellet, PD0325901 at 10 μg per pellet, and SP600125 at 20 μg per pellet. ZA was dosed at 1 μg per pellet, HA was 2 % w/v, and the co-delivery (HA + ZA) group had these doses combined. The anti-catabolics PS1145 was used a 10 μg per pellet and AFG495 at 25 μg per pellet. Doses were selected based on extrapolations from prior *in vitro* experiments (data not shown), and were limited by the loading capacity of the injectable material.

Laboratory synthesis of SAIB

A magnetic stirrer, 60 mM acetic anhydride and 240 mM isobutyric anhydride were added to a dry round-bottom flask fitted to a reflux condenser, and this was stirred at 500 rpm for the duration of the synthesis. 10 g of sucrose and 2.1 g of sodium acetate, were added to the flask, and heated to 120 °C for 30 min. The temperature was then dropped to 90 °C, where excess ethanol was added (approximately 150 mL) for 20 min. The flask was then allowed to cool, and the contents poured into a 1 L Schott bottle filled with ice cold water. The bottle was shaken and allowed to chill in the refrigerator overnight, whereby the product precipitated and fell to the bottom. The water was decanted and the contents transferred to a 50 mL Falcon tube. Water was added, the suspension shaken vigorously, and then centrifuged at 6000 rpm for 3 min. Water was decanted again the wash process repeated 4 to 5 times until the product was sufficiently viscous. The final product was allowed to dry at 50 °C in a vacuum oven.

Animal purchase, housing and ethics

8-week-old female C57BL6 mice were purchased from the Animal Resources Centre (Perth, Australia) kept in an on-site specific pathogen free (SPF) animal facility. Mouse chow and water were provided *ad libitum*. Animals were allowed to acclimatise for a week prior to intervention. Ethics was approved by the CHW/CMRI Animal Ethics Committee (K294).

Intramuscular injection of SAIB

An SAIB:Ethanol (85:15) stock solution was prepared and allowed to mix overnight. Prior to surgery, rhBMP-2 and any other agents were mixed into the stock solution of SAIB. Animals receiving an injection were pre-dosed with buprenorphine at least 30 min prior to surgery, where they were anaesthetised using Isoflurane gas. Using a 0.5 mL 30-gauge insulin syringe, 20 µL of the SAIB mixture was injected into the quadriceps of the mouse.

Mice were monitored over the three week experiment, and euthanised using a CO_2 chamber. Sites of interest were harvested and x-rayed. Specimens were preserved with 4 % paraformaldehyde for 4 h at room temperature, and at 4 °C overnight, and then changed to 70 % ethanol.

All *in vivo* experiments, bar the final fluorescent ZA biodistribution study, were conducted using groups of five mice injected bilaterally (n = 10/group). The fluorescent ZA distribution study was conducted in groups of five mice that were injected unilaterally (n = 5/group). In this study, animals receiving systemic bisphosphonate were dosed at 0.1 mg/kg (equivalent to about 2 µg per mouse) 1 week post-surgery and sacrificed as normal (3 weeks post-surgery).

Surgical implantation of collagen

Collagen pellets were prepared from collagen sponges (1 mm height) provided in the rhBMP-2 INFUSE™ kits using a 3 mm diameter biopsy punch in a sterile environment. 10 µL of a 0.5 mg/mL rhBMP-2 solution was applied slowly to the sponge in a drop-wise fashion, and allowed to sit for 20 min prior to implantation. Animals (n = 5) were anaesthetised by 35 mg/kg Ketamine and 0.5 mg/kg Xylazine. The operative site was shaved and wiped with povidine-iodine solution. An incision was made in the quadriceps of the mouse, in which the collagen pellet was placed. The incision was sutured closed, the animals were allowed to recover on a heat pad, and given post-operative analgesics and saline.

Radiography

Bone nodule placement was monitored and checked by digital X-ray (Faxitron X-ray Corp, Tucson, AZ, USA) relative to the whole limb (25 kV, 2x magnification). Samples were then scanned by micro-computed tomography (microCT) using a SkyScan 1174 compact microCT scanner (Skyscan, Kontich, Belgium). All samples were scanned in 70 % ethanol, using a 0.5 mm aluminium filter, 50 kV X-ray tube voltage, and 800 µA tube electric current. Bone nodules and tibiae were scanned at a pixel resolution of 14.8 µm. The scanned images were reconstructed using NRecon, version 1.6.1.7 (SkyScan), and analysed using CTAnalyser software, version 1.11.8.0 (SkyScan). A global threshold to define bone tissue in pellets was set at a mineral density of 0.3 g/cm^3, and for trabecular analysis at 0.4 g/cm^3. Samples that had fused to the femur were excluded, resulting in final test items of n = 5-10 specimens in each group for all analyses.

Fluorescent tracking of *in vivo* ZA

To determine the release and subsequent biodistribution of bisphosphonate *in vivo,* pamidronate was fluorescently tagged with a commercially available Alexa Fluor (555) according to the manufacturer's protocol. The resultant fluorescent bisphosphonate was termed AlexaPam. The cross-linking of the dye moiety interferes with the biological activity of pamidronate and diminishes its interaction with farnesyl diphosphate synthase, but it does not compromise the 'bone hook' involved with mineral avidity. To allow for both bisphosphonate tracking and osteoclast inhibition, the AlexaPam was admixed with ZA in a 1:1 (w/w) ratio. Following fixation, samples were cut using a diamond saw, either bisected down the centre of the pellet or along the growth plate for the femurs. Samples were imaged directly on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), AlexaPam was detected using the 561 laser line and with 570-670 nm emission bands, and muscle autofluorescence was detected using 488 laser line and with 500-560 nm emission bands. Bone surfaces were scanned with a 5× objective using the Tile Scan (9-12 images) and Z stack (4-5 slices of a 6 µm optical depth that spanned between 30-200 µm) functions. High Z stacks were to account for uneven bone surfaces, with maximum intensity projections used to reconstruct a view of the bone surface in two dimensions. Images were processed with Leica Application Suite Advanced Fluorescence software (Version 2.5.1-6757) and Adobe Photoshop CS5 (Version 12.1x32).

