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Article

Live Fluorescent Staining Platform for Drug-Screening and Mechanism-Analysis in Zebrafish for Bone Mineralization

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Abstract: Currently, drug screening is primarily based on high-throughput screening, within cell-based experiment or on animal model to confirm the biological effects. The mammalian system is considered time-consuming and expensive and difficult to perform a high-throughput drug screening. It creates a gap between *in vitro* cell-based models and the *in vivo* mammalian models. On the contrast, zebrafish is an efficient model that could link preclinical toxicity screening with the drug development pipeline. Zebrafish, due to many advantages, such as highly conservative genomic, rapid development, short life span, large number of offspring, low cost, easy manipulation, is an excellent animal model for disease-based research. In this study, zebrafish embryos were incubated with small molecular compounds that affected bone mineralization in 96-well microplates. Among 24 screened compounds in the kinase inhibitor library, we identified three compounds, pentamidine, BML-267, and alendronate, which showed increased embryonic mineralization; while six compounds, RWJ-60475, levamisole HCL, tetramisole HCL, fenvalerate, NSC-663284, and BML-267ester, were inhibitory to bone mineralization. The level of osteogenic mineralization was evaluated by fluorescent dye staining and quantified by image analysis software. The system was validated by the biological response of alendronate and dorsomorphin in zebrafish, and consisted to results in the mouse model. In addition, quantitative real time-PCR was performed to evaluate the biological pathways involved in bone metabolism at the molecular level. We found that alendronate enhanced the level of bone mineralization by inhibiting osteoclast-related genes. In summary, our research showed that zebrafish may have the potential to be a drug-screening and mechanism-analysis platform for bone mineralization.

Keywords: drug screening; bone mineralization; osteoclast; zebrafish

1. Introduction

Bone, a dynamic organ that serves mechanical and homeostatic functions, undergoes a continual self-regeneration called remodeling [1]. Bone remodeling is a process specified by a balance between bone formation by osteoblasts and bone resorption by osteoclasts [2]. An imbalance in bone remodeling contributes to several pathologic conditions, including osteosclerosis, osteopetrosis, and osteoporosis [3]. Osteosclerosis is a bone disorder characterized by an abnormal thickening and progressive increase in bone mass of the skeleton owing to an increased number of osteoblasts. In contrast, osteopetrosis results from a primary decrease in osteoclastic function [4].

With the aging of the population, the cost of osteoporosis is an increasingly significant public health concern. Estimably, annual cost of medical costs for osteoporosis in the U.S. range from $10 to $22 billion. There are also indirect costs including a reduction of quality of life and productivity [5-

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9]. Estimates showed that by 2010, about 12 million people at age of 50-year old will be afflicted with osteoporosis, while more than 40 million people will suffer from low bone mass. By 2020, there is an expectation of 14 million cases of osteoporosis and more than 47 million cases of low bone mass. By 2025, the cost of osteoporosis may rise to nearly $25.3 billion annually in the U.S. [10]. In worldwide, osteoporosis causes more than 8.9 million fractures annually. Osteoporosis is estimated to affect 200 million women. Osteoporosis is a huge personal and economic expense. In Europe, osteoporosis is greater than all cancers except lung cancer. It is comparable or greater than chronic non-communicable diseases, such as rheumatoid arthritis, asthma and high blood pressure related heart disease [11].

Unlike other diseases, there are few agents that promote bone formation in patients with substantial bone loss. Drugs that promote reabsorption remain scarce. Therefore, the development of a relatively simple, quick, effective animal platform, to screen for anabolic and catabolic therapeutic compounds and to evaluate the role of bone-related drug utility is very important. Research on drugs that promote osteogenesis mainly uses *in vitro* cell culture or *in vivo* mouse model as drug screening platforms. Human mesenchymal stem cells (hMSCs) are cultured in multi-well plates. After adding a variety of drugs, respectively, osteogenic differentiation is measured in terms of the survival rate of osteoblasts and the degree of osteogenic differentiation, such as alkaline phosphatase and cell proliferative activities [10-12] while thickness of the sections of the mice are measured [13]. *In vitro* cell-based models are suitable for high-throughput drug screening. However, they lack relevant whole organism physiology to further validate the findings. In contrast, mammalian models provide relevant *in vivo* information but they are not suited for high-throughput screening, and hence they could not cope with validating the large number of 'hits' generated from *in vitro* screening.

