Ixazomib enhances parathyroid hormone-induced β-catenin/T-cell factor signaling by dissociating β-catenin from the parathyroid hormone receptor.

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Ixazomib enhances parathyroid hormone–induced β-catenin/T-cell factor signaling by dissociating β-catenin from the parathyroid hormone receptor

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Abbreviations used: CREB, cAMP response element–binding protein; CRE-luc, cAMP response element–luciferase; GSK3β, glycogen synthase kinase 3β; GST, glutathione-S-transferase; Izb, ixazomib; PKA, protein kinase A; PLC, phospholipase C; PTH, parathyroid hormone; PTHR, PTH receptor; TCF, T-cell factor.

INTRODUCTION

Long after Bauer and colleagues discovered the anabolic effect of parathyroid hormone (PTH) in 1929 (Bauer et al., 1929), recombinant PTH(1-34) (teriparatide) was approved as the first anabolic agent for the treatment of osteoporosis in the United States in 2002, and no other anabolic drugs are on the market. The optimal use of PTH therapy in osteoporosis depends in part on our understanding of the regulation of PTH signaling to maintain the anabolic actions of PTH while mitigating its catabolic effects. PTH action in bone is mediated by type 1 PTH receptor (PTHR), a member of the G protein–coupled receptor superfamily. Stimulation of PTHR by PTH on osteoblasts leads to activation of Gαs and Gαq, with consequent induction of cAMP/protein kinase A (PKA) and phospholipase C (PLC)/PKC signaling pathways, which results in both osteoblast formation and osteoclast resorption (Qin et al., 2004; Cheloha et al., 2015). Whereas anabolic PTH effects in bone are mediated mostly through the cAMP/PKA signaling pathway, PLC/PKC signaling has been shown to be inhibitory to the osteoanabolic actions of PTH (Ogata et al., 2011). Wnt/β-catenin signaling controls bone formation and homeostasis by increasing osteoblast differentiation and inhibiting osteoclastogenesis (Gaur et al., 2005; Glass et al., 2005;
transmembrane domain (Nelson and Nusse, 2004; Stepniak
findings highlight the role of
some inhibitor bortezomib has been used as an effective therapy for
2014; Muz
differentiation and bone-resorptive activity to mitigate the catabolic
reported that carfilzomib suppressed PTH stimulation of osteoclast
2015) reported that N-cadherin modulated LRP6-PTHR interaction,
questration at the plasma membrane (Stepniak
β
cadherin abundance on the cell surface and results in
2016). The interaction of N-cadherin with
cadherins in cell adhesion (Nelson and Nusse, 2004; Stepniak et
2009; Marie et al., 2014). Cadherins, which have a single-pass trans-
membrane domain, bind to β-catenin through their carboxy-terminal
domain at the cytoplasmic tail. Thus β-catenin serves as a link
between cadherins and the actin cytoskeleton to mediate the role of
cadherins in cell adhesion (Nelson and Nusse, 2004; Stepniak et
2009; Marie et al., 2014). In addition, β-catenin also binds to numerous
other proteins in a cadherin-independent manner (Bienz and Clevers, 2003; Yano et al., 2013; Yang and Wang, 2015). In bone,
N-cadherin associates with β-catenin at the cell membrane to regulate
osteoblastogenesis by limiting Wnt signaling. Revollo et al.
2015) reported that N-cadherin modulated LRP6-PTHR interaction,
restrained the intensity of PTH-induced β-catenin signaling, and reduced bone formation in response to intermittent PTH administration.
Moreover, N-cadherin restrains PTH's repressive effects on sclerostin/SOST by regulating LRP6-PTHR interaction (Yang et al., 2016). The interaction of N-cadherin with β-catenin causes increased cadherin abundance on the cell surface and results in β-catenin sequestration at the plasma membrane (Stepniak et al., 2009; Marie et al., 2014). The outcome reduces β-catenin nuclear translocation and decreases TCF/lymphoid enhancer factor–dependent transcriptional activity. Accordingly, blockade of β-catenin degradation may enhance PTH stimulation of β-catenin/TCF signaling.

