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A NOVEL LIBRARY SCREEN IDENTIFIES IMMUNOSUPPRESSORS THAT PROMOTE OSTEOBLAST DIFFERENTIATION

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Abstract

Bone homeostasis can be compromised by an increase in osteoclast-mediated resorption and/or a decrease in osteoblast-mediated bone deposition. While many efforts have focused on treating osteoclast resorption, there has been less emphasis on identifying strategies for promoting osteoblast function. Herein, we describe a high-throughput screening assay to select for small molecules that augment bone morphogenetic protein-2 (BMP-2)-mediated osteoblast lineage commitment. After an initial screen of 5405 compounds; consisting of FDA-approved drugs, known bioactives, and compounds with novel chemical makeup, we identified 45 small molecules that promoted osteoblast commitment. Of the 45 candidates, there was a broad array of classes that included nine retinoid analogs/derivatives and four immunosuppressants, notably rapamycin and FK-506, which were chosen for further study. Treatment of osteoblast precursor cells with rapamycin or FK-506, either alone, or synergistically with BMP-2, increased levels of phospho-Smad1/5/8 protein and transcription of Runx-2, Osx and Smad-7, consistent with a role in promoting osteoblast differentiation. Only FK-506 was able to enhance osteocalcin transcripts and alizarin red staining, both late markers for differentiation. When osteoblast differentiation was suppressed with exogenous TGF- β 1 treatment, rapamycin (but not FK-506) was able to rescue expression of differentiation markers, indicating distinct but overlapping activity of these compounds. Collectively, these data add to an understanding of pathways engaged in osteoblastogenesis, support a role for non-redundant immunosuppressant signaling, and provide a novel approach for the discovery of potentially therapeutic compounds that affect bone remodeling.

INTRODUCTION

Loss of bone mass is an increasingly common morbidity in our aging society and is associated with an increased risk of fracture and frailty and, alarmingly, increased prevalence in chronic but treated disorders such as long-term HIV-1 infection and diabetes mellitus [1] [2]. Understanding the mechanism through which bone mass is regulated and the risk factors for bone dysregulation are critical challenges for developing new and

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effective therapeutics. There are substantial data supporting crosstalk between bone forming osteoblasts and bone resorbing osteoclasts, which allow for dynamic bone remodeling necessary for bone maintenance, strength and structural integrity ([3–5]. Net bone loss can occur if there is a loss in osteoblast activity or if there is an increase in osteoclast activity [6, 7]. The therapeutic focus has more recently been on blocking osteoclast activity (e.g., antibody to RANKL, Denosumab,[8]) and while these advances are promising, efforts to promote osteoblast activity may also be useful in establishing an arsenal of therapeutic options in countering bone loss.

High-throughput screens are becoming increasingly more common in their use to identify novel compounds as therapeutic. A clear advantage of this approach is that small molecule libraries have the capacity to probe cellular pathways to identify novel modulatory nodes with desirable effects. For example, a screen was recently done to identify anti-inflammatory compounds for use in cystic fibrosis and several compounds were discovered [9]. We adopted a similar strategy to identify osteoblast promoting compounds using a complex set of chemical libraries that range from FDA approved compounds to small molecules with unknown function.

Bone homeostasis is maintained by the proliferation and differentiation of osteoprogenitor cells, which will eventually guide the production of mineralized bone [10, 11]. Osteoblast differentiation can be induced by bone morphogenetic protein (BMP)-2, initiating a signal cascade that promotes osteoblast specific genes; including the transcription factor Runx2, a critical regulator, as well as the downstream transcription factor osterix (Osx) [12–15]. Mature osteoblasts then produce alkaline phosphatase (ALP) that can be measured via cell staining [16] and was used in this study to identify compounds that promote osteoblastogenesis. Late differentiation is induced by another transcription factor, osteocalcin (Ocn) and this allows the cells to eventually secrete proteins that form the mineralized extracellular matrix [12–15].

To find novel drugs that promote osteoblast differentiation, we began with the C2C12 cell line that is biased to become muscle but can be induced to differentiate along the osteoblast lineage in the presence of BMP-2 [17], with essentially no background ALP staining, allowing us to find drugs that would enhance this efficiency and therefore potentially enhance osteoblast differentiation of pre-osteoblasts. Once the libraries were screened, 45 compounds were identified as “positive hits” that enhanced ALP expression. We chose two hits; FK506 and rapamycin, for further study because their effect on the BMP-2 pathway was unclear. Both of these immunosuppressive drugs have previously been implicated in osteoblastogenesis, however the results have been contradictory and the mechanisms of action remain unclear [18–23]. Additionally, transforming growth factor (TGF)- β 1 is an anti-inflammatory cytokine that can function as both an antagonist [24–27] and agonist [28–30] on bone differentiation, depending on context. We therefore utilized TGF β 1 under antagonistic conditions to further explore rapamycin and FK506 activity under conditions that attenuate bone.

MATERIALS AND METHODS

Cell Culture

C2C12—The C2C12 myoblast cell line (ATCC) was maintained in growth medium (GM) that consisted of High Glucose DMEM (Gibco), 10% Fetal Bovine Serum (Gibco), and 1% pen/strep (Invitrogen). To induce osteoblast formation, cells were allowed to reach 50% confluency, washed with PBS (1x) and switched to differentiation medium (DM) that consisted of Low Glucose DMEM, 2% Horse Serum (Gibco), 1% pen/strep and lyophilized Bone Morphogenetic Protein-2 (BMP-2) (Genscript #Z00327), reconstituted in 20mM acetic

acid, at a concentration range of 20–200ng/mL [17]. Negative controls were switched to DM but only received 20mM acetic acid. Cells were passaged approximately every two days and were kept below 70% confluency, as per manufacturer instructions.

MC3T3-E1—MC3T3-E1 Subclone 4 pre-osteoblast cells (ATCC #CRL-2593) were maintained in Minimum Essential Medium, Alpha modification (Invitrogen #A10490-01) containing 10% FBS and 1% penicillin/streptomycin. Osteoblast induction was performed by supplementing the medium with 100ng/mL BMP-2, as previously described [31]. All cell lines used were maintained in a 37°C incubator at 5% CO₂.