Although approaches to multiplex cell-based assay designs that select specific cell types, signaling pathways and reporters have been developed and some *in vitro* designs are optimized for high throughput to benefit screening efficiencies, *in vitro* cell-based assays and subsequent preclinical *in vivo* studies do not yet provide sufficient pharmacological and toxicity data or reliable predictive capacity for understanding drug candidate performance *in vivo.* The drug development process is costly and inefficient [14]. A crucial gap exists between *in vitro* cell-based models and the *in vivo* mammalian models. Zebrafish could enhance preclinical drug screening by its strategic placement between *in vitro* cell-based and mammalian models along the drug development pipeline. In addition to its small size, the availability of a large number of zebrafish embryos allows drug screening to be performed in micro-well plates; this makes automation in a high-throughput manner possible. The low throughput of mammalian models creates a major bottleneck to evaluating the numerous 'hits' identified from *in vitro* cell-based screening. With lower maintenance cost and less space required for a zebrafish facility compared to a mammalian facility, it is more cost-effective to employ zebrafish for early preclinical drug screening.

Zebrafish have been well known as useful animal models for studying vertebrate development [15,16]. The benefits of applying zebrafish to study vertebrate biology, physiology, pathology and toxicology are based on high genomic conservation (compared to mammals) and rapid development and differentiation. Many advantages of zebrafish include short life span, large number of offspring, and low cost. Compared to mammalian models, they easily manipulate for generating transgenic species. They also greatly speed up disease-based research in clinical studies [17]. To this end, the zebrafish animal model was used to screen for drugs that affect mineralization [18].

In this study, we have developed a high throughput screening of anti-osteoporosis drug in zebrafish. Calcein was adopted as an indicator dye for bone mineralization. The stained area on the spinal cord was used as a quantitative standard, to evaluate the impact of the drugs on bone mineralization. After the efficacy of the compounds is evaluated, molecular analysis was used to determination the effects of drugs on osteoblasts, osteoblasts and calcium ions in the regulation of mineralization. We have a better mechanistic understanding of the mode of action by which drugs affect the final degree of mineralization. Zebrafish screening provides a simple high throughput screening for bone anabolic and catabolic compounds. And at the same time, it provides further analysis at the molecular level.

2. Results

Validation of small molecular screening platform in zebrafish with positive and negative control compounds

Previous studies have shown that alendronate promotes bone formation with a less anisotropic microstructure in mouse and rat models [20,21]. It also served as a potential treatment for patients who suffered from bone loss [22,23]. To investigate the effect of alendronate on the embryonic skeletal development, we treated 3 dpf (day-post-fertilization) embryos for 4 days with alendronate at different concentrations (10, 20, and 30 μM) and compared to a mock control in 0.1% DMSO. We first evaluated the spinal development of 7 dpf embryos with calcein staining. We found that the notochord number and mineralization area of alendronate-treated larvae was significantly increased compared to mock treatment group (Fig. 1A-B).