Cell culture

MC3T3-E1 cells (sub-clone 4), a commonly used pre-osteoblastic cell line (Kartsogiannis and Ng, 2004), were cultured in standard tissue culture flasks using α-Minimal Essential Medium (MEM) supplemented with 10 %

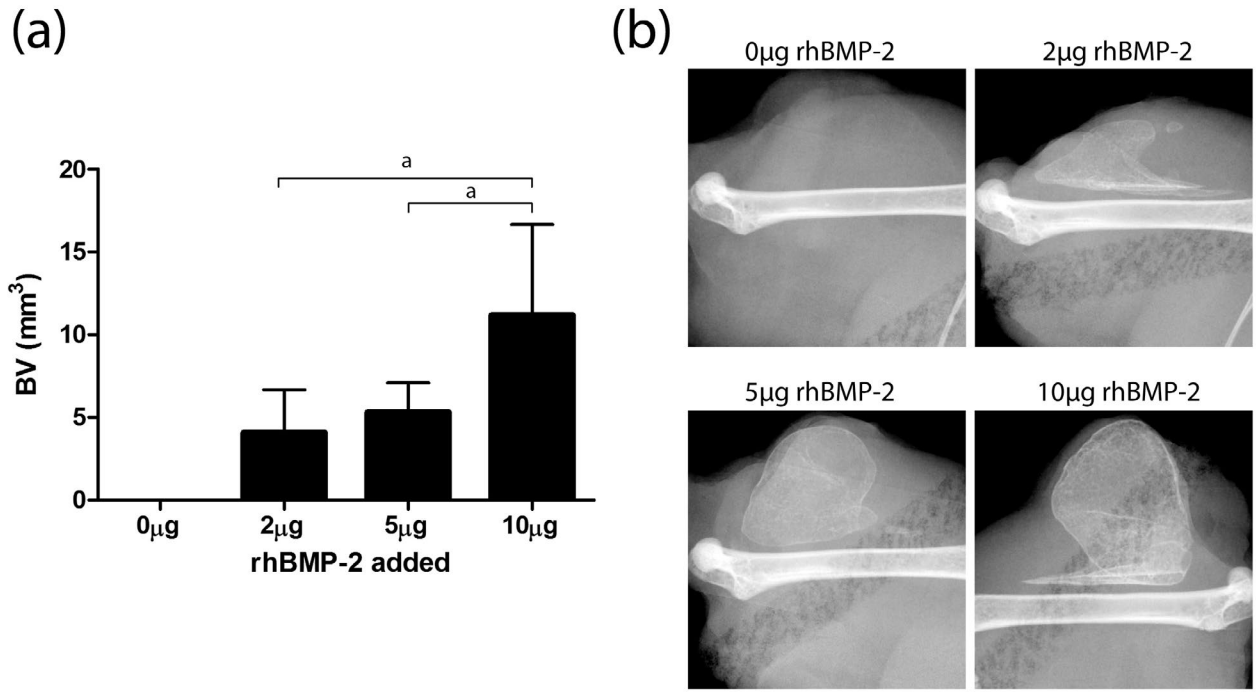

Fig. 1. Increasing doses of rhBMP-2 delivered in the SAIB construct into the quadriceps of the mouse. (**a**) Quantified bone volume formation in the ectopic nodule model of the mouse. Error bars represent SD; [a], $p < 0.05$. (**b**) Representative samples from each group as shown by X-ray.

foetal bovine serum, 1 % L-glutamine and 2 % penicillin/streptomycin. Media was changed every 4 days and cells were removed from flasks using trypsin-EDTA solution. Cell number was calculated using a haemocytometer. To determine the cytotoxic dose response to ZA, cells were seeded in 24-well plates at a density of $2x10^4$ cells per well in 500 µL and cultured in a range of ZA concentrations (1-200 µM). A dose of 50 µM was found to be highly cytotoxic and used for subsequent HA rescue experiments. Transwell inserts (1 µm pore size) were inserted into each well containing 500 µL of media of the following treatments: media alone, 2 % HA, 100 µM ZA or 100 µM ZA + 2 % HA. Preliminary experiments where 10 µg (2 %) HA was added directly to cells showed high cell death and this was overcome by physically containing the HA within Transwell inserts.

Viability was measured at 4 days and 7 days post seeding. Cellular viability was assessed using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were incubated with the viability solution for 30 min at 37 °C and read using a spectrophotometer at 595 nm. All samples were assayed in triplicate and the results are representative of $n = 4$. Data is represented as a percentage of the control cell viability value for that time point.

Quantification of ^{14}C-ZA

For the *in vitro* binding assays, a range of doses (1-50 µM) of carbon-14 bound ZA (^{14}C-ZA) was added to 2 % HA particles in saline and agitated. After 2 h, samples were spun down in a centrifuge, and the supernatant carefully extracted and added to scintillation fluid. Readings from the precipitate were taken by dissolving in 10 N HCl, neutralising with equal amounts of 10 N NaOH, and added to scintillation fluid. Radio counts were read in triplicate using a 1900CA Tri-carb liquid scintillation analyser (Packard Bioscience, Meriden, CT, USA).

For quantification of ^{14}C-ZA in tibia, tibiae were carefully stripped of any soft tissue and then dissolved in 10 N HCl and homogenised using a TissueRuptor homogeniser (Qiagen, Hilden, Germany). This was then neutralised using equal amounts of 10 N NaOH. This was added to scintillation fluid, and read in triplicate using the 1900CA Tri-carb liquid scintillation analyser.

Statistical analysis

Statistical analyses on cell culture results were carried out using student's *t*-test or ANOVA. Unlike cell culture assays, the *in vivo* ectopic bone formation have not been demonstrated to follow a normalised distribution; moreover, exclusion of fused samples that are unable to be accurately quantified has the potential to skew data in this model. Thus non-parametric testing (Mann Whitney U and Kruskal Wallis) was used to analyse these data. All tests were performed using GraphPad Prism (Version 5.00 for Windows, GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $\alpha < 0.05$.

Results

SAIB delivery of rhBMP-2 produces ectopic bone in a dose-dependent manner

Three rhBMP-2 doses (2, 5 and 10 µg) were compared to SAIB alone delivered by direct injection. The ectopic