Chemical Libraries

Chemical libraries used in this study were prepared by the Center for Molecular Discovery at Boston University Medical Center. The libraries consisted of 640 FDA approved compounds (Enzo Life Sciences), 480 ICCB known bioactive compounds (Enzo Life Sciences), 446 compounds from the NIH Clinical Collection (BioFocus DPI), ~2,000 Natural-like compounds from the Center for Chemical Methodology and Library Development at Boston University, and a diversity subset of 2,000 compounds (Chembridge).

BMP-2 and Compound Stimulation

384-square well plates (BD) were used for primary screening. PBS was added to the outermost wells to reduce edge effects. In each plate, 40 wells were used as positive controls, 28 wells were used as negative controls, and the center 240 wells were used for compound testing. All plates were screened in duplicate. The inner 308 wells received 750 cells per well and were allowed to adhere in GM for 24 hours. GM was then removed, wells were washed once with PBS, and DM was added to the inner 308 wells. Negative control wells received DM. Positive control wells received BMP-2 in DM. Test wells received BMP-2 and compounds at a target concentration of 1uM. DMSO was used as the vehicle for compound addition in this study. DMSO was also added to positive and negative control wells in equimolar amounts.

ALP and DAPI Staining

Cells were initially fixed with a fixative solution that consisted of a 3:10:26 ratio mixture of 37% formaldehyde:citrate:acetone. Colorimetric detection of osteoblasts was achieved using the Alkaline Phosphatase (ALP) kit obtained from Sigma-Aldrich (Catalog #86C). Cell number was determined based on nuclei staining with DAPI nucleic acid stain as per manufacturers instructions (Invitrogen).

Image Acquisition and Analysis

Well images identifying ALP+ cells were acquired using a MIAS-2 plate reader, as per manufacturer instructions (Digilab, Holliston, MA).

Search Strategies (ImageJ, Digilab, Visual inspection)

Acquired images were assessed using three different search strategies (ImageJ software, Digilab software, and systematic scanning by eye); each having different sensitivities and specificities with regard to this assay.

ImageJ Analysis

Well images were opened with the image software platform ImageJ. Images were normalized on a per plate basis and, since ALP positive cells become darker in a gray scale

image, the image darkness was assessed. Images were analyzed using an Area Fraction method whereby a minimum pixel darkness threshold was applied for all image wells. All pixels that were as dark or darker than the threshold applied were converted to black. All pixels that did not meet the threshold were converted to white. The binary image was then assessed to see percentage of pixels of the image that were black (i.e. Area Fraction). Average Area Fraction from duplicate compound wells was divided by average duplicate nuclei count (as measured by DAPI count in ImageJ) to get Area Fraction on a per-cell basis. This ratio (Area Fraction: Nuclei count) was then compared to positive control wells. Ratios for the positive control wells, per plate, were determined and any compound wells that were greater than three standard deviations above the average ratio of positive control wells were considered potential augmenters of bone formation and were compared with the two other search strategies.

Digilab Analysis

Well images were analyzed using eaZYX Image Analyzer software (Digilab, Holliston, MA). Cell number was determined by counting the number of DAPI stained nuclei per image. Fluorescent nuclei images were overlaid with the bright-field images to determine the number of ALP positive cells per well.

Visual inspection Analysis

The image of a representative positive control was kept open for comparison while visually scanning the images of wells that had compounds added. Wells were rated categorically on a four-point scale: (1) clear augments of ALP expression, (2) potential augments that appeared to have ALP expression above the representative positive control, (3) changes in the morphology of the cells, and (4) did not change or suppressed ALP expression. Duplicate wells that were independently confirmed by eye for increased ALP expression were compared with the other two search strategies.

Quantitative RT-PCR

Total RNA was extracted by the TRIzol method as recommended by the manufacturer (Invitrogen). Isolated RNA was cleaned up using the Rneasy Kit (Qiagen) and 500ng of RNA was reverse-transcribed using the High Capacity cDNA Synthesis Kit (Applied Biosystems #4368813). Taqman expression assays were used for detection. The expression of 18S (Applied Biosystems #4319413E) was used for normalization of gene expression values. Real-time primers Runx2, Sp7 (Osx), Bglap1 (Ocn) and Smad7 were obtained from Applied Biosystems (Mm00501580_m1, Mm00504574_m1, Mm03413826_m1 and Mm00484742_m1 respectively). Quantification was determined using the $\Delta\Delta CT$ method and normalized to the untreated sample.

Western Blot

MC3T3 cells were lysed, on ice, for 20 minutes using lysis buffer containing 10mM Tris, pH 7.6, 150mM NaCl, 2mM EDTA, 1% Triton, 0.1% SDS, 0.1g deoxycholic acid, 1X protease inhibitor cocktail (Roche), 500mM sodium fluoride, 100mM sodium pyrophosphate, and 400mM β -glycerophosphate and centrifuged at 16,000rpm for 10min at 4°C. Protein concentrations were determined using the BCA protein assay kit (Pierce). Equal amounts of protein (20ug) were resolved by SDS polyacrylamide gel electrophoresis. Gels were transferred to Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (Bio-Rad #162-0115) and probed with either Phospho-Smad 1/5/8 (Cell Signaling 9511S) or Smad-1/5/8 (Santa Cruz SC-6031R), as primary antibodies overnight at 4°C in 5% milk. After washing, membranes were probed with the corresponding secondary anti-rabbit HRP-conjugated (Cell Signaling 7074S) for 1 hour at room temperature. Bands were visualized

by chemiluminescence using Amersham ECL Western Blotting Detection Reagents (GE Healthcare RPN2106). To observe correlating amounts of total protein and phosphoprotein, the same blot was stripped for 30 minutes at 65°C in 62.5mM Tris pH 6.8, 2%SDS and 0.6% β -mercaptoethanol. Stripped blots were washed for 30 minutes and then re-blocked before the primary antibody. Bands were analyzed for density with ImageJ and normalized to loading control Smad 1/5/8. Values represent fold change compared to untreated samples.

Alizarin Red Staining

Cells were fixed with 2.5% glutaraldehyde after 21 days of stimulation and washed with PBS adjusted to a pH of 4.2. They were stained with 2% Alizarin Red S (Sigma-Aldrich A5533-25G) for 20 minutes at 37°C. After being washed with PBS four images were captured for each well.