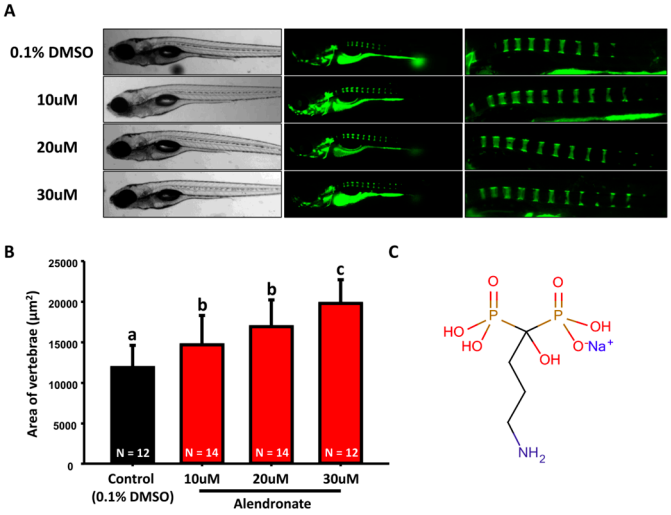

Figure 1. Evaluation of mineralization in alendronate-treated zebrafish. (A) Different concentration of alendronate (10, 20, and 30 μM) were treated on 3 dpf zebrafish. Calcein staining on mock and alendronate-treated embryos at 7 dpf. (B) Quantification of mineralization degree detecting the fluorescence intensity at the area of centrum form ring in the notochord. (C) Structure of alendronate. (mean ± SD; tested by one-way ANOVA; N = fish number).

Dorsomorphin inhibits Smad-dependent BMP (Bone morphogenetic protein) signaling, which has been shown to be a key promoter during osteogenesis [11,24]. We hypothesized that dorsomorphin may serve as a negative regulator for bone mineralization. As the data showed in Fig. 2, when we treated 3 dpf zebrafish with dorsomorphin at various concentrations (10, 20, and 30 μM), we found that dorsomorphin caused decreased number of notochord and degree of mineralization in zebrafish larvae in a dose-dependent manner. Therefore, we validated our system with alendronate and dorsomorphin, respectively, by demonstrating that response in zebrafish are comparable to results in the rodent model.

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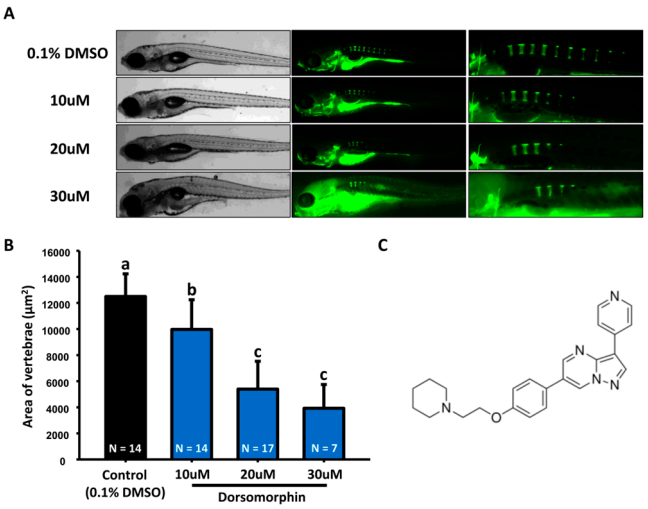

Figure 2. Evaluation of mineralization in dorsomorphin-treated zebrafish. (A) Different concentration of dorsomorphin (10, 20, and 30 μM) were treated on 3 dpf zebrafish. Calcein staining on mock and dorsomorphin-treated embryos at 7 dpf. (B) Quantification of mineralization degree detecting the fluorescence intensity at the area of centrum form ring in the notochord. (C) Structure of dorsomorphin. (mean ± SD; tested by one-way ANOVA; N = fish number).

High-throughput screening on small molecular library

In our study, we aimed to evaluate the small molecular compounds in our library that affect bone mineralization. Initially, we selected alkaline-phosphatase-inhibitor-like compounds in our chemical library and treated 10 μM of each compound to 3 dpf embryonic zebrafish for 4 days. Compared to mock treatment control, 10 out of 24 compounds showed embryonic toxicity. In addition, 3 compounds increased embryonic mineralization, while 6 compounds were inhibitory to bone mineralization (Fig. 3). Three compounds that caused increased vertebral area were, pentamidine (CYCU-1140), BML-267 (CYCU-1147), and Alendronate (CYCU-1152). Our finding (Fig. 1) was consistent with previous studies that alendronate is an activator for bone development [20,21]. Therefore, we choose alendronate as a positive control for our study. Pentamidine was validated with calcein staining. Significant increased bone mineralization was observed within zebrafish vertebral region (Fig. 4A and B). Moreover, zebrafish treated under 10 μM treatment of pentamidine showed more pronounced mineralization than 30 μM treatment of alendronate. Therefore, we hypothesized that pentamidine is more potent to promote bone mineralization than alendronate. Next, we also examined the mineralization effect on BML-267. Consistent with the increasing vertebral area observed at 10 μM of BML-267, the mineralization level of embryonic zebrafish was induced at 10 and 20 μM of BML-267 (Fig. 5A and B). Interestingly, we noticed an inhibitory effect of BML-267 at higher concentration. The inhibitory effect may be due to some negative feedback. Further studies are needed to clarify the issue.