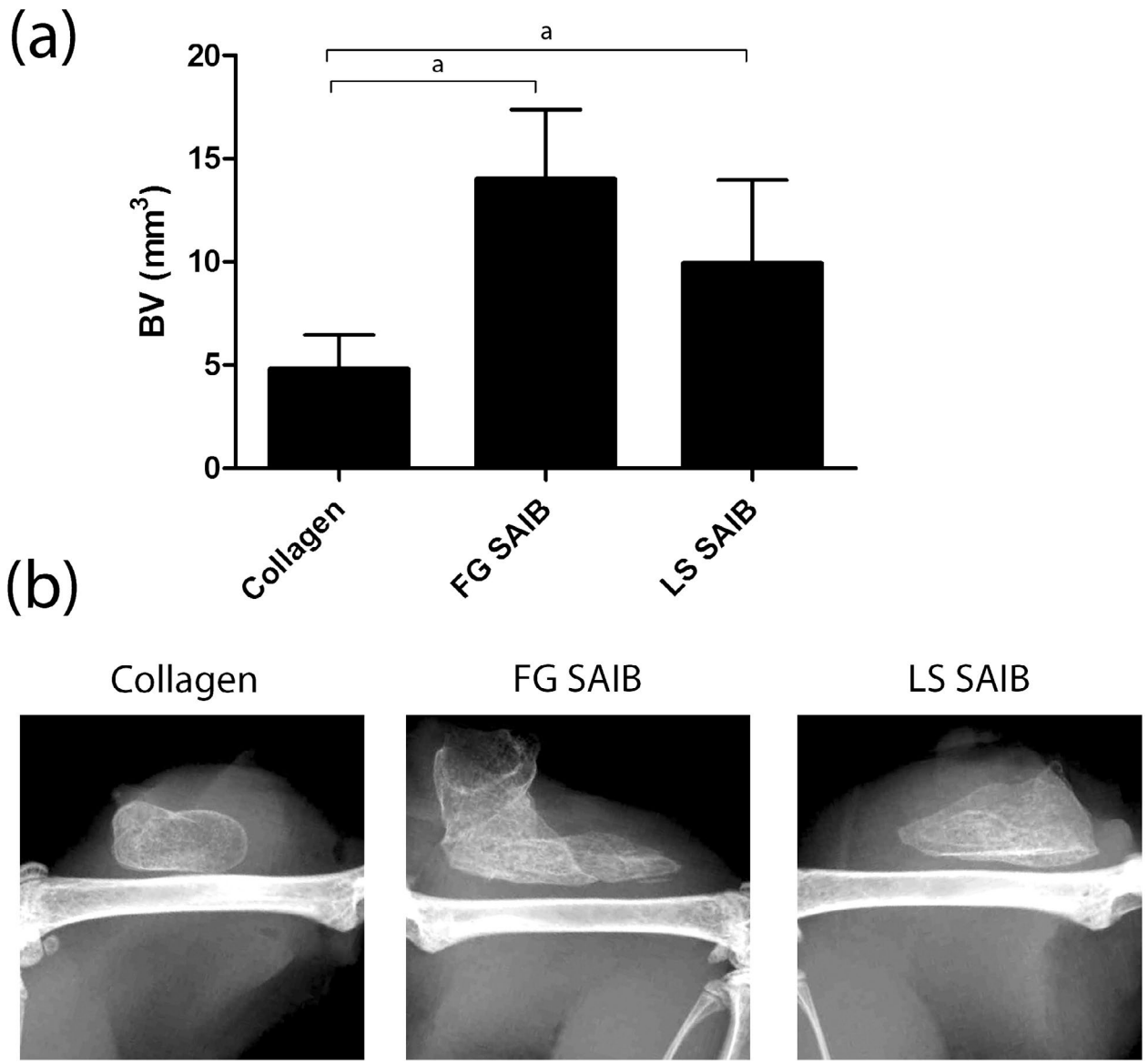

Fig. 2. rhBMP-2 induced bone growth delivered by collagen sponge, commercially purchased SAIB (Food grade, FG), and SAIB synthesised in the lab (Laboratory synthesised, LS). (**a**) Bone volume of the resultant pellet quantified by microCT. [a], $p < 0.05$. (**b**) Representative radiographs of specimens with the median bone volume.

nodules were harvested after three weeks *in vivo*, X-rayed and bone volume quantified by microCT. An increasing dose of rhBMP-2 led to increased bone formation (Fig. 1), however even the lowest dose of 2 µg rhBMP-2 led to robust formation of a bone nodule. SAIB is thus able to deliver rhBMP-2 percutaneously resulting in ectopic bone formation after three weeks. From this result, 5 µg of rhBMP-2 was chosen for following experiments.

SAIB delivery of rhBMP-2 produces more bone than delivery by porous collagen

In this study SAIB was compared head to head against the commercial porous collagen sponge as the current clinical gold-standard for rhBMP-2 delivery. Ectopic bone formation was induced by a single local dose of 5 µg rhBMP-2 delivered either by SAIB or collagen. For SAIB, two preparations were compared: (a) commercially-available food grade quality grade (FG SAIB) and (b) laboratory-synthesised SAIB prepared in-house (LS SAIB).

After 3 weeks, specimens were harvested and X-rayed, and bone volume of the ectopic bone nodules quantified by microCT. The primary outcome measure was bone volume formation for each group (Fig. 2a). With an equivalent dose of rhBMP-2, commercial SAIB produced increased bone volume (+190 %, $p = 0.002$) *versus* collagen sponge. SAIB synthesised in house also led to increased BV (+100 %, $p = 0.0001$). Representative X-rays (Fig. 2b) reveal that nodules produced by the collagen sponge were more regularly shaped than those produced by SAIB, and this was seen across all samples.

The effect of adjunctive agents on bone formation

The combination of other agents that can increase bone progenitor differentiation, mature osteoblast function, or decrease bone resorption may reduce the effective dose