RESULTS

High-throughput screen identifies osteoblast-inducing compounds

In the course of our studies on muscle differentiation using the C2C12 cell line, we observed incomplete BMP-2-mediated conversion of C2C12 cells to osteoblasts based on ALP staining with virtually no background in untreated cells, which prompted us to use this system to screen a library of compounds that augment BMP-2 mediated osteoblast conversion. C2C12 cells were plated at 750 cells per well in a 384-square well plate and were allowed to adhere for 24 hours in Growth Media (GM). The GM was replaced with either low serum Differentiation Media (DM) (Negative control), DM + BMP-2 (Positive control), or DM + BMP-2 + small molecules (Figure 1a). After 72 hours, cells were fixed and stained for Alkaline Phosphatase (ALP) and DAPI for nuclei visualization. We found that the negative control wells expressed close to zero ALP, reinforcing the specificity of this assay. Positive control wells consistently had ALP expression in 20–30% of the cells. Initially, we performed a pilot study of 240 FDA approved compounds on our system; a number of the small molecules added in combination with BMP-2 strikingly enhanced conversion to osteoblast (ALP+) cells. Notably, there were several phenotypes observed including: no obvious effect on conversion, attenuated conversion to osteoblasts, morphological changes and enhancement or suppression of proliferation of cells. Encouraged by these initial findings, we were interested in screening a larger set of small molecules from more diverse backgrounds to identify enhancement of osteoblast conversion.

Results from screen of 5405 compounds

We were most interested in those compounds that enhanced osteoblast formation beyond BMP-2 alone due to their therapeutic potential to promote bone formation. To ensure a robust detection of augmenters (i.e., those that promoted osteoblast differentiation), we applied three search strategies to measure the effects of added compounds' on ALP expression. After screening 5405 compounds in duplicate from five different chemical libraries, we then applied a cutoff filter that revealed 45 compounds with enhanced ALP expression. From these 45 hits (18 strongest hits and 27 potential hits, Table 1 and Supplementary Figure 1) we decided to further evaluate two unexpected compounds, rapamycin and FK-506 (FK-520 was also identified but was not among the top hits), for their effect on the pre-osteoblast mouse cell line MC3T3 (Figure 1b). We chose rapamycin and FK-506 to validate our screen because they are not widely recognized as osteoblast potentiators, and because, based on prior studies, their role in osteoblasts is mixed. Collectively, we found that screening a large number of compounds allowed us to discover several expected and unexpected compound hits among the libraries tested.

Comparison and characteristics of results from three different search strategies (ImageJ, Digilab, visual inspection)

To ensure true positivity, images of wells from the initial screen of chemical libraries were processed using three different search strategies based on method of analysis (ImageJ, Digilab and visual inspection) to ensure reproducibility and reduce false positives. Resultant images were analyzed using ImageJ software, Digilab eaZYX Image Analyzer software, and systematically scanned by eye. **ImageJ:** Analysis using ImageJ software (see methods section) indicated 211 compounds with ALP expression > three standard deviations above the average (99%) of positive controls. **Digilab:** Analysis using the eaZYX Image Analysis software from Digilab indicated 31 compounds that had ALP expression in the 95th percentile above the positive controls (see methods). **Visual inspection:** Image scanning by eye revealed 44 compounds that appeared to enhance ALP expression above representative positive control wells. There were also 32 compounds that were noted by eye to have morphologic changes as compared to positive controls (data not shown). If a compound well was detected as an augmenter of ALP expression in at least two of the three analysis search strategies, the compound was considered for further analysis. Cross validation of the results from the three analyses revealed thirty-one compounds that appeared in only two out of three searches and eighteen compounds that were identified in all three searches (Figure 2 and Table 1). Therefore, using three different methods of analysis became the most stringent way to determine the strongest positive hits.

We were also interested in the functional class types of compounds represented in the top 18 hits and whether they included known and/or novel effectors. Therefore, we conducted a literature search on the eighteen compounds that were identified in all three analyses and organized them into groups based on common features. Of the eighteen compounds, eight were retinoid derivatives/analogues (13-*cis* retinoic acid, bexarotene, TTNPB, etc.), four known immunosuppressant drugs (FK-506, mycophenolate mofetil, mycophenolic acid, and rapamycin), two prostaglandins (prostaglandin B2 and prostaglandin E1), one fatty acid (C2 dihydroceramide), one platelet-activating factor (PAF) receptor antagonist (PCA 4248), one anticonvulsant medication (phenytoin), and one antimalarial medication (quinine) (Figure 2). There were also notable hits among the top 45 compounds including Reseveratrol, which has been implicated in pro-osteogenic activity [32, 33] and supplementary Figure 1.

Validation of Rapamycin and FK-506 induced osteoblastogenesis using MC3T3 pre-osteoblast cells

To validate our screening assay with functional assays, we utilized an osteoblast precursor cell line, MC3T3, with the two compounds rapamycin and FK506. These compounds were initially added in addition to BMP-2, which upon engagement with BMPR-I/II promotes phosphorylation of Smad 1/5/8 (P-Smad 1/5/8) and downstream activation of osteoblastogenesis such as Runx2 and Osx [4, 10, 14]. As shown in figure 3a, rapamycin was added to MC3T3-E1 cells in addition to BMP-2 and protein was collected 5 and 10 minutes after stimulation. Phospho-Smad 1/5/8 levels were measured via western blot and compared to total Smad 1/5/8 levels. Rapamycin significantly increased phosphorylation (lanes 3 and 6). The same experiment was carried out with FK-506 and an increase in phosphorylation was also observed (Figure 3b, lanes 3 and 6), indicating that both immunosuppressants increase signaling of BMP-2. To determine whether or not osteoblast specific genes were increased with rapamycin and FK-506, the compounds were added to the cells with BMP-2 and then RNA was collected 6 hours and 24 hours after stimulation to quantify *Runx-2* and *Osx* transcripts using quantitative realtime-PCR (qRT-PCR). Both compounds significantly increased *Runx2* and *Osx* transcripts, although *Osx* had a much more robust change, especially at 24 hours, both transcription factors were up regulated in reproducible experiments (Figure 4). This result is somewhat expected since Runx2 levels