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A

Code	Name	Code	Name
CYCU-1122	Endothall	CYCU-1136	BN-82002
CYCU-1123	Benzylphosphonic acid	CYCU-1138	NSC-663284
CYCU-1124	L-p-Bromotetramisole oxalate	CYCU-1139	Cyclosporin A
CYCU-1125	RK-682	CYCU-1140	Pentamidine
CYCU-1126	RWJ-60475	CYCU-1141	BVT-948
CYCU-1127	RWJ-60475 (AM)3	CYCU-1142	B4-Rhodanine
CYCU-1128	Levamisole HCl	CYCU-1143	BML-268
CYCU-1129	Tetramisole HCl	CYCU-1146	PD-144795
CYCU-1132	Fenvalerate	CYCU-1147	BML-267
CYCU-1133	Tyrfphostin 8	CYCU-1148	BML-267 Ester
CYCU-1134	CinnGel	CYCU-1149	OBA
CYCU-1135	CinnGel 2 Me	CYCU-1152	Alendronate

B

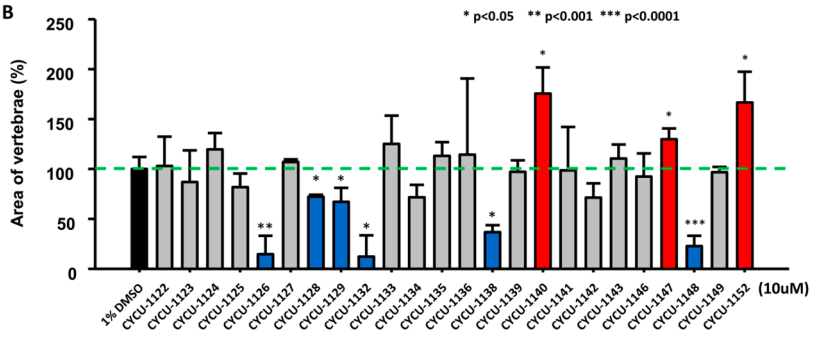

Figure 3. High-throughput small molecular screening on bone development. (A) List of small molecules in the library. (B) Relative quantification of mineralization. 10 μM of each compound was treated to 3 dpf zebrafish. The quantification was determined with fluorescence microscopy within vertebrae area after calcien staining. The blue column showed decreased mineralization after treating the specific compound; whereas red showed increased. (mean ± SD; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$; tested by student's T test with 1% DMSO control group).

Among the six compounds tested, RWJ-60475 (CYCU-1126), levamisole HCL (CYCU-1128), tetramisole HCL (CYCU-1129), fenvalerate (CYCU-1132), NSC-663284 (CYCU-1138), and BML-267ester (CYCU-1148) showed the most inhibitory effect on mineralization (Fig. 3B). A gradient of BML-267ester was chosen for validation of mineralization after treatment. As expected, BML-267ester showed a dose-dependent mineralization in embryonic zebrafish (Fig. 6A and B).