The ubiquitin-proteasome pathway plays an important role in regulating and controlling bone metabolism (Murray et al., 1998; Garrett et al., 2003; Yang et al., 2015). The first-generation proteasome inhibitor bortezomib has been used as an effective therapy for the treatment of multiple myeloma, a disease characterized by an increase in the activity of osteoclasts and a decrease in the function of osteoblasts adjacent to tumor cells in the bone marrow (Pennisi et al., 2009). Carfilzomib, a next-generation selective proteasome inhibitor, exhibits potent antmyeloma efficacy compared with bortezomib (Hemdon et al., 2013; Berenson et al., 2014). We previously reported that carfilzomib suppressed PTH stimulation of osteoclast differentiation and bone-resorptive activity to mitigate the catabolic effects of PTH (Yang et al., 2015). A significant limitation of treatment with bortezomib and carfilzomib is that both drugs are administered intravenously or subcutaneously, which is inconvenient for patients and increases treatment cost. Ixazomib (lzb) is a small-molecule proteasome inhibitor that overcomes these limitations. In November 2015, lzb became the first orally administered proteasome inhibitor approved in the United States as an effective therapy for multiple myeloma. This effect is mediated in part through inhibition of pathologic bone destruction (Kupperman et al., 2010; Garcia-Gomez et al., 2014; Muz et al., 2016). Proteasome inhibitors such as bortezomib and carfilzomib directly stabilize β-catenin protein and increase free cytosolic β-catenin and β-catenin nuclear translocation (Qiang et al., 2009; Hu et al., 2013). However, how proteasome inhibition regulates the complex interplay between PTHR and β-catenin to in turn regulate β-catenin/TCF signaling has not been elucidated.

In the present study, we show that lzb reverses the β-catenin–mediated PTHR signaling switch by enhancing PTH-induced cAMP formation and cAMP response element-luciferase (CRE-luc) reporter gene activity and reducing PTH-stimulated intracellular calcium mobilization in osteoblasts. Izb enhances PTH-induced β-catenin/TCF signaling by separating β-catenin from the PTHR and promoting β-catenin translocation.

**RESULTS**

**Knockout of β-catenin increases PTH-induced cAMP formation and reduces intracellular calcium in osteoblasts**

Recent findings show that β-catenin switches the PTHR signaling by binding to the intracellular carboxyl-terminal region of the PTHR in both chondrocytes and HEK293 cells (Yano et al., 2013; Yang and Wang, 2015). In an initial set of experiments, we examined whether β-catenin also switched the PTHR signaling in osteoblasts. Saos2 osteoblastic cells endogenously express both β-catenin and the PTHR (Wang et al., 2009). These cells were transfected with a clustered regularly interspaced short palindromic repeats (CRISPR) construct or CRISPR control plasmid. After 48 h of transfection, the cells were transferred into a 96-well plate with one cell per well by a serial dilution. Two single colonies were identified for knockout of β-catenin expression in Saos2 cells (hereafter referred to as Saos2-β-Cat-KO-3 and Saos2-β-Cat-KO-10 cells; Figure 1A), whereas no β-catenin expression was reduced in cells transfected with control plasmid (Saos2-β-Cat-Ctr; Supplemental Figure S1).

To assess the effect of β-catenin on PTH-induced Gs/cAMP signaling, we conducted PTH stimulation of cAMP generation in Saos2-β-Cat-KO-3 cells (β-Cat KO) and their control cells (Saos2-β-Cat-Ctr-1, WT Ctr). Knockout of β-catenin significantly increased PTH(1-34) (hereafter referred to as PTH) stimulation of cAMP formation (Figure 1B). To evaluate PTHR-mediated Gs/GLC signaling, we measured intracellular calcium mobilization ([Ca2+]i), an index of PLC activity, in Saos2-β-Cat-KO-3 cells and Saos2-β-Cat-Ctr-1 cells loaded with the calcium-sensitive dye Fluo-4 AM. Knockout of β-catenin markedly inhibited PTH-induced [Ca2+]i (Figure 1C). Similar results also occurred in Saos2-β-Cat-KO-10 and Saos2-β-Cat-Ctr-2 cells (unpublished data). Collectively these data clearly demonstrate that knockout of β-catenin reverses the PTHR signaling switch to increase Gs/cAMP signaling and reduce Gs/GLC activation, which favors the anabolic PTH action in bone.

**lzb enhances PTH-induced cAMP formation in a time- and concentration-dependent manner**

We previously reported that proteasome inhibitors stabilized β-catenin by the ubiquitin-proteasome pathway (Qiang et al., 2009; Hu et al., 2013). Because β-catenin switches PTHR signaling, we next determined whether lzb regulated PTHR activation. lzb alone was not able to stimulate cAMP formation (Supplemental Figure S2). However, pretreatment of lzb enhanced PTH stimulation of cAMP formation in Saos2 cells, which exhibited time and concentration dependence. Maximal stimulation by lzb was achieved at 3 h (Figure 2A), but prolonged lzb treatment tended to reduce its effect on PTH-induced cAMP formation. At the 3 h, lzb elicited a concentration-dependent increase in PTH-induced cAMP formation over the range 12.5–100 nM (Figure 2B), but higher concentrations of lzb also decreased its effect on PTH stimulation of cAMP production.
Materials and Methods