are tightly regulated and must be shut down at certain time points soon to allow for differentiation to continue and therefore it is possible that at 6 hours there is less of a change compared to 24 hours. We were also interested in whether rapamycin and FK-506 require BMP-2 to augment osteoblastogenesis. To address this, we evaluated bone markers in response to rapamycin and FK-506 alone. As shown in Figure 5, both FK506 and rapamycin were sufficient to increase P-Smad 1/5/8 ratios (Figure 5a, lanes 4,8,12) and *Osx* and *Runx2* mRNA levels (Figure 5b). However, the level of induction was not as dramatic as it was in the presence of exogenous BMP-2 but did reach levels similar to BMP-2 alone. Since the compounds had an effect on early differentiation capability, we wanted to assess their influence on late differentiation. Osteocalcin (*Ocn*) is an osteoblast-specific marker of late differentiation and is often observed at later time points, *e.g.*, 14 days and later. Similar to previous experiments, FK-506 and rapamycin were added to the cells with BMP-2, with new media and compounds added every two days. We observed that *Ocn* transcripts were significantly increased when FK-506 was present, with or without BMP-2 (Figure 6a), but not with rapamycin (data not shown). To assess late differentiation Alizarin-red staining was conducted to measure mineralization. Staining was done after 21 days and images were captured demonstrating an increase in the level of mineralization with FK-506 treatment, with or without BMP-2 (Figure 6b).

Rapamycin but not FK506 rescues TGF β 1 mediated inhibition of BMP-2 induced Runx2 and Osx and augments Smad 7 mRNA induction via BMP-2

We were then interested in exploring whether rapamycin and FK506 were equivalently capable of attenuating TGF β 1 mediated decline in osteoblastogenesis, an antagonistic effect that has been well documented [7, 25, 34]. When TGF β 1 was added to MC3T3 cells, it reduces basal levels of Runx2 and *Osx* mRNA as well as preventing the induction of these transcripts by BMP-2, as expected (Figure 4). However, when rapamycin was added with BMP-2 to cells after 24 hours of TGF β 1 pre-treatment, the ability of TGF β 1 to inhibit osteoblast formation was attenuated (Figure 7a). Interestingly, by contrast, when FK506 was added after TGF β 1 stimulation, there was no apparent rescue of TGF β 1 mediated suppression (data not shown). These data suggest that rapamycin interferes with the TGF β 1 consistent with these compounds having different mechanisms of action.

We were then interested in whether Smad-7, a suppressor of Smad-2/3 downstream of TGF β 1, might be a target of rapamycin and/or FK-506. As shown in Figure 7b, rapamycin augmented Smad 7 expression in the context of BMP-2 and TGF β 1; whereas TGF β 1 had no apparent effect on Smad-7 levels (similar results were seen with FK-506, data not shown). Because BMP-2 alone induces Smad-7, BMP-2 signaling may involve activation of protective factors that negatively regulate Smad-2/3, further ensuring expression of bone markers, and augmented by rapamycin/FK-506. Collectively, these data suggest that while both immunosuppressants are capable of inducing bone markers, they nevertheless differ in detail.

DISCUSSION

In this study, we describe a novel high-throughput screening approach to identify bone-promoting compounds that induce osteoblastogenesis *in vitro*. Among over 5000 compounds from several chemical libraries that were screened, 45 compounds were identified and cross-validated using three different criteria. Two compounds were of particular interest to us due to their notable role as immunosuppressive drugs (*i.e.*, rapamycin and FK506, [35]). Our results support prior but mixed studies suggesting an osteogenic role for rapamycin and FK506. After we were able to validate the use of FK506 and rapamycin in promoting osteoblastogenesis, we evaluated the capacity for these compounds to attenuate bone growth antagonists. Because TGF β 1 has a prominent role in bone loss studies and has been

previously shown to inhibit osteoblastogenesis, this antagonist was chosen. Our data support a capacity for rapamycin but not FK-506 to rescue TGF β ₁ mediated inhibition. By studying these compounds in the context of antagonist perturbation that share features with common bone disease models, we were able to gain further insights into the pathways involved in osteoblast differentiation. Notably, TGF β ₁ can promote osteoblastogenesis, often in the very early stages of commitment [36]. In this study, however, we chose to focus on the inhibitory role due to previous studies linking increased TGF β ₁ levels with decreased bone mass [37].

Among the most potent compounds identified in this screen were the retinoids, metabolically active forms of Vitamin A. Reports of *in vivo* experiments clearly show enhanced bone healing after injury in mice with increased Vitamin A in their diet and those studies have suggested that retinoic acid enhances osteoblast differentiation by increasing BMP2 mRNA expression [38].

Notable compounds among the strongest hits and potential hits (Table 1, Supplementary Figure 1) included five commonly used immunosuppressant drugs (rapamycin, FK-506, FK-520, mycophenolate mofetil and mycophenolic acid) that are reported to function differently but ultimately suppress B and/or T cell proliferative responses [39–41]. We also identified four different DNA topoisomerase inhibitors as well as two actin polymerization inhibitors. Another group of interest that enhanced ALP expression were four prostaglandins (prostaglandins B1, E1, E2 and 13,14-Dihydro-prostaglandin E1). It is interesting to note that our high-throughput screen identified different compounds that fell into specific functional classes, suggesting common mechanisms of action affecting osteoblast lineage commitment. An unexpected observation for the majority of compound hits that enhanced ALP expression in our screen was their effect on cell proliferation. A general model for an inverse relationship between proliferation and differentiation has been proposed, possibly explaining why our hits would induce differentiation at the same time they prevent proliferation [42].

It has been observed that different immunosuppressant regimens used for organ transplant have varying effects post-transplantation bone loss [43, 44]. *In vitro* and *in vivo* data show conflicting results, possibly due to the wide variation of conditions and concentrations of immunosuppressant drugs used [45]. *In vitro*, FK-506 has been shown to enhance osteoblast differentiation at low concentrations (10nM-1 μ M) [46]. At higher concentrations (>25 μ M), FK-506 has been shown to inhibit osteoblast differentiation [47]. Interestingly, rat studies have indicated that FK-506 treatment was shown to decrease bone mineral density whereas rapamycin was shown to be bone sparing [48]. However, one study shows that FK-506 increased bone formation in alveolar bone of rats [49]. Recently, several studies have shown novel therapeutic roles for rapamycin, making it important to note that it should no longer just be thought of as an irrelevant immunosuppressive drug. One study showed that rapamycin was able to increase longevity in mice [50], whereas another recent study has shown that rapamycin can reverse the phenotype of Hutchinson-Gilford Progeria Syndrome cells [51]. For our screen, the immunosuppressant drugs were tested at relatively low doses (i.e. <1 μ M).