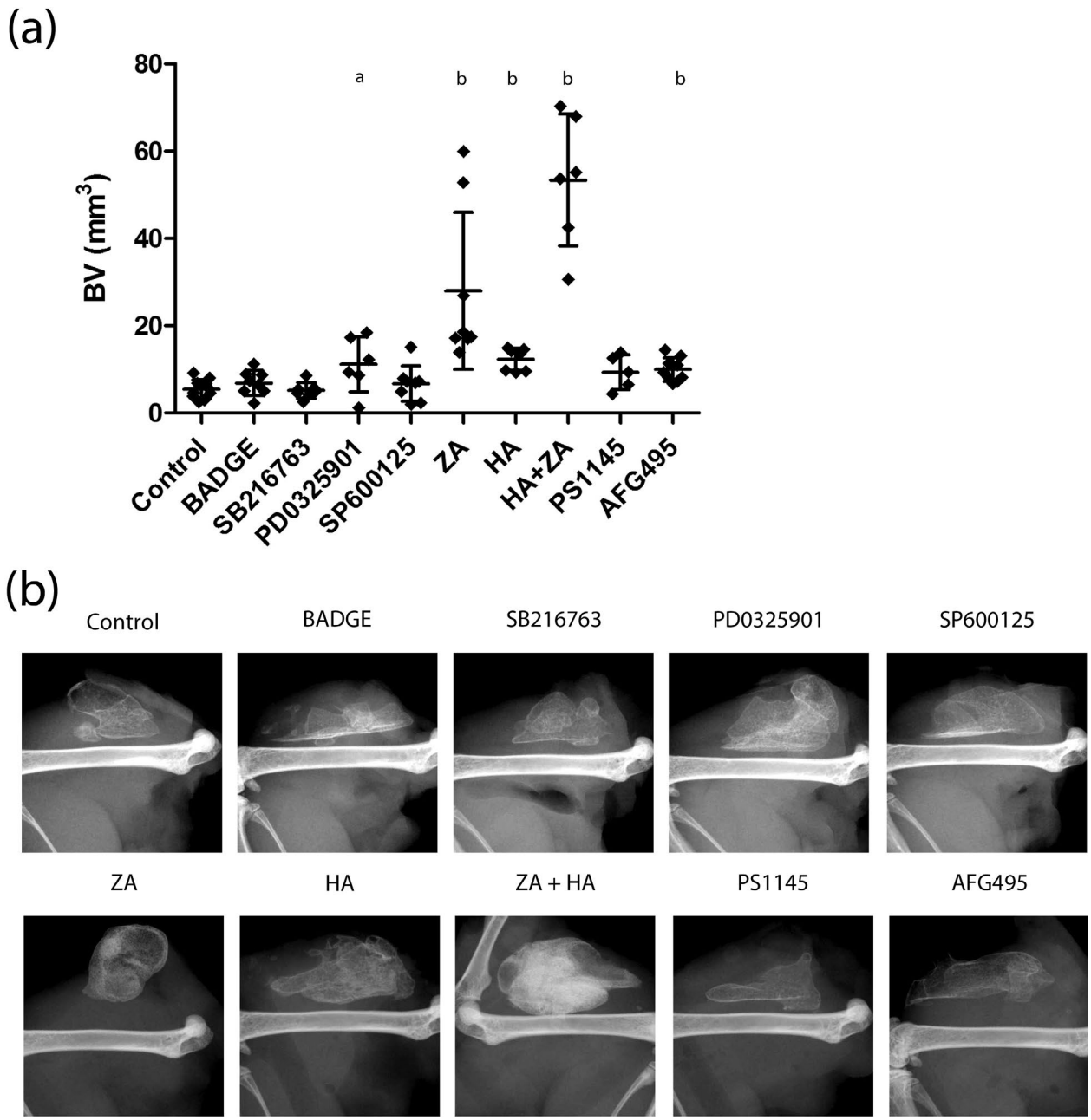

Fig. 3. Bone formation induced by 5 µg rhBMP-2, and a range of potential adjunctive agents including BADGE (15 µg), SB216731 (15 µg), PD0325901 (10 µg), SP600125 (20 µg), ZA (1 µg) and/or HA (2 % w/v), PS1145 (10 µg) and AFG495 (25 µg). (**a**) Bone volume (BV) of the ectopic pellets was quantified after three weeks *in vivo* by microCT. [a], $p < 0.05$; [b], $p \leq 0.01$, increased compared to the control group. (**b**) Representative X-rays showing the samples with the median BV from each group.

of rhBMP-2. A variety of small molecule drugs were trialled in a method for rapid *in vivo* screening. Drugs were delivered in FG SAIB by direct injection in combination with rhBMP-2 as an osteogenic stimulus.

The agents tested could be broadly split into two groups; those affecting cell fate decisions (BADGE, SB21676, PD0325901, and SP600125) and anti-resorptives (ZA, PS1145, and AFG-496). BADGE is a competitive PPARγ antagonist, an inhibitor of the adipogenic pathway that may encourage progenitors to adopt a bone rather than a fat cell lineage (Wright *et al.*, 2000). SB216763 is a Wnt agonist (Adachi *et al.*, 2011), PD0325901 is a MEK inhibitor (Zhang *et al.*, 2012), and SP600125 is a JNK inhibitor (Doan *et al.*, 2012); all of these pathways have been implicated in the modulation of osteoblast differentiation. PS1145 is an IKK inhibitor (Carr *et al.*, 2012), and AFG-496 is an inhibitor for Cathepsin K (rodent). These inhibitors both inhibit vital osteoclast enzymes, in turn inhibiting their actions. The human Cathepsin K inhibitor AFG-495 was reported to be in development for clinical applications (Podgorski, 2009). Both Cathepsin K and IKK inhibitors have been shown to suppress bone resorption (Stroup *et al.*, 2001; von Metzler *et al.*, 2007).

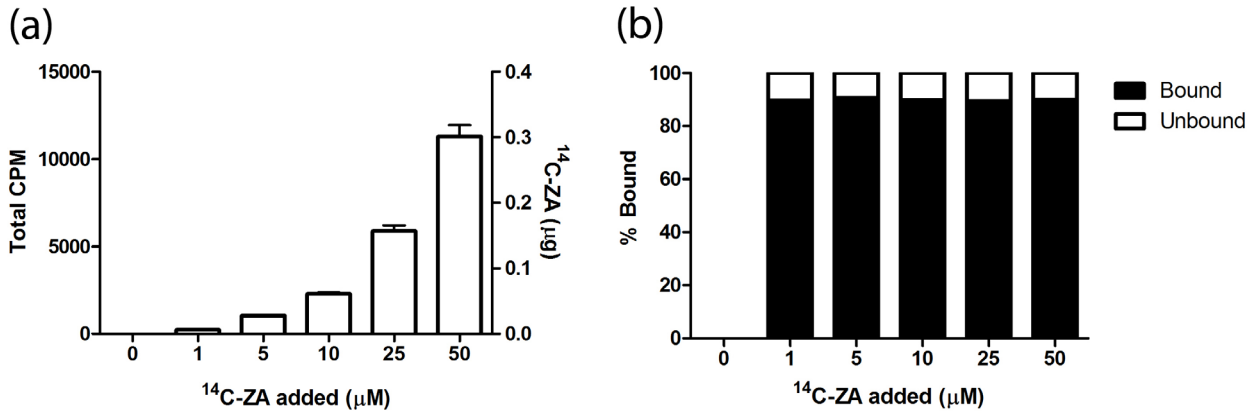

Fig. 4. Detection of ^{14}C-ZA in the supernatant after binding with 2 % HA in saline for 2 h. (**a**) Total unbound ^{14}C-ZA detected at increasing doses and (**b**) percentage of ^{14}C-ZA found bound to HA.

The primary outcome measure was bone volume formation in the ectopic bone pellets as measured by microCT. The most striking result was the +900 % increase in bone volume in the rhBMP-2/HA/ZA group ($p = 0.0002$) over the rhBMP-2 controls (Fig. 3a). The ZA group had an increase of +400 % in bone volume ($p < 0.0001$), and the HA group increased by +125 % ($p = 0.0001$). To confirm that HA particles were not contributing to the increased bone volume, SAIB containing 2 % HA was injected into animals and immediately scanned. The HA was not detectable by microCT, using the settings utilised for subsequent bone scanning (data not shown). For the other anti-catabolic agents, PS1145 gave a +70 % increase (N.S.), and AFG495 resulted in a +82 % increase ($p = 0.001$) over rhBMP-2 alone.

The agents that affected cell fate had more modest results. BADGE and SP600125 increased BV by +25 % and +23 %, respectively, while SB216763 decreased BV by -5 % (all N.S.). Only the MEK inhibitor PD0325901 gave a significant +105 % increase in BV ($p = 0.042$). Fig. 3b displays the representative X-rays from each group. Most notable is the ZA + HA group, which shows an increase in both radio-opacity and bone pellet size.

ZA rapidly and avidly binds to HA *in vitro*

Using ^{14}C-ZA, the binding to HA was examined *in vitro*. A range of ZA doses were tested and the amount of unbound ZA was determined after 2 h. While the absolute amount of unbound ^{14}C-ZA increased with increasing ^{14}C-ZA doses, the ratio of bound/total ZA remained constant at ~90 % (Fig. 4). The constant rate of binding suggests that HA and ZA binding has reached equilibrium, with on and off binding rates being equal. These data suggest that with the doses ranges used in prior and subsequent *in vitro* and *in vivo* experiments, the binding capacity of the HA was not saturated by the bisphosphonate concentration.

Successive experiments examining the binding of 50 μM ZA to HA over a time series revealed that an equivalent proportion of ZA was bound at all time-points (15 min-24 h) (data not shown). These data suggest a rapid and stable binding equilibrium between HA and ZA *in vitro*.

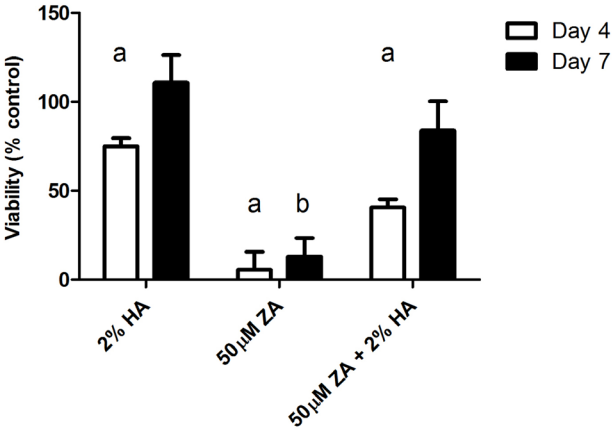

Fig. 5. Mouse derived MC3T3 cell viability in the presence of ZA and/or HA. At two time points, 2 % HA has a protective effect against 50 μM ZA on cell proliferation. Data are normalised to cell viability values of control cells cultured in normal conditions at the relevant time point. [a], $p < 0.05$ compared to day 4 control; [b], $p < 0.05$ compared to day 7 control.