After 24 h of culture, cells were serum starved overnight. PTH (100 nM) stimulation of intracellular cAMP accumulation (B) or PTH (1 µM) induction of intracellular calcium (C) was performed with primary antibodies of mouse monoclonal β-catenin (β-Cat) antibody plus rabbit polyclonal actin antibody and then with secondary antibodies (goat anti-mouse antibody [red] plus goat anti-rabbit antibody [green]). Actin was used for loading control. (B, C) Saos2-β-Cat-KO-3 cells (β-Cat KO) and control Saos2-β-Cat-KO-1 cells (WT Ctr) were set up in a 24- or 96-well plate. After 24 h of culture, cells were serum starved overnight. PTH (100 nM) stimulation of intracellular cAMP accumulation (B) or PTH (1 µM) induction of intracellular calcium (C) was measured as described in Materials and Methods. Data are summarized as mean ± SE of triplicate measurements. n = 4. *p < 0.05, **p < 0.01, compared with WT Ctr cells treated with vehicle; †p < 0.05, ‡p < 0.01, compared with WT Ctr cells treated with PTH; ††p < 0.05, ‡‡p < 0.01, compared with β-Cat KO cells treated with vehicle.

Similar to other proteasome inhibitors (Qiang et al., 2009; Hu et al., 2013), Izb but not PTH caused slight cytotoxicity when the cells were treated with it for a prolonged time or at higher concentrations (Supplemental Figure S3, A–C). To reduce the cytotoxicity of Izb, we pretreated Saos2 cells with Izb or vehicle for the first 3 h, followed by an additional 5 h of culture in Izb-free medium, which mimics the structure. Because Izb increases active forms of β-catenin and promotes β-catenin translocation, we asked whether Izb was able to separate β-catenin from the PTHR at the plasma membrane. Saos2 cells were transfected with pCDNA3.1 vector, hemagglutinin (HA)-PTHR, and/or Flag–β-catenin as indicated. After 48 h of transfection, the cells were treated with vehicle or Izb (100 nM) for 3 h, followed by an additional 5 h of culture in Izb-free medium. The membrane proteins were isolated, and the interaction of Flag–β-catenin with HA-PTHR was performed by immunoprecipitation assay. The result in Figure 3C shows that treatment with Izb reduced the interaction of β-catenin with the PTHR at the plasma membrane, further confirming that Izb promotes β-catenin translocation to cytosol and nucleus. As a complementary experiment, Saos2 cells were transiently transfected with green fluorescent protein (GFP)-PTHR. After 48 h of transfection, the cells were treated with vehicle or Izb as before. There was a colocalization of β-catenin with the PTHR at the plasma membrane in the absence of Izb, whereas Izb reduced this colocalization (Figure 3D). Collectively, Izb enhanced PTH-induced cAMP generation due to the separation of β-catenin from the PTHR.

Izb regulation of the PTHR signaling is β-catenin dependent

To test whether the specificity of Izb effect on PTH-induced cAMP production is due to separation of β-catenin from the PTHR, we used both wild-type and β-catenin–knockout Saos2 cells. Indeed, Izb failed to enhance PTH-induced cAMP formation and reduce intracellular calcium mobilization in β-catenin–knockout cells compared with that of wild-type Saos2 control cells (Figure 4).

Izb promotes PTH-induced CRE-luc activity and cAMP response element–binding phosphorylation

We next determined the effect of Izb on cAMP/PKA downstream signaling in osteoblasts. The active catalytic subunit of PKA stimulates CRE-luc activity (Castellone et al., 2005). Wild-type Saos2 control cells or Saos2-β-Cat-KO cells were infected with lentiviral particles containing CRE-luc reporter gene. The cells were pretreated with Izb (100 nM for 1 h) before PTH (100 nM) was added to the culture for another 2 h. The CRE-luc activity was then measured. As shown in Figure 5A, Izb itself was not able to stimulate CRE-luc activity in wild-type control or β-catenin–knockout cells. However,
Knockout of β-catenin significantly increased PTH-induced CRE-luc activity. Izb failed to enhance PTH stimulation of CRE-luc activity in β-catenin-knockout cells compared with wild-type control cells. cAMP response element–binding (CREB) protein signaling plays a key role in regulating osteoblast activity and bone formation (Long et al., 2001). PTH can induce phosphorylation of CREB through the cAMP/PKA signaling pathway (Pearman et al., 1996; Revollo et al., 2008). Saos2 wild-type cells were pretreated with Izb (100 nM) for 2 h before PTH (100 nM) was added to the culture for another 1 h. PTH markedly induced CREB phosphorylation (Figure 5B). Izb by itself had no effect on phosphorylation of CREB but facilitated PTH stimulation of CREB phosphorylation. Total CREB abundance was unaffected in these cells (Figure 5C), further confirming that effects of Izb on PTHR downstream events are β-catenin dependent and mediated by the cAMP/PKA signaling pathway.