Although rapamycin and FK-506 share common signaling targets (i.e., Smad-1/5/8), they also have compound specific effects. FK-506 is a well-recognized calcineurin inhibitor and rapamycin inhibits the mammalian target of rapamycin (mTOR). Both appear to act through FKBP12 and are indicated in our graphical model (Figure 8). Calcineurin is a phosphatase that acts upon nuclear factor of activated T cells (NFAT) allowing it to translocate into the nucleus and act as a transcription factor [52]. Constitutively active NFATc1 has been shown to inhibit osteoblast differentiation and function [53]. mTOR's role in osteoblastogenesis is not as clear but it has been shown to be critical in the differentiation of mesenchymal stem

cells [18, 20, 54]. Whether rapamycin's suppression of mTOR contributes to the rescue from TGF β ₁ suppression will require further study.

The capacity for both rapamycin and FK-506 to enhance osteoblastogenesis independently of BMP-2 may be due to low but detectable levels of BMP-2 present in unstimulated cells (see Figure 3). A model describing the augmentation of BMP-2 consistent with our observations is shown in figure 8. In this model, both FK-506 and rapamycin promote phosphorylation of Smad 1/5/8. This, then, allows for the downstream signaling leading to activation of osteoblast specific genes. Either alone or in the presence of exogenous BMP-2, these compounds, or more potent derivatives, show promise for promoting osteoblast differentiation. Interestingly, only FK-506 was observed to enhance late differentiation, indicating that FK-506 and rapamycin signaling are non-redundant. One possible explanation may be differential effects on *Runx2* levels during late differentiation, explaining why rapamycin did not enhance *Ocn* and mineralization. Although FK-506 also enhances *Runx2*, it is likely that it enhances osteoblastogenesis through a *Runx2*-independent mechanism as well.

TGF β ₁ has been demonstrated to decrease osteoblast differentiation [27, 55] and *in vivo* experiments have shown that blockade of TGF β ₁ results in increased bone mineral density accompanied by increased osteoblast numbers [24]. Elevated TGF β ₁ has been implicated in several disease states. For example, TGF β ₁ is increased in the serum of HIV positive patients compared to HIV negative patients. These HIV positive patients also have increased loss of bone density compared to their HIV negative age matched controls [56, 57]. Although FK-506 and rapamycin both acted to increase osteoblast formation in our system, they were not equally efficacious in attenuating TGF β ₁ mediated decline in osteogenic signaling. Only rapamycin was able to attenuate the loss of differentiation. Figure 8 shows a hypothetical model for TGF β ₁ inhibition and the ability of rapamycin to rescue. Previous studies indicate that TGF β ₁ can signal through two different pathways; a canonical pathway that induces Smad 2/3, which blocks osteogenesis, and a non-canonical pathway that induces mTOR, also blocking osteogenesis [58, 59]. We hypothesize that when rapamycin is introduced to pre-osteoblasts blockage of mTOR leads to downstream effects on the non-canonical TGF β ₁ signaling pathway. The rapamycin-FKBP12 complex is known to bind mTOR and, thereby, block p70s6kinase (p70s6K) activation [60]. Rapamycin has been shown to potentiate osteoblast differentiation via a p70s6K dependent manner [61]. It has also been proposed that mTOR signaling affects Sp1 transcriptional activity [62]. Sp1 has previously been implicated in TGF β ₁ signaling [63] and overexpression of Sp1 resulted in six-fold increase of basal Smad7 promoter activity [64], indirectly enhancing Smad7 activity, a TGF β ₁ inducible antagonist [65, 66]. Therefore, Smad7 upregulation may assist but is insufficient for rapamycin to attenuate TGF β ₁ induced repression of osteoblast differentiation, since FK-506 also induces Smad-7. FK-506 did not appear to significantly rescue TGF β ₁ mediated decline in osteoblast differentiation. Further studies are clearly needed to dissociate the effects of rapamycin and FK-506 on TGF β ₁ signalling.

Although this screen was focused on the identification of bone promoting compounds, an added advantage of our high-throughput approach is the utility for discovery of novel pathways with desirable outcomes. For example, our screen identified a platelet activation factor (PAF) receptor antagonist as a hit that increases osteoblast formation. Having this hit allows for the further study of the role of PAF receptors in the mechanism through which bone is formed. An isolated study has reported a potential role of PAF in bone metabolism [67] but there has not been follow up on this observation, making the need for pathway analysis even more clear. High-throughput screens may also allow for the identification of multiple alternatives for use in patient oriented approaches by providing multiple alternative pathways that may overcome host specific deficits, such as genetic diseases in one pathway.

Thus, high throughput screening is a powerful technology in translational medical research (i.e., targeted therapeutics). The more obvious benefit to high throughput screens is the ability to discover new drugs quickly and efficiently. An additional benefit is the ability to use the compound hits to explore mechanisms. Through these benefits, we were able to identify an uncommon role for two common immunosuppressants. Further studies will need to be done to understand either their true potential, or derivatives thereof, as potential therapeutics in mitigating bone loss.