HA yields protection against the cytotoxic effects of ZA *in vitro*

The mouse derived MC3T3 pre-osteoblast cell line was cultured with a dose range of ZA (0 μM, 1 μM, 5 μM, 10 μM, 50 μM and 200 μM) and the effects on cell viability were measured. Doses of 50 μM and 200 μM ZA were cytotoxic, with over 90 % cell death at days 4 and 7 (data not shown).

It was hypothesised that the presence of HA could act as a sink for ZA to prevent cytotoxic effects on cultured cells. Again, in the presence of 50 μM ZA there was significant cell death, but this was found to be attenuated by the presence of 2 % HA (Fig. 5). By day 7, cells treated with 50 μM ZA showed an 87 % drop in viability compared to normal media controls, whereas 50 μM ZA/2 % HA treated cultures were not significantly different from controls. These data suggest that HA can have a protective effect against ZA *in vitro*.

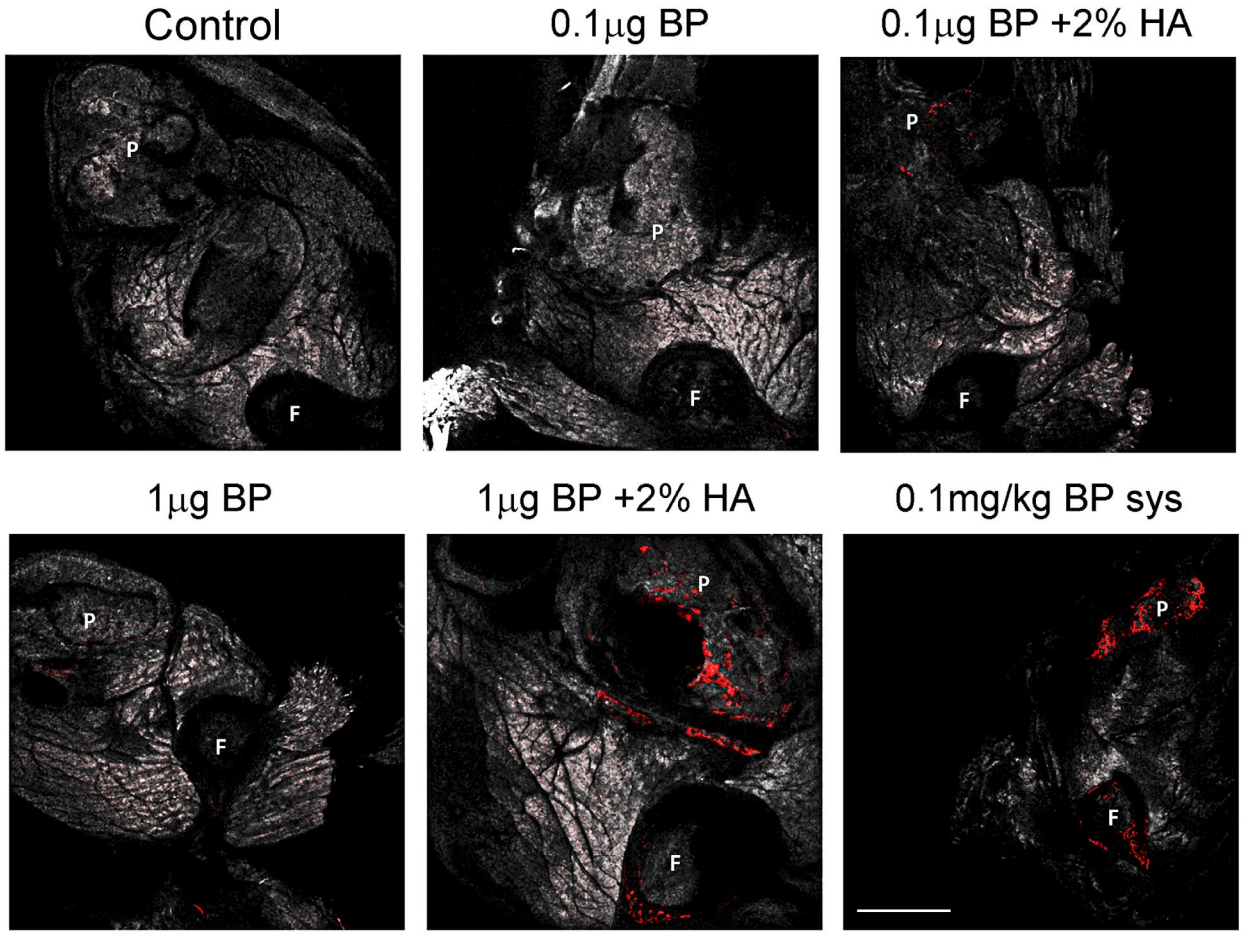

Fig. 6. Equal amounts of fluorescently tagged pamidronate (AlexaPam) and radiolabelled zoledronic acid were delivered in SAIB with and without 2 % HA into the mouse quadriceps. Images are representatives, with the panels showing local delivery of the agents and the final panel shows bisphosphonates delivered by systemic injection (0.1 mg/kg, equivalent to about 2 μg per mouse) one week following implantation of SAIB/rhBMP-2/HA. Images were taken from specimens bisected through the bone pellet and femur. Images show fluorescent AlexaPam (red) overlaid over background muscle autofluorescence (white). P = Pellet, F = femur, all panels at same magnification, scale bar indicates 1 mm.

The effect of HA in delivery of ZA, *in vivo* study

We hypothesised that sequestration of bisphosphonate by HA was a key mechanism underlying the synergistic effects of ZA+HA on rhBMP-2 induced bone. This effect was further investigated using fluorescently-labelled pamidronate to track the bio-distribution of bisphosphonates after implantation. Equal amounts of AlexaPam and ^{14}C-ZA were implanted with 5 μg rhBMP-2 in SAIB/2 % HA. Two doses of 0.1 μg AlexaPam and 0.1 μg ^{14}C-ZA and 1 μg Alexa-Pam and 1 μg ^{14}C-ZA were trialled. One group received a systemic dose of 0.1 mg/kg Alexa-Pam and 0.1 mg/kg ^{14}C-ZA one week post-surgery. With the average size of the mice, this equated to a systemic dose of about 2 μg of each bisphosphonate per mouse.

Three weeks after implantation, the distribution of bisphosphonate in the ectopic bone pellet and at distal sites (contralateral femora or tibiae) were examined by fluorescent confocal microscopy. Confocal images were taken from cross sections through centre of the pellets. A significant retention of AlexaPam in the SAIB/2 % HA groups was observed but not for the SAIB alone groups (Fig. 6). In the SAIB/2 % HA groups the staining was shown to be focal, with retention at the pellet and on the borders of the local femur. A higher fluorescent signal was observed in the high bisphosphonate groups, although this was not quantified.