**Izb enhances PTH stimulation of GSK3β phosphorylation at Ser-9 and β-catenin phosphorylation at Ser-675**

The stability of β-catenin is regulated by a multiprotein complex, which includes adenomatous polyposis coli, GSK3β, and axin. PKA phosphorylates GSK3β at the amino acid Ser-9, which inhibits its kinase activity and then inactivates the β-catenin destruction complex (Castellone et al., 2005; Suzuki et al., 2008). PTH induces β-catenin/TCF signaling via inactivation of GSK3β in osteoblasts (Suzuki et al., 2008). Saos2 cells were pretreated with Izb (100 nM) for 2.5 h, and PTH (100 nM) was then added for another 30 min. GSK3β functions upstream of β-catenin activation. As expected, Izb by itself has no effect on GSK3β phosphorylation at Ser-9 (Figure 6A). PTH treatment increased GSK3β phosphorylation, and Izb further enhanced PTH stimulation of GSK3β phosphorylation.

PKA-phosphorylated β-catenin at Ser-675 increases β-catenin accumulation in the cytosol, thereby promoting β-catenin downstream signaling (Taurin et al., 2006; Revollo et al., 2015). We hypothesized that Izb enhanced PTH-induced phosphorylated β-catenin at this site. To test this idea, we pretreated Saos2 cells with ibz for 1 h. Then we added 100 nM PTH to culture for another 2 h. The data in Figure 6B show that PTH stimulated β-catenin phosphorylation at Ser-675. Izb but not PTH slightly increased total β-catenin abundance. However, Izb and PTH synergistically increased β-catenin (Ser-675) phosphorylation, although Izb on its own had no notable effect on the phosphorylation of β-catenin.

**Izb promotes PTH-induced β-catenin/TCF signaling in osteoblasts**

PTH-facilitated β-catenin/TCF signaling is cAMP/PKA dependent (Kulkarni et al., 2005; Suzuki et al., 2008). Our data show that Izb enhanced PTH-induced cAMP formation and promoted PTH stimulation of GSK3β and β-catenin phosphorylation. These findings suggest an additional mechanism by which Izb enhances PTH-induced β-catenin/TCF signaling in osteoblasts. To test this idea, we transfected Saos2 cells with TOPFlash or FOPFlash plasmid. The cells were treated with Izb for the first 3 h, followed by an additional 5 h culture in Izb-free medium in the presence of PTH (100 nM) in Saos2 cells. Cytotoxicity was assessed by MTT assay as described in Materials and Methods. Data are summarized as mean ± SE of triplicate measurements. n = 3. *p < 0.05, **p < 0.01, compared with cells treated with vehicle; †p < 0.05, ‡p < 0.01, compared with cells treated with PTH.
synergistically increased nuclear \(\beta\)-catenin levels and \(\beta\)-catenin/TCF activation. Similar results were also identified in mouse osteoblasts (Supplemental Figure S7). PKA inhibitor H89 but not PKC inhibitor Bis I inhibited PTH-stimulated TCF reporter activity (Figure 7B), confirming that PTH-stimulated \(\beta\)-catenin/TCF signaling is cAMP/PKA dependent. However, H89 only partially reversed the effect of Izb on PTH stimulation of \(\beta\)-catenin/TCF activation. Furthermore, H89 failed to affect Izb increase of free cytosolic \(\beta\)-catenin level, nuclear \(\beta\)-catenin expression (Supplemental Figure S8, A and B), and \(\beta\)-catenin/TCF signaling (Supplemental Figure S8D). H89 also had no effect on \(\beta\)-catenin phosphorylation at Ser-675 in the presence of Izb (Supplemental Figure S8C). These data clearly demonstrate that the effect of Izb alone on \(\beta\)-catenin translocation and \(\beta\)-catenin/TCF signaling is not related to a cAMP/PKA pathway. Together the results show that, in addition to being capable of promoting \(\beta\)-catenin translocation, Izb enhances PTH stimulation of \(\beta\)-catenin/TCF signaling via an increase in cAMP-dependent signaling.

**DISCUSSION**

Although PTHR signaling pathways are being studied in increasing detail, the regulation of PTHR functions has not been fully elucidated. The anabolic PTH effects on bone are mostly mediated by the G\(\alpha_s/cAMP\) signaling pathway, whereas G\(\alpha_q/PLC\) activation may antagonize these osteoanabolic actions (Datta and Abou-Samra, 2009;...
signaling (Datta and Abou-Samra, 2009; Romero s/cAMP activation and increasing α to G Wnt. Therefore manipulating PTHR signaling by shifting Gvolves the canonical
NHERF1 increases PTH-stimulated cAMP accumulation (Wheeler amino acids, which interact with NHERF1 and NHERF2 (Romero has a PSD-95/Discs large/ZO-1 (PDZ) motif located in the last four
αq/PLC activation in chondrocytes and HEK293 cells (Yano gion of the PTHR and switches the PTHR signaling from G
2007). In contrast to the aforementioned regulatory proteins, kCat KO cells treated with PTH; k
-Cat KO cells treated with Izb (100 nM) for 3 h. PTH stimulation of cAMP formation (A) and intracellular calcium (B) were measured. Data are summarized as mean ± SE of triplicate measurements. n = 3. *p < 0.05, t
-Cat KO cells treated with vehicle; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with vehicle; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with PTH; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with Izb; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with vehicle; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with PTH; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with Izb.