Highlights

- High-throughput screen identifies osteogenic compounds.
- Two immunosuppressors among many selected for secondary analysis.
- Rapamycin and FK-506 promote osteogenesis.
- Pathway analysis supports different role.
- Novel approach for identifying potential factors mitigating bone loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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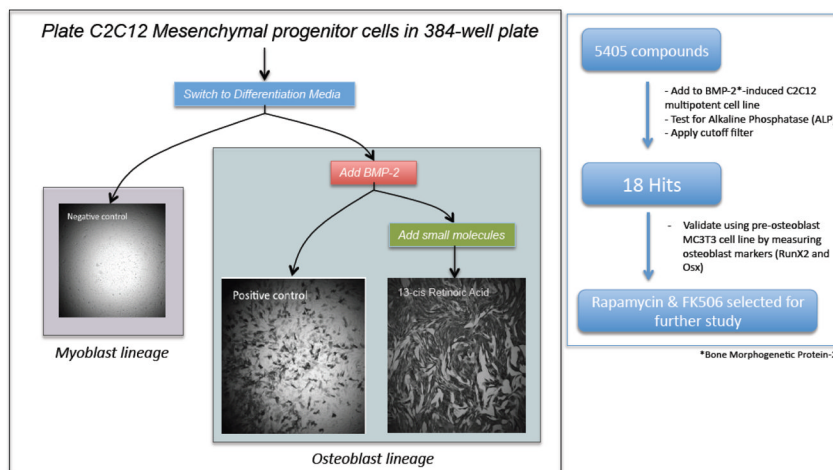


Figure 1. Protocol for high-throughput screening of small molecules to investigate BMP-2 induced promotion of osteoblast formation

A: Overview of C2C12 cells switched to DM media (low serum) Media and that received either no BMP-2 (Negative Control), BMP-2 only (Positive Control), or BMP-2 + Compound*. The level of alkaline phosphatase (ALP) activity measured colorimetrically was scored in all wells using three criteria (see methods). Compounds that enhanced ALP expression were considered for further analysis. **B:** Schematic of 5405 compounds tested on C2C12 cells in a 384-well plate format, in duplicate for BMP-2 induced ALP expression*. ALP intensity images were acquired using a Digilab Plate reader. Images were analyzed using three independent criteria and considered for secondary screening and validation in the MC3T3 pre-osteoblast cell line. Enhancement of the mRNA and protein levels of the mature osteoblast markers RunX2 and Osterix was tested. DM = differentiation media. *Plates were run in duplicate.

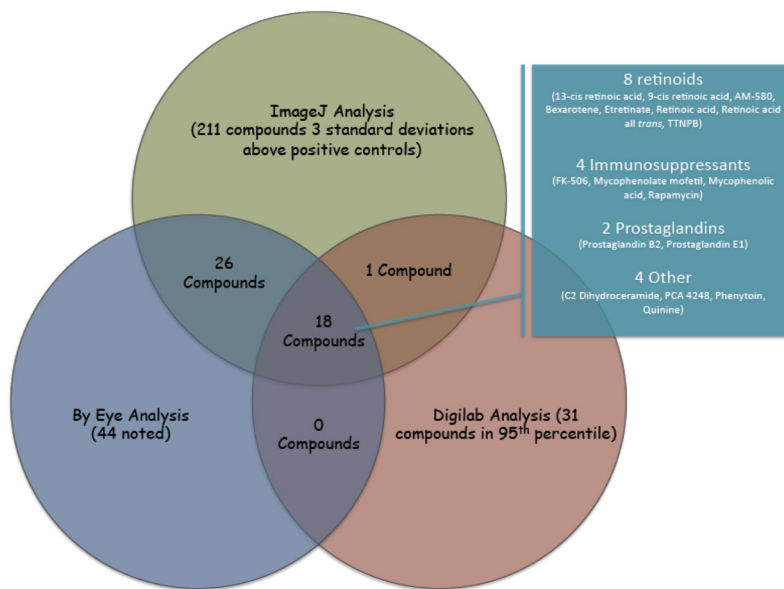


Figure 2. Venn diagram depiction of the 3 analysis approaches
 ImageJ analysis was used to find compounds that were three standard deviations above the positive controls. 211 compounds were identified with ImageJ. Digilab analysis was used to find compounds that were in the 95th percentile and 31 compounds were identified under this category. The compounds were also analyzed by visual inspection with 44 noted. Of these, 18 compounds were common to all three analyses. Functional categories of the 18 compounds are indicated in the inset box.

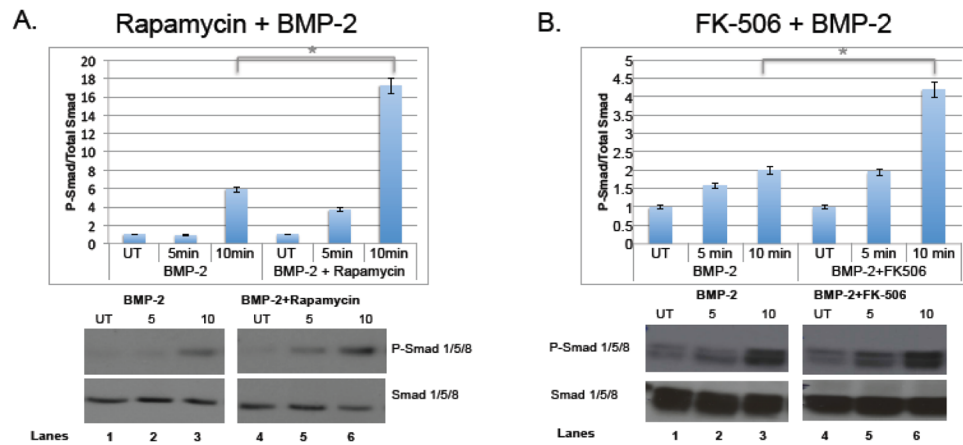


Figure 3. Rapamycin and FK-506 increase BMP-2 induced phosphorylation of Smad 1/5/8
 MC3T3 pre-osteoblast cells were plated at a density of 8×10^5 cells per 9.6 cm^2 well. BMP-2 was added to differentiate the cells with rapamycin (A) or FK-506 (B) at a concentration of 100ng/mL. Total protein was collected at 5 and 10 minutes and analyzed by western blot with antibodies to phospho-Smad 1/5/8 and total Smad 1/5/8. Graphs are shown as a ratio of phosphorylated and total Smad 1/5/8 and compared to untreated samples. Shown are representative results (average of duplicates) of at least three independent experiments. (* indicates p -value=0.001)