MicroCT analysis of the proximal trabecular bone architecture was undertaken on the contralateral tibia as a measure of effects of systemic effects on bone turnover. The 0.1 μg bisphosphonate dose groups had comparable bone volumes to the control group. The 1 μg dose locally delivered bisphosphonate groups showed elevated bone volumes in the proximal tibia comparable to the 0.1 mg/kg systemic bisphosphonate dose (Fig. 7a).

The contralateral tibiae were also examined for binding of ^{14}C-ZA by scintillation counting. While ^{14}C-ZA counts were only found to be significantly increased in the systemic dosing group (Fig. 7b), analysis of AlexaPam distribution by fluorescent confocal microscopy showed evidence of bisphosphonate from the SAIB implant reaching the contralateral tibiae in the 1 μg dose locally delivered bisphosphonate groups.

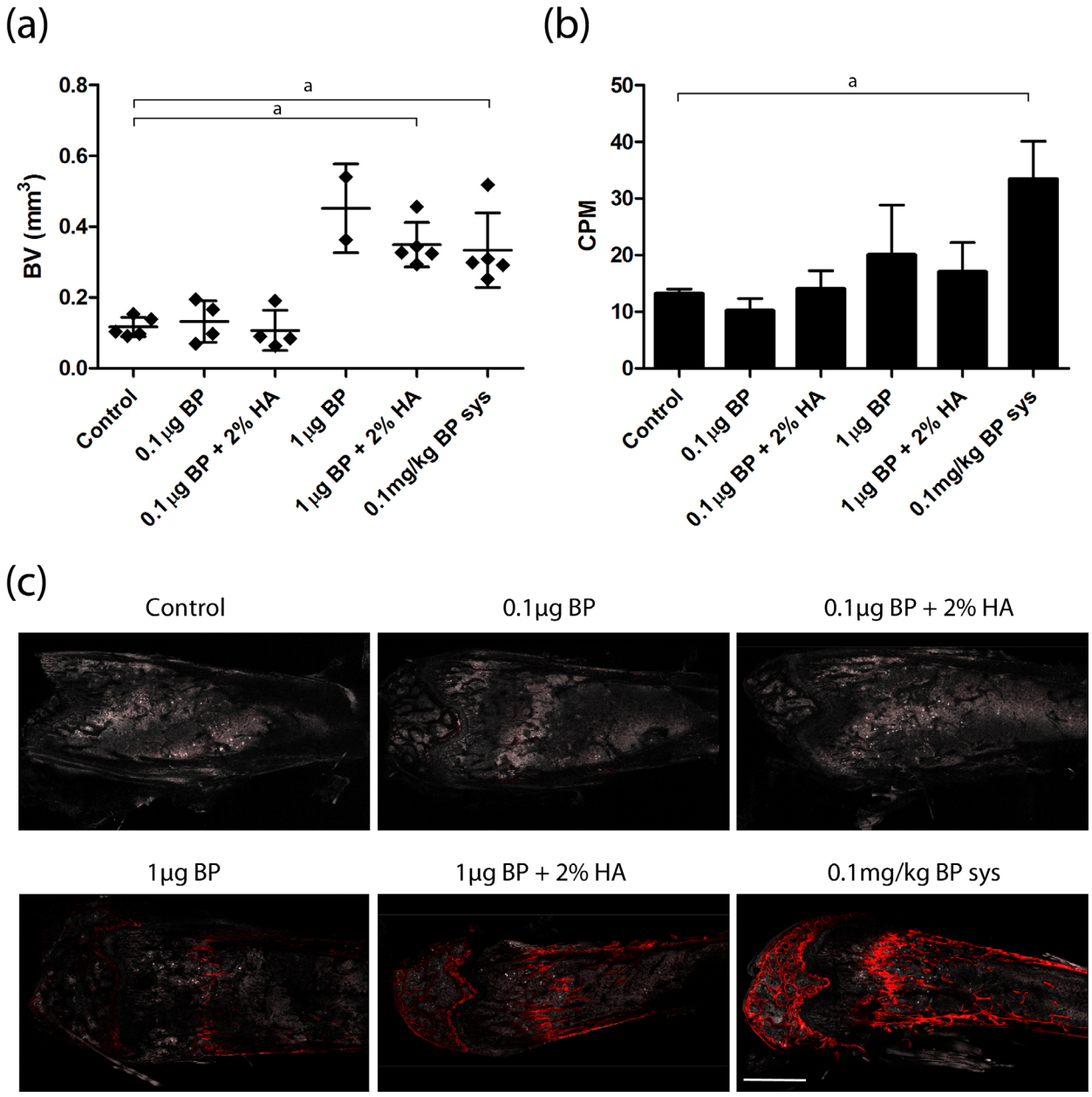

Fig. 7. SAIB was used to deliver rhBMP-2, as well as 2 % HA and fluorescently tagged and radiolabeled bisphosphonate to the muscle pouch of the mouse. (**a**) Quantified bone volume data of the proximal trabecular of the contralateral tibia from µCT. (**b**) ^{14}C-ZA counts detected in the whole contralateral tibia, and (**c**) representative images of fluorescent labels detected in cross sections of contralateral femur. Scale bar indicates 1 mm. [a], $p < 0.05$.

Discussion

rhBMP carriers are critical determinants of the utility of a bone tissue engineering approach, which has led to significant research into carriers with specific properties (Giannoudis *et al.*, 2007a). Carriers are required to deliver the rhBMP-2, create a potential space for new bone formation, support cell proliferation, as well as provide an environment for nutrient, oxygen and waste transfer. Currently, surgical intervention is required for the incorporation of traditional implantable scaffolds made of collagen, ceramic or polymer (Bose *et al.*, 2012). Alternative treatment approaches, including bone grafting, also require open surgery. The drawbacks of invasive interventions include the cost of operation, complications ranging from donor site morbidity, and in the case of autologous bone grafts, they may lead to more general issues arising from anaesthetics and infection (Seiler and Johnson, 2000; Ebraheim *et al.*, 2001). The ability to negate the need for surgery is highly desirable, especially in cases of delayed union where surgical intervention could be avoided completely. An injectable bone tissue engineering construct can be contemplated as an alternative approach for bone tissue engineering. However, a scaffold system is only one element of the construct, and considerations must be made for the loading environment of the bony defect. This study describes the evaluation and development of SAIB as an injectable bone tissue engineering scaffold,

and as a carrier system for rhBMP-2. In addition, the inclusion of adjunctive agents including anti-catabolic bisphosphonates and their biodistribution in the construct was investigated.

