Inhibition of AXL receptor tyrosine kinase enhances osteoblast function and bone mass in mice

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INTRODUCTION: Osteoporosis is characterized by reduced bone formation and increased fracture risk, with current anabolic therapies limited by safety concerns and high cost. Identifying small-molecule targetable regulators of osteoblast differentiation could provide new therapeutic opportunities. Using a kinome-wide RNAi screen in primary murine calvarial osteoblasts, we identified AXL receptor tyrosine kinase (Axl) as a negative regulator of osteoblast differentiation. Axl is expressed at high levels in immature osteoblasts and declines with maturation, suggesting a role in suppressing osteoblast development. We hypothesized that genetic or pharmacological inhibition of Axl would enhance osteoblast activity and bone formation *in vitro* and *in vivo.*

METHODS: Animal procedures were approved by the local regulatory authority (Regierungspräsidium Tübingen, Germany; License No. 1245). Primary murine osteoblasts were isolated from calvariae of 3-5-day-old neonatal mice and from long bones of 12-week-old mice. Cells were subjected to siRNA-mediated *Axl* knockdown or treated with the selective Axl inhibitor BGB324 (14-100 nM). Differentiation was evaluated by alkaline phosphatase (Alp) activity, osteogenic gene expression, and Alizarin Red S (ARS) staining. Cell viability was assessed using PrestoBlue. For *in vivo* experiments, 11-week-old female BALB/c mice (n=7/group) received BGB324 (50 mg/kg, twice daily) or vehicle by oral gavage for 2 weeks. Femurs and vertebrae were analyzed by high-resolution microcomputed tomography (µCT) to quantify trabecular and cortical parameters. Static histomorphometry assessed osteoblast, osteoclast and osteocyte parameters, while dynamic histomorphometry with calcein labeling quantified mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR). Plasma markers of bone turnover, N-terminal propeptide of type I procollagen (PINP) and C-terminal telopeptide of type I collagen (CTX-I) were measured by ELISA. Transcriptomic profiling was performed by RNA sequencing (RNAseq) after *Axl* knockdown in primary murine calvarial osteoblasts, with candidate genes validated by qPCR and functional assays. One-way ANOVA with Tukey's test was used, *P < 0.05.

RESULTS: The unbiased high-throughput RNAi screen using Alp activity as a functional readout identified *Axl* as a potent suppressor of osteoblast differentiation. *In vitro*, siRNA-mediated *Axl* knockdown increased Alp activity (+398%±135.97), upregulated osteogenic genes (*Runx2, Sp7 and Alpl*), and enhanced matrix mineralization (+459%±116.96 by ARS staining) compared to controls. Pharmacological inhibition with BGB324 (100 nM) similarly increased Alp activity (+47%±24.75) and matrix mineralization (+165%±111.97), while lower concentrations (14 nM) had modest effects. *In vivo*, µCT of femurs showed significantly higher trabecular bone volume fraction (BV/TV) **(Fig. 1A)**, due to increases in trabecular thickness (Tb.Th) and number (Tb.N) in BGB324-treated mice versus controls (*$P < 0.05$), while cortical thickness remained unchanged. In vertebrae, BV/TV increased by 29%±3.01. Dynamic histomorphometry revealed significantly elevated MS/BS, MAR, and BFR **(Fig. 1A)**. Static histomorphometry showed a reduction in osteoblast surface (Ob.S/BS, -57%±1.16) but increased osteocyte density (+21%±137.86), suggesting accelerated osteoblast-to-osteocyte transition. Plasma PINP was elevated (+33%±0.88), whereas CTX-I levels were unchanged, indicating an anabolic but no anti-resorptive effect. RNAseq revealed 1425 down regulated and 757 upregulated genes after *Axl* knockdown **(Fig. 1B)**, including interferon-stimulated gene 15 *(Isg15* **(Fig. 1C)***)*. Functional validation demonstrated that siRNA-mediated *Isg15* knockdown impaired Alp activity (-60%±18.40) **(Fig. 1D)**, reduced matrix mineralization (-92%±6.03), and decreased Erk1/2 phosphorylation, positioning Isg15 as a key downstream effector of *Axl* signaling in osteoblasts.

DISCUSSION: Axl inhibits osteoblast differentiation and bone formation by repressing the Isg15-Erk signaling axis. Pharmacological inhibition with BGB324 enhanced osteoblast activity, indicates an osteoblast-to-osteocyte transition, and increased trabecular bone mass in adult mice without affecting cortical bone or systemic toxicity. The apparent paradox of reduced osteoblast surface despite increased bone formation suggests that Axl inhibition accelerates terminal differentiation towards osteocytes, thereby sustaining bone matrix deposition.

SIGNIFICANCE: This study identifies Axl as a novel osteoanabolic target. Repurposing BGB324, a clinically advanced small-molecule Axl inhibitor, may provide a cost-effective, accessible therapy to stimulate bone formation in osteoporosis and other conditions of impaired skeletal regeneration.

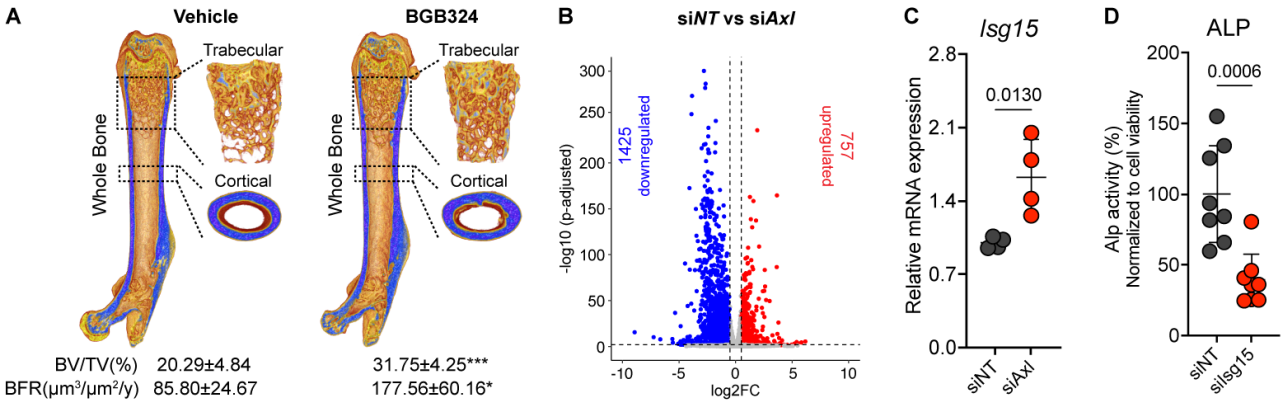

Figure 1: Inhibition of Axl with BGB324 increases bone mass via modulation of *Isg15* expression. (A) Representative µCT images of femurs from mice treated with either vehicle or BGB324 for 2 weeks, highlighting trabecular parameter such as percent bone volume fraction - BV/TV (%) (n=7/group), and bone formation rate - BFR ((µm³/µm²/y) (n=4-5/group). (B) Volcano plot illustrating differentially expressed genes (DEGs) following siRNA-mediated knockdown of *Axl* after day 6 of osteogenic differentiation. (C) *Isg15* expression levels after siRNA-mediated *Axl* knockdown, measured 8 days post-transfection (n=4/group). (D) Cellular Alp staining after siRNA-mediated knockdown of *Isg15* assessed 8 days post-transfection. Statistical differences between the two groups were determined using one-way ANOVA, followed by Tukey's test.