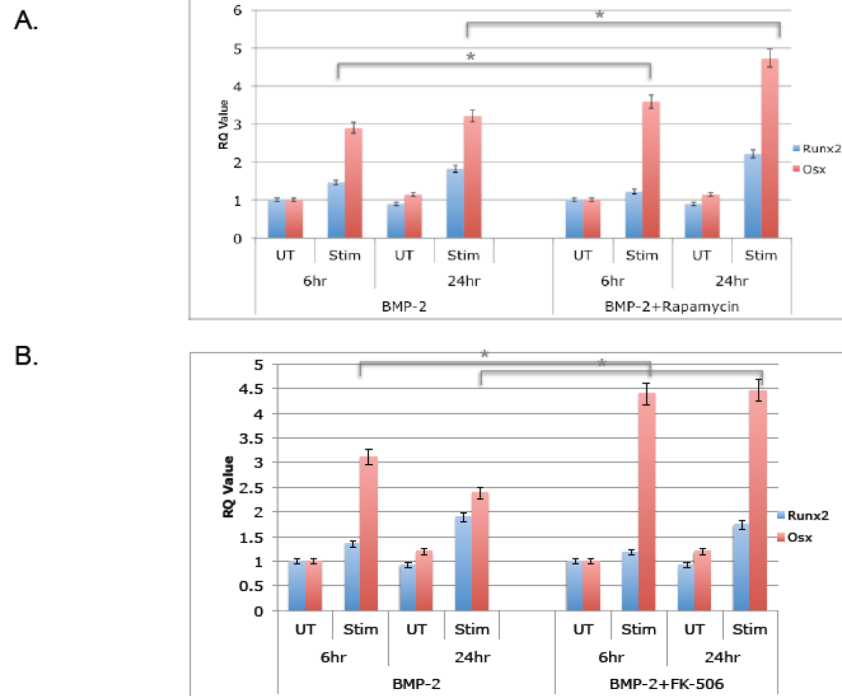


Figure 4. Rapamycin and FK-506 increase BMP-2 induced *Runx2* and *Osx* transcripts. MC3T3 pre-osteoblast cells were plated at a density of 8×10^5 cells per 9.6 cm^2 well. BMP-2 was added to differentiate the cells with rapamycin (A) or FK-506 (B) at a concentration of 100ng/mL. Cells were harvested at 6hr and 24hr time points, RNA was purified and target transcripts *Runx-2* and *Osx* were analyzed by qRT-PCR. UT=untreated and Stim=stimulated with the indicated compound. Values represent fold change compared to untreated samples after normalization using the $\Delta\Delta\text{CT}$ method. Shown are representative results (average of duplicates) of at least three independent experiments. (* Indicates p-value=0.001 for both *Runx2* and *Osx*)

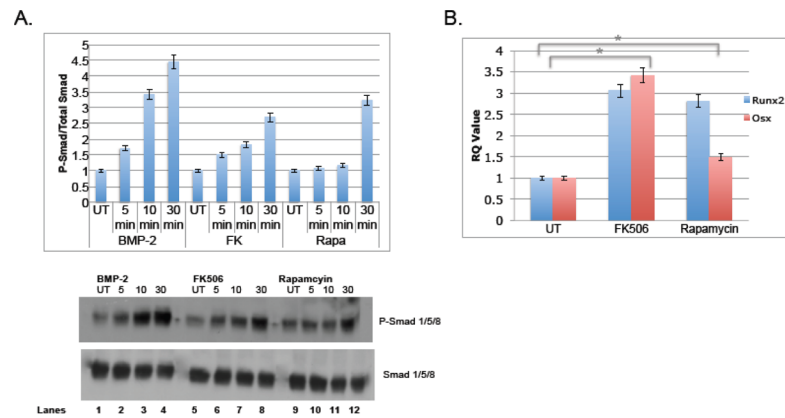


Figure 5. Rapamycin and FK-506 induce osteoblast differentiation independently of BMP-2

The same experiments were done as shown in figures 3 & 4, except rapamycin and FK-506 were added to the MC-3T3 cells without BMP-2. (A) Phosphorylation of Smad 1/5/8 was observed at 5, 10 and 30 minutes after stimulation and compared to total Smad 1/5/8 levels via western blot. The graph represents the ratio of P-Smad to total Smad and is representative of 3 independent experiments. (B) RNA was collected 24 hours after stimulation and analyzed by qRT-PCR for *Runx2* and *Osx* levels. The values for the graph were determined by $\Delta\Delta\text{CT}$ and compared to the untreated sample and were normalized to 18S. This was representative of three independent experiments (* indicates p-value=0.02 for both *Runx2* and *Osx*).

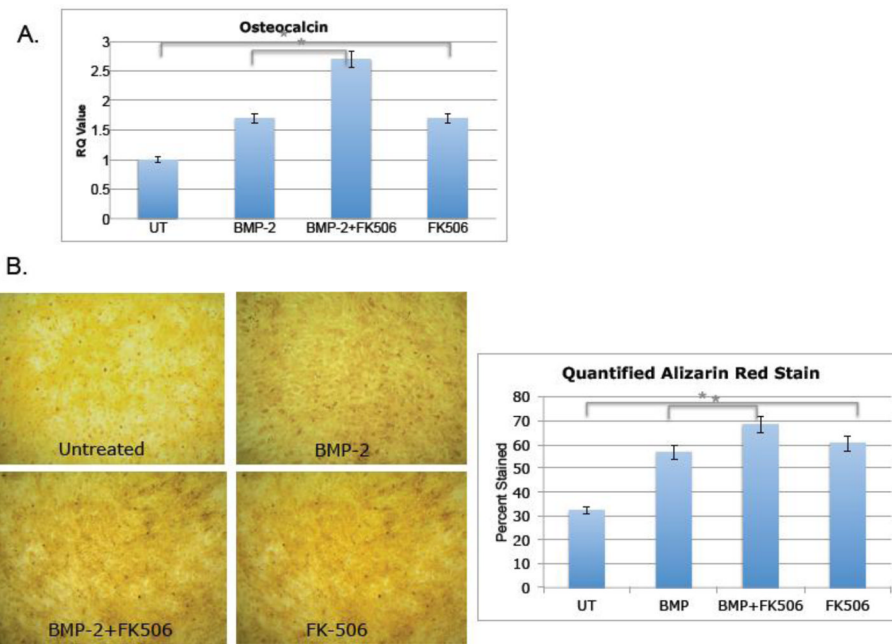


Figure 6. FK-506 induces late differentiation markers

MC-3T3 cells were plated at a density of 8×10^5 cells per 9.6 cm^2 well and treated with 100ng/ml FK-506 in the presence or absence of 100ng/ml BMP-2. (A) Media was replaced with fresh compound stimulation every two days and then RNA was collected and purified for qRT-PCR of *Ocn* mRNA transcripts on day 14. Values in the graph represent fold change compared to untreated samples after normalization using the $\Delta\Delta\text{CT}$ method. Shown are representative results (average of duplicates) of at least three independent experiments. (* indicates p -value=0.001). (B). Media was replaced with fresh compound stimulation every two days and then stained on day 21 with Alizarin-red. Pictures are representative of three independent experiments. Quantification was done using ImageJ (* indicates p -values=0.02).