Firstly, an injected SAIB scaffold was shown to be able to act as a carrier for rhBMP-2 and result in ectopic bone nodules after three weeks *in vivo* (Fig. 1). In mouse models, 10 μg rhBMP-2 is a standard dose to induce robust formation of ectopic bone, when delivered *via* porous collagen (Stoeger *et al.*, 2003). For alternative delivery systems such as poly-(α hydroxy acid) polymers, higher doses are required (Schindeler *et al.*, 2010). However, bone growth has been seen with as little as 1 μg of rhBMP-2 on a collagen sponge in a mouse (Kato *et al.*, 2006). SAIB was shown to result in robust bone nodule formation with as little as 2 μg rhBMP-2, and bone volume was increased in an rhBMP-2 dose dependent manner. However, there are some limitations to an injectable construct. The current injection system offers limited control over the shape of the resultant bone formation. In the mouse heterotrophic ossification model, the bone nodules that formed were highly irregular and likely affected by the forces they receive within the muscle (Figs. 1b and 2b). If a regular shape is required, some method of containing the injected SAIB may be required. Once an optimised formulation for an SAIB construct is attained, a next step would be to utilise a more challenging orthopaedic model, such as a rat critical defect. It will also be necessary to assess the benefits and limitations of using a containment system.

Being minimally invasive is one of the most important advantages of an injectable delivery system for bone tissue engineering. Such a system could be utilised to deliver bone growth agents in difficult or awkward surgical situations and also percutaneously. The ability to deliver rhBMP-2 non-invasively could reduce the need for open surgery, reducing infection risks, costs and pain to the patient.

When compared to a porous collagen sponge, the current clinical standard of rhBMP-2 delivery, SAIB exhibited simpler implantation and superior ectopic bone volume formation (Fig. 2). We hypothesise that SAIB may have resulted in more bone formation due to a different rhBMP-2 release profile than collagen. It has been reported that SAIB releases drugs in a burst release fashion (Lu *et al.*, 2007), while rhBMP-2 adsorbs onto collagen resulting in a more sustained release (Friess *et al.*, 1999). In a study investigating various formulations involving rhBMP-2 delivered on collagen pellets, it was found that the rhBMP-2 release profile has an effect on ectopic bone formation (Maeda *et al.*, 2004). They suggested that the most effective profile was that of an initial burst with sustained release. It may be that our SAIB more fits this profile than the collagen sponge. Infection is a major issue for many situations where rhBMP-2 is currently used. The use of a collagen sponge requires the creation of an open wound to deliver rhBMP-2, which carries with it the risk of infection (Carragee *et al.*, 2011). An injectable scaffold could mitigate this risk.

For an equivalent dose of rhBMP-2, SAIB delivery increased the resultant bone by up to 3-fold when compared to collagen. While there was no significant difference between bone volume formation between the commercially purchased food grade SAIB and the laboratory synthesised version, there appeared to be a trend towards increased bone formation in the FG SAIB. This may be attributed to residual solvents in the laboratory synthesised SAIB, which will require further optimisation of the process. However, the success of this in-house manufactured SAIB further validates the synthesis process.

The avoidance of open wounds associated with the SAIB construct also has practical benefits with respect to bone drug screening in animal models. Compared to an injectable matrix, the implantation of a solid scaffold in an animal model typically requires anaesthesia, increased surgical and recovery time, as well as more intensive post-surgical monitoring and a higher infection risk. It also eliminates the use of sutures in animal studies and subsequent re-suturing. The development of SAIB construct in this study has allowed for high throughput screening of putative adjunctive agents. Our group has previously described a solid, polymer delivery system that contained a dose of 10 μg rhBMP-2 per pellet (Schindeler *et al.*, 2010; Carr *et al.*, 2012). This construct has also enabled a reduction in the dose of rhBMP-2 required, increasing the economy of these studies. Thus, injectable SAIB has provided a low infection risk, minimally invasive bone tissue engineering system that expedites high throughput screening studies.

In screening of potential adjunctive agents to rhBMP-2, significant increases in bone formation were found with the addition of the MEK inhibitor PD0325901, the Cathepsin K inhibitor AFG495, and HA, ZA, and their combination (Fig. 3). In this study, we chose to pursue and optimise the combination of HA and ZA, which increased bone volume by the greatest magnitude. The performance of other adjunctive agents may be further improved by dose optimisation, although in most cases the doses used were maximal as limited by the loading capacity of the SAIB carrier.

The greatest synergism was found with the addition of 2 % HA and ZA (Fig. 3). The addition of HA to the formulation resulted in an increase in bone volume formation, both on its own (+125 % increase) and in combination with the bisphosphonate ZA (+900 % increase). In part, this response can be explained by the fact that HA may act as a nidus for bone formation. HA is highly osteoconductive, and induces appositional bone growth, where osteoid is laid down directly onto the mineral surface (Vaccaro, 2002). This effect can be seen in the increase in bone formation in the HA treatment group. However, we hypothesise that HA also acts to sequester locally delivered bisphosphonate, retaining it for action at the local site as well as reducing the local cytotoxic effects of ZA.

An *in vitro* binding assay using radiolabelled ZA revealed that ZA binds to HA immediately and at a constant rate (Fig 4b). Thus, the methods of the previous *in vivo* experiment would have been sufficient to bind 90 % of the available ZA to the 2 % HA that was added. Further, when this binding was examined in cell culture, it was found that HA was able to rescue ZA mediated cell death (Fig 5). It appears that the strong affinity of ZA for HA is able to protect against its cytotoxic effects to surrounding cells. These data further confirm previous data from this

laboratory showing that when ZA has been bound to HA, it is toxic to only osteoclasts, and not osteoblasts (Schindeler and Little, 2005).

Finally, this sequestration was investigated *in vivo*, using fluorescently labelled bisphosphonate (Fig. 6). While some non-labelled functional PAM may have been present, the lower potency of this drug would suggest that most of the anti-osteoclastic effect would be due to co-delivered ZA. Confocal images showed that after three weeks, bisphosphonate was only retained locally in the dual administered HA/bisphosphonate groups. Thus HA can sequester locally delivered bisphosphonate, as evidenced by our *in vitro* and *in vivo* biodistribution studies. This sequestration may improve the efficacy and availability of bisphosphonate at the desired site of action (Nancollas *et al.*, 2006). While HA acts to keep any delivered bisphosphonate to the local site, it does not totally prevent its release into the circulation (Fig. 6). While BP was not detected using the ^{14}C-ZA assay (Fig. 7), this could be due to insufficient specific activity of the radiolabelled agent or due to subtle differences in affinity between the BPs. Despite the effects of locally delivered bisphosphonate on ectopic bone formation, in the absence of HA there is no evidence of high levels of bisphosphonate retention at the site. It is possible that these increases are due to low levels of bisphosphonate retention that are below levels of detection. This is in contrast to the high levels of retained bisphosphonate observed with HA co-delivery. Moreover, this is associated with the large increases in ectopic bone volume seen in these groups. It is clear that the combination of rhBMP-2, HA and ZA demonstrates strong synergy.

The use of rhBMP-2 in excessive doses can lead to side effects that range from pathological inflammation and/or heterotrophic ossification to premature bone resorption (Axelrad and Einhorn, 2009; Argintar *et al.*, 2011; Carragee *et al.*, 2011). The ability to reduce the amount of rhBMP-2 needed may ameliorate these side effects without sacrificing the resulting bone formation. For the same dose of rhBMP-2, SAIB results in almost three times the bone production when compared to the current clinical standard for rhBMP-2 delivery (Fig. 2a). With the addition of some adjunctive agents, most notably the combination of HA and ZA, this bone formation is even further boosted (Fig. 3a). This is highly relevant clinically, as a reduction in rhBMP-2 needed for a procedure reduces the overall cost of the procedure, the potential side effects, as well as infection risk all whilst using currently approved agents.