FIGURE 4: Izb blocks β-catenin–mediated PTHR signaling switch. Saos2-β-Cat-KO-3 cells (β-Cat KO) and their control cells (Saos2-β-Cat-Ctr-1, WT Ctr) were seeded onto a 24- or 96-well plate. After confluence, the cells were serum starved overnight and then pretreated with Izb (100 nM) for 3 h. PTH stimulation of cAMP formation (A) and intracellular calcium (B) were measured. Data are summarized as mean ± SE of triplicate measurements. n = 3. *p < 0.05, t
-Cat KO cells treated with vehicle; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with vehicle; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with PTH; *p < 0.05, *p < 0.01, compared with WT Ctr cells treated with Izb; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with vehicle; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with PTH; *p < 0.05, *p < 0.01, compared with β-Cat KO cells treated with Izb.

Ogata et al., 2011). Additional anabolic signaling by PTH action involves the canonical β-catenin pathway, which is independent of Wnt. Therefore manipulating PTHR signaling by shifting Gαq/PLC to Gαs/cAMP activation and increasing β-catenin/TCF signaling represents a means of maintaining the anabolic actions of PTH.

Sequences located at the carboxyl terminus of PTHR control its signaling (Datta and Abou-Samra, 2009; Romero et al., 2011). PTHR has a PSD-95/Discs large/ZO-1 (PDZ) motif located in the last four amino acids, which interact with NHERF1 and NHERF2 (Romero et al., 2011; Vilaradaga et al., 2011). As originally described by Mahon et al. (2002), NHERF2, a NHERF1 homologue, markedly inhibits adenylyl cyclase by stimulating inhibitory Gαi proteins in PS120 cells transfected with the PTHR. In contrast, no differences of PTH-stimulated cAMP formation were noted between wild-type and NHERF1-null proximal tubule cells (Cunningham et al., 2005) in the presence or absence of NHERF1. In ROS17/2.8 cells or primary osteoblasts, NHERF1 increases PTH-stimulated cAMP accumulation (Wheeler et al., 2008; Wang et al., 2013). Moreover, truncation of the carboxyl-terminal region of the PTHR that lacks determinants for stable β-arrestin association (PTHR-480stop) enhances PTH stimulation of adenylyl cyclase but not PLC (Iida-Klein et al., 1995; Wang et al., 2007). In contrast to the aforementioned regulatory proteins, β-catenin specifically binds to the intracellular carboxyl-terminal region of the PTHR and switches the PTHR signaling from Gαs/cAMP to Gαq/PLC activation in chondrocytes and HEK2923 cells (Yano et al., 2013; Yang and Wang, 2015). As an initial step, we determined whether this signaling switch by β-catenin also occurred in osteoblasts. To that end, we generated a β-catenin–knockout cell line in osteoblasts using CRISPR/Cas9 genome-editing technology. Our data showed that knockout of β-catenin (i.e., zero expression of β-catenin) significantly increased PTH-induced cAMP formation, whereas PTH-stimulated PLC activity was markedly reduced in these knockout cells compared with cells transfected with CRISPR/Cas9 control plasmid (Figure 1). These data are consistent with a previous study, using small interfering RNA, demonstrating that knockdown of β-catenin (i.e., reduction of β-catenin expression) inhibited the PTHR signaling switch (Yano et al., 2013). These findings raised an important question of whether the intervention of β-catenin interaction with PTHR in osteoblasts has a similar effect to alter PTH-induced PTHR signaling. Proteasome inhibitors such as bortezomib and carfilzomib, which have been used to treat multiple myeloma patients, were shown to directly stabilize β-catenin protein and increase β-catenin nuclear translocation (Qiang et al., 2009; Hu et al., 2013). In the present study, we chose Izb, the first orally administered proteasome inhibitor for the treatment of multiple myeloma, because it has the potential to treat resorptive bone diseases such as osteoporosis. We found that Izb increased active β-catenin expression and translocation (Figure 3 and Supplemental Figure S5). Most importantly, we found, using coimmunoprecipitation (coIP) and confocal microscopy, that Izb was able to separate β-catenin from the PTHR at the cell membrane. Pretreatment of Izb increased PTH-stimulated cAMP production and reduced PTH-induced PLC activity (Figure 4 and Supplemental Figure S4), demonstrating that Izb can inhibit the PTHR signaling switch to increase cAMP formation in osteoblasts by promoting the dissociation of β-catenin from the PTHR. To the best of our knowledge, this is the first report showing how a proteasome inhibitor regulates PTHR signaling by dissociating β-catenin from the PTHR.