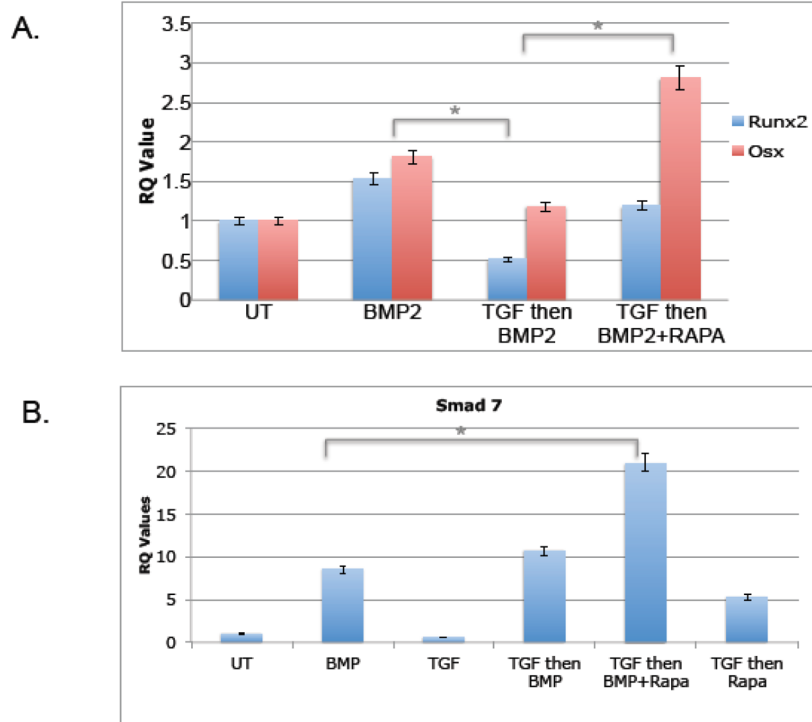


Figure 7. TGF β inhibits osteoblast differentiation and Rapamycin rescues this inhibition while increasing induction of Smad7 transcripts. MC3T3 cells were treated with media containing 1ng/mL of TGF β ₁ for 24 hours and then the media was replaced with BMP-2 or BMP-2 with 100ng/ml rapamycin. RNA was collected 24 hours afterwards and analyzed by qRT-PCR and the $\Delta\Delta$ CT method. (A) Values of Runx2 and Osx transcripts are represented as fold change compared with untreated samples (* indicates p-value=0.02). (B) Values of Smad 7 transcripts. Shown are representative results (average of duplicates) of at least three independent experiments. (* indicates p-value=0.03)

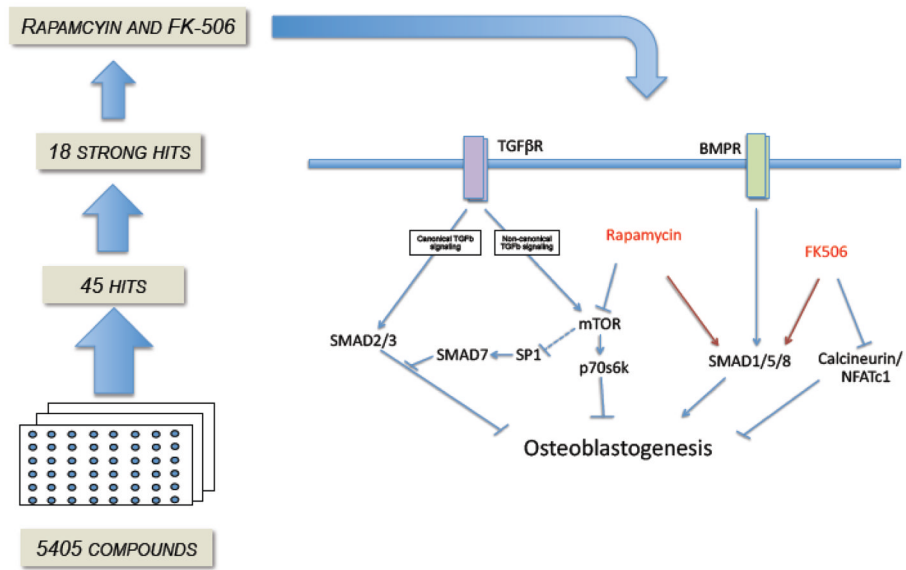


Figure 8.
Graphical model of role for Rapamycin and FK-506 promoting osteoblastogenesis.

Table 1**Small compound libraries**

5 different chemical libraries were used and the number of compounds in each library is indicated in the table. The number of hits per library is broken down into “strongest hits” and “potential hits.” Strongest hits were characterized as compounds that were positive in all three analyses, whereas potential hits were characterized as compounds that were positive in two of the three approaches. The **BU-CMLD** is composed of stereochemically and structurally complex chemical libraries (sprioketal, epoxyquinol, oxime, macrodiolide, diketopiperazine, cyclic ether). This library uniquely probes three-dimensional space by employing stereochemical and positional variation within the molecular framework as diversity elements for library design. The **NIH** and **FDA** approved drug library, and **ICCB** are comprised of small molecules that are all known bioactives. These collections were assembled to affect a wide variety of biological pathways. The **Chembridge** represent drug-like small molecules, rationally selected based on 3D pharmacophore analysis to cover the broadest part of biologically relevant pharmacophore diversity space.

Library Name	Number of Compounds	Strongest Hits	Potential Hits
NIH	446	1	6
ICCB	480	13	13
FDA	640	4	3
BUCMLD	1839	0	1
ChemBridge	2000	0	4
Total:	5405	18	27