Other groups have attempted to develop injectable delivery systems for bone tissue-engineering and while many show promise, they have not yet translated into conventional usage. The majority of injectable materials are water-based hydrogels. Some of these hydrogels require a curing process, such as poly(ethylene glycol)/fibrinogen that requires UV light to gel (Peled *et al.*, 2007), which prevents their use in non-invasive applications. Others are able to be injected. Hyaluronic acid (Martinez-Sanz *et al.*, 2012), gelatin (Yamamoto *et al.*, 2006) and silk (Zhang *et al.*, 2011) based hydrogels have all shown the capacity to deliver BMP-2 and produce bone in animal models. However, many of these hydrogels comprise of animal-derived proteins that can bring associated costs and disease risk.

Alternatively, injectable calcium phosphates have been explored in bone tissue-engineering (D'Este and Eglin, 2013). A water-free tricalcium phosphate formulation was found to induce ectopic bone formation in a canine muscle pouch model (Davison *et al.*, 2012). SAIB also uses water-free carriers and has been reported to support a range of alternative solvents including benzyl alcohol (Hadj *et al.*, 2009), ethanol, ethyl lactate and N-methyl-2-pyrrolidone (Lu *et al.*, 2007). Some organic solvents can be poorly tolerated and may cause pain at the injection site (Packhaeuser *et al.*, 2004), making solvent selection a key consideration.

Lastly, injectable synthetic polymers, such as phase transitioning poly (-lactic-co-glycolic acid), have been suggested as a method for creating an injectable but still porous scaffold (Krebs *et al.*, 2009). However, a common problem with synthetic polymers is the generation of adverse biodegradation products that can create an acidic microenvironment, which can limit osteoconduction (O'Brien, 2011).

In summary, SAIB presents with many advantages when compared with alternate injectable constructs in current development. SAIB allows for percutaneous delivery and has biocompatible breakdown products. As a water-free carrier, it utilises small amounts of well-tolerated organic solvents. SAIB is also cheaply and easily manufactured and stored, with established large scale manufacture already in place. As a food additive, and now trialling as a carrier, it has a long standing safety record with regulatory agencies around the world. These practical considerations, alongside its impressive bone formation seen in this study, mean that SAIB has strong promise as a bone tissue engineering scaffold of the near future.

Conclusion

Our work has found that an SAIB carrier can outperform collagen as the current clinical standard of rhBMP-2 delivery in terms of bone volume formation and versatile delivery. In addition, the co-delivery of ZA and HA particles can further enhance bone production up to 10-fold. We have presented evidence supporting the concept that ceramic particles can enhance the efficacy of bisphosphonates in a tissue engineering setting by sequestering them to the site of delivery.

Overall, the SAIB formulation has the potential to allow for robust bone formation with reduced doses of rhBMP-2, while the use of a percutaneous delivery system can reduce the risk of infections. The use of SAIB as an injectable construct for the delivery of rhBMP-2 is a promising avenue for the bone tissue engineering field.

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Discussion with Reviewers

M. Krebs: Could you elaborate on the mechanism of the phase transition of SAIB? And comment on this? Have you tried studying release over time of the BMP-2 from the SAIB matrix?

Authors: The mechanism of phase transition is that of solvent diffusion. When the SAIB/solvent solution is injected into the muscle, the solvent diffuses away rapidly, leaving behind a semi-solid depot. This is described in more detail in the text.

M. Krebs: What is your hypothesis for why the SAIB system produced more ectopic bone than the collagen sponge system?

Authors: We cannot definitively say why SAIB resulted in more bone formation than collagen, but hypothesise it

may be due to the different release profiles of the rhBMP-2 from the two different systems. Alternatively, SAIB or its breakdown products may provide some additional benefit compared to collagen.

M. Krebs: It was mentioned that SAIB retains BMP-2, but actually this was not demonstrated. Could you comment on this? Have you tried studying release over time of the BMP-2 from the SAIB matrix?
Authors: We have re-evaluated our use of the term 'retain' based on the Reviewer's suggestions. While others have reported data from *in vitro* elution assays using SAIB, it is hydrophobic and it is highly likely that elution *in vivo*, where SAIB is biologically broken down, will be substantively different. This makes *in vitro* assays of limited value. *In vivo* elution with fluorescently tagged rhBMPs is a possible method to address this, but not within the scope of these studies.

I. Kramer: Commercial bovine collagen sponge for BMP2 is known to lose consistency after BMP2 solution is spread on it, and it also leaks upon handling. Is it possible that the inferior bone formation is due to BMP2 solution leaking during handling?
Authors: With regard to commercial bovine collagen, we added a volume that was able to adequately wet the sponge without oversaturating it, and without allowing any added volume to drip off prior to implantation. In this process, we are confident that we are consistent with the amount of rhBMP-2 added to each animal. However, once the collagen sponge has been implanted, movement and pressure can potentially cause deformation and/or bulk loss of solution. Notably though, these are the conditions that are seen for the current clinical use of rhBMPs in collagen sponges, making it a relevant comparison.

M. D'Este: The aim of this study was to assess the utility of SAIB as a non-invasive delivery system for rhBMP in combination with different drugs thought to promote ossification in a mouse model of ectopic bone formation. Using this model the authors screened different pharmacological principles added as potential adjunctive agents to the SAIB/rhBMP2 mixture. Out of the tested agents addition of ZA and HA as well as the MEK inhibitor PD0325901 and the Cathepsin K inhibitor AFG495 resulted in significantly augmented ossification compared to control. It remains unclear whether the lack of promotion of bone formation with the other agents is related to inappropriately chosen doses (e.g., the doses need to be mentioned in the text), suboptimal PK with the SAIB delivery system (absence of slow release, low binding etc.) or lack of a sufficient PD effect *in vivo* or a combination thereof. The authors should discuss how the chemical nature of SAIB might affect the release of different drugs (proteins and chemicals) in relation to the drug's physicochemical properties.
Authors: The doses chosen were extrapolated from *in vitro* data gathered previously (data not shown). However, in many cases the doses were limited by the maximum capacity of SAIB to hold the drugs. There was no evidence of inhibitory effects with any of the agents tested, so it is possible that if a higher loading was achievable a greater effect might be seen. Alternatively, it is possible that pharmacokinetics of the SAIB delivery system is suboptimal for these agents. Or that they simply are poorly effective for promoting bone formation. This study was a screen of available drugs, and while only one combination was chosen to be taken forward, it does not mean that the other agents are completely ineffective. Some discussion of this has been added to the text.

Reviewer IV: What specific bone tissue engineering applications do you envisage percutaneous injection systems such as SAIB to be most applicable, as opposed to surgical intervention?
Authors: The bone tissue engineering applications that we have in mind for SAIB are the percutaneous treatment of atrophic non-unions of long bones, and minimally invasive spinal fusions. These are situations where the use of rhBMP-2 has the potential to increase bone formation, and healing outcomes. They also represent indications where open surgeries could be avoided or lesion sizes greatly reduced with the use of our construct.