It is established that PTH-induced β-catenin/TCF signaling is cAMP/PKA dependent (Kulkarni et al., 2005; Suzuki et al., 2008). CRE-luc activity and CREB phosphorylation are downstream events that depend on cAMP/PKA activation. We demonstrated that Izb by itself had no effect on CRE-luc activity and CREB phosphorylation but enhanced PTH stimulation of CRE-luc activity and phosphorylation of CREB (Figure 5). Furthermore, Izb and PTH synergistically increased GSK3β phosphorylation at Ser-9 and phosphorylated β-catenin at Ser-675 (Figure 6), the outcome of which results in β-catenin translocation to the nucleus. It is known that PTH-induced β-catenin/TCF activation takes >6 h. Similar to other proteasome inhibitors, Izb treatment in cells for >3 h has some cytotoxicity (Boissy et al., 2008). To maintain Izb biological activity and avoid its toxicity, Saos2 cells received Izb for the first 3 h, followed by an additional 5 h of culture in Izb-free medium. Our findings show that Izb enhanced PTH-induced β-catenin/TCF signaling without inducing cytotoxicity (Figures 2 and 7). Therefore our work reveals a novel role for Izb in regulating PTH-induced β-catenin/TCF signaling via the cAMP/PKA-dependent pathway.

The increase of cytosolic and nuclear β-catenin by proteasome inhibition is via its specific inhibition of β-catenin degradation but independent of Wnt ligands and not related to GSK3β phosphorylation (Qiang et al., 2009). In the present study, we demonstrated that Izb by itself does not affect GSK3β phosphorylation at Ser-9 and β-catenin phosphorylation at Ser-675. Thus the underlying mechanisms by which Izb induces β-catenin/TCF signaling are not fully understood. Proteasome inhibition can stabilize numerous proteins. PTHR directly interacts with Dishevelled to regulate β-catenin signaling (Romero et al., 2010). Zhou et al. (2016) recently reported that ubiquitin-specific protease 4 strongly inhibited Wnt/β-catenin signaling by removing lysine-63 linked polyubiquitin chain from Dishevelled and antagonized osteoblast differentiation.
Cell treatment with PTH plus Izb. (C) Izb fails to affect PTH stimulation of CREB expression in four independent experiments presented as mean ± SE. a   p < 0.01, compared with cells treated with vehicle; b   p < 0.05, compared with WT Ctr cells treated with PTH; c   p < 0.05, compared with β-Cat KO cells treated with vehicle; d   p < 0.05, compared with β-Cat KO cells treated with PTH; e   p < 0.05, compared with cells treated with Izb; f   p < 0.01, compared with cells treated with PTH plus Izb. 

**FIGURE 5:** Izb enhances PTH-induced CRE-luc activity and CREB phosphorylation. (A) Knockout of β-catenin increases PTH-induced CRE-luc activity. Saos2-β-Cat-KO-3 cells (β-Cat KO) and their control cells (Saos2-β-Cat-Ctr-1, WT Ctr) were seeded onto a 24-well plate. After cells reached 80% confluence, the cells were infected with lentiviral particles of CRE-luc reporter and cultured for 48 h. The cells were serum starved overnight and then pretreated with Izb (100 nM for 1 h) before PTH (100 nM) was added to the culture for another 2 h. PTH induction of CRE-luc activity in each group was measured as described in Material and Methods. Data are summarized as mean ± SE of triplicate measurements. n = 3. * p < 0.05, ** p < 0.01, compared with WT Ctr cells treated with vehicle; † p < 0.05, ‡ p < 0.01, compared with WT Ctr cells treated with PTH; § p < 0.05, ¶ p < 0.01, compared with WT Ctr cells treated with Izb; †† p < 0.05, ‡‡ p < 0.01, compared with WT Ctr cells treated with PTH plus Izb; ††† p < 0.05, ‡‡‡ p < 0.01, compared with WT Ctr cells treated with β-Cat KO cells treated with vehicle; †††† p < 0.05, ‡‡‡‡ p < 0.01, compared with β-Cat KO cells treated with PTH; ††††† p < 0.05, ‡‡‡‡‡ p < 0.01, compared with β-Cat KO cells treated with Izb. (B) Izb enhancement of PTH-induced CREB phosphorylation is cAMP/PKA dependent. Saos2-β-Cat-Ctr-1 cells (WT Ctr) were pretreated with Izb (100 nM for 2 h) before PTH (100 nM) was added to the culture for another 1 h in the presence or absence of PKA inhibitor H89 (10 µM) or PKC inhibitor Bis I (10 µM) for 1 h and 15 min. Left, PTH-induced CREB phosphorylation and total CREB expression (loading control). Right, quantified CREB phosphorylation and total CREB expression in four independent experiments presented as mean ± SE. * p < 0.05, ** p < 0.01, compared with cells treated with vehicle; † p < 0.05, ‡ p < 0.01, compared with cells treated with PTH; § p < 0.05, ¶ p < 0.01, compared with cells treated with Izb; †† p < 0.05, ‡‡ p < 0.01, compared with cells treated with PTH plus Izb. (C) Izb fails to affect PTH stimulation of CREB phosphorylation in β-catenin-knockout cells. Saos2-β-Cat-KO-3 cells (β-Cat KO) cells were pretreated with Izb (100 nM for 2 h) before PTH (100 nM) was added to the culture for another 1 h. Left, PTH stimulation of CREB phosphorylation. Right, quantified CREB phosphorylation and total CREB expression in three independent experiments presented as mean ± SE. * p < 0.05, ** p < 0.01, compared with cells treated with vehicle; † p < 0.05, ‡ p < 0.01, compared with cells treated with PTH; § p < 0.05, ¶ p < 0.01, compared with cells treated with Izb; †† p < 0.05, ‡‡ p < 0.01, compared with cells treated with PTH plus Izb.
and osteogenesis in vitro and therapeutic efficacy in animal models of osteoporosis.

MATERIALS AND METHODS

Human [Nor8,18,Tyr34]PTH(1-34) was purchased from Bachem California (Torrance, CA). Carfilzomib was purchased from LC Laboratories (Woburn, MA), and Izb was obtained from Selleck Chemicals (Torrance, CA). Carfilzomib was purchased from LC Laboratories (Woburn, MA), and Izb was obtained from Selleck Chemicals (Torrance, CA). Calbiochem (San Diego, CA). The Cyclic AMP Direct EIA Kit was purchased from Arbor Assays (Ann Arbor, MI). Ionomycin was from EMD Millipore (Billerica, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell culture

Saos2 cells, a human osteoblast–like cell line, were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM/F-12 with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. MC4 osteoblastic cells (subclone 4 of MCT3-E1; ATCC) were cultured in α-MEM with 10% FBS in the presence of 50 μg/ml ascorbic acid for 5 d and then used in the experiments. All cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Primary osteoblast culture

All of the experiments using mice for generation of primary osteoblasts were performed according to the protocol approved by the Animal Care and Use Committee of Thomas Jefferson University. Generation of primary osteoblast cultures was described previously (Yang et al., 2015). Briefly, calvariae were removed from 2- to 3-old C57BL/6 mice and digested with 1 mg/ml collagenase type II and 0.25% trypsin-EDTA. Cells released from the second and third digestions were grown in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After trypsinization of the confluent cells, differentiating osteoblasts were cultured in the presence of 50 μg/ml ascorbic acid for 7 d and used in the experiments.

CAMP formation and detection

Saos2 cells were transfected with Glosensor CAMP reporter plasmid (pGS-22F) in a six-well plate by Lipofectamine 2000 following the manufacturer’s instruction. After 36 h of transfection, cells were transferred into a 96-well plate. The confluent cells were then treated with vehicle or Izb (100 nM) at the indicated time points. After treatment, cells were incubated with luciferin for 20 min at room temperature, and then PTH or vehicle was added. Luminescence arising from intracellular CAMP binding to the Glosensor reporter protein was measured in real time at 2-min intervals for 60 min using a plate reader (Synergy-2; Gidion et al., 2014; Carter et al., 2015). The peak response time occurred by 16 min.

Additional CAMP measurements were performed in primary osteoblasts and Saos2-β-Cat-KO cells and Saos2-β-Cat-Ctr cells. These cells were treated with vehicle or Izb (100 nM for 3 h). PTH (100 nM for 15 min)-induced CAMP formation was detected using the Cyclic AMP Direct EIA Kit (Arbor Assays) following the supplier’s instructions. The samples were analyzed by using a CAMP standard curve.
Data are summarized as mean ± SE of triplicate measurements. *p < 0.05, **p < 0.01, compared with cells treated with vehicle; *p < 0.05, *p < 0.01, compared with cells treated with PTH; *p < 0.05, **p < 0.01, compared with cells treated with Izb. (B) Izb promotes PTH-induced TCF reporter activity. Saos2 cells were seeded into a 96-well plate. After 90% confluence, the cells were transfected with TOPflash or FOPflash plasmid. At 36 h after transfection, the cells were serum starved for 12 h and then treated with Izb for the first 3 h, followed by an additional 5 h of culture in Izb-free medium in the presence or absence of PTH (100 nM) for 8 h. The β-catenin/TCF activation was measured as described in Materials and Methods. Data are summarized as mean ± SE of triplicate measurements. n=3. *p < 0.05, **p < 0.01, compared with cells treated with vehicle; *p < 0.05, *p < 0.01, compared with cells treated with PTH; *p < 0.05, **p < 0.01, compared with cells treated with PTH plus Izb.

**Cell viability assay**

Cell viability was measured as described previously (Yang et al., 2015). Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 500 µg/ml. The cells were further incubated for 1 h at 37°C in a 5% CO2 atmosphere, and the liquid in the wells was subsequently removed. DMSO was then added to each well, and the absorbance was measured at 570 nm.

**Knockout of β-catenin gene expression in Saos2 cells**

Constitutive β-catenin expression in Saos2 cells was knocked out using the CRISPR/Cas9 genome-editing technique. Table 1 lists the double-stranded oligonucleotides encoding a CRISPR targeting RNA (crRNA) of β-catenin that allows sequence-specific targeting of the Cas9 nuclease. The double-stranded oligonucleotides were cloned into linearized GeneArt CRISPR nuclease vectors with an OFP reporter following the manufacturer’s protocol. Saos2 cells were seeded into a six-well plate. After cells reached 90% confluence, CRISPR plasmid or control plasmid was transfected into the cells using Lipofectamine 2000 following the manufacturer’s protocol. Two days after the transfection, the cells were counted and seeded into a 96-well plate with one cell per well by a serial dilution. OFP reporter was applied to monitor transfection efficiency in cells for fluorescence-based tracking using an Evos machine (Invitrogen). After ~3 wk, Western blotting was used to screen β-catenin expression in cells. Zero expression of β-catenin was identified in two single colonies when the cells were transfected with CRISPR construct, whereas β-catenin expression was not changed in cells transfected with the control plasmid.
FIGURE 8: Model of Izb regulation of PTHR signaling cascade in bone. Both PTH and Izb are able to induce β-catenin (β-Cat)/TCF signaling. Izb stabilizes β-Cat by inhibiting its proteasome degradation. β-Cat–mediated PTHR signaling switch is blocked by Izb due to its promoting of β-Cat translocation, thereby dissociating β-Cat from the PTHR. Izb facilitates PTH stimulation of CREB phosphorylation, β-Cat (Ser-675) phosphorylation, GSK3β (Ser-9) phosphorylation, and TCF/Leff activity through a cAMP/PKA signaling pathway. Collectively these findings suggest that the combination of PTH and Izb favors the anabolic PTH action in bone.

protease inhibitor cocktail set I. HA beads were added to equal amounts of soluble membrane proteins in each group. Immunoprecipitated proteins, nuclear proteins, or total lysates (Wang et al., 2008; Hu et al., 2013) were analyzed by SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semidy method (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in Tris-buffered saline/Tween 20 buffer at room temperature in 10% goat serum in PBS. Blocking was performed by incubating the cells for 1 h at room temperature and in 10% goat serum in PBS. Anti-β-catenin mouse mAb diluted (1:250) in blocking buffer was applied to the specimens for 1 h at room temperature. Alexa Fluor 546–tagged goat anti-mouse secondary antibody diluted 1:1000 was applied under the same conditions as the primary antibody. Nucleus staining was performed with DRAQ5. Coverslips were mounted for confocal microscopy (Wang et al., 2008).

TOPflash/FOPflash activity assay
Saos2 cells or MC4 cells were transfected with TOPflash or FOPflash in a 12-well plate by using Lipofectamine 2000 according to the manufacturer’s protocol. After 36 h of transfection, cells were serum starved for 12 h and then treated with the indicated reagents. Eight hours after treatment, cells were lysed with reporter lysis buffer (Promega). The cell lysates were then drawn four times through a 21-gauge needle attached to a 1-ml syringe. The supernatants were transferred to a 96-well plate. The luminescence of each well was recorded after addition of BioGlo Luciferase substrate (Promega). The ratio of TOP/FOP signal was calculated and normalized to control conditions.

Statistical analysis
The data are presented as mean ± SE of triplicate measurements. In the figures, n indicates the number of independent experiments. Statistical analysis between control and treated groups was performed using Student’s t test. Multiple comparisons in one or two types of cells were evaluated by one-way or two-way analysis of variance followed by Bonferroni’s posttest (Prism; GraphPad). p < 0.05 was considered sufficient to reject the null hypothesis.

Table: CRISPR sequences for knockout of human β-catenin.

<table>
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<tr>
<th>Double-stranded cloning Oligo</th>
<th>Sequence</th>
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<td>β-Catenin</td>
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<td>Reverse: 5'-AACGCTGGACATTAGTGGGA</td>
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<tr>
<td></td>
<td>Reverse: 5'-TCTATAGACCTGAGAAATG</td>
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</tr>
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</table>

REFERENCES
Replacement of bortezomib with carfilzomib for multiple myeloma patients progressing from bortezomib combination therapy. Leukemia. 28, 1529–1536.


Ixazomib regulates PTH signaling | 1803