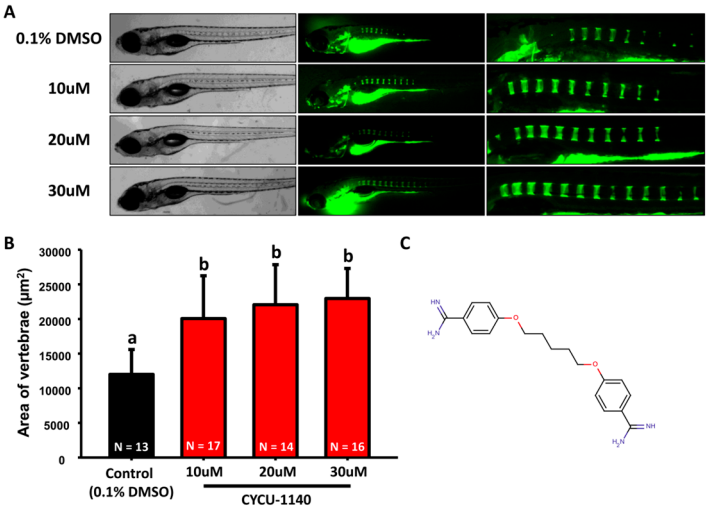

Figure 4. Increase of mineralization in pentamidine-treated zebrafish. (A) Different concentration of pentamidine (CYCU-1140, 10, 20, and 30 μM) were treated on 3 dpf zebrafish. Calcein staining on mock and pentamidine-treated embryos at 7 dpf. (B) Quantification of mineralization degree detecting the fluorescence intensity at the area of centrum form ring in the notochord. (C) Structure of pentamidine. (mean ± SD; tested by one-way ANOVA; N = fish number).

Zebrafish embryos are a potential drug-screening and mechanism-analysis platform for bone mineralization

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Two progresses are involved in bone formation: osteoblastic differentiation and osteoclastic inhibition. During osteoblastogenesis, mesenchymal stem cells (MSCs) are induced into osteoblasts, which proliferate and maturate into mineralized bone cells [25]. We next selected several genes that associated with bone development and validated the activities of these marker genes: osteoclast-associated markers (*ctsk*, *mmp9*, *rank*, and *acp5b*); calcium absorption-related markers (*trpv6*, *vdra*, and *vdrb*); osteoblast progenitor markers (*runx2a*, *runx2b*, and *sp7*); preosteoblast markers (*alp*, *bmp2b*, *bmp4*, and *colla1a*); mature osteoblast markers (*osteopontin*, *phex*, and *osteonectin*) by real time-PCR to confirm the affecting osteogenesis processes. Compared with the mock treatment group, *ctsk*, *mmp9*, *rank*, and *acp5b* were significantly down-regulated in alendronate treated embryos (Fig. 7). This suggested to us that osteoclast-related pathway was generally inhibited. On the other hand, *bmp2b* and *col1a1a* were up-regulated, which were consistent with increased bone formation seen in the clinical settings [26]. In addition, *osteonectin* was significantly upregulated, which has been reported to link to bone mineralization [27]. Taken together, alendronate triggered bone formation through inhibiting on osteoclast-associated signaling, and activate bone mineralization through *bmp2b-osteonectin* pathway.

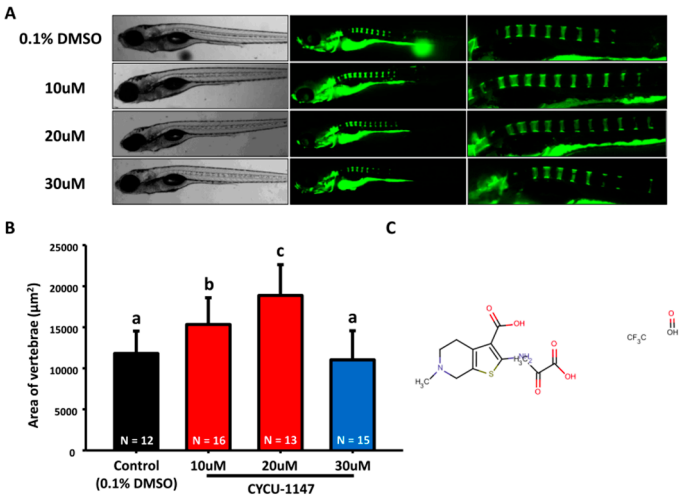

Figure 5. Increase of mineralization in BML-267-treated zebrafish. (A) Different concentration of BML-267 (CYCU-1147, 10, 20, and 30 μM) were treated on 3 dpf zebrafish. Calcein staining on mock and BML-267-treated embryos at 7 dpf. (B) Quantification of mineralization degree detecting the fluorescence intensity at the area of centrum form ring in the notochord. (C) Structure of BML-267. (mean ± SD; tested by one-way ANOVA; N = fish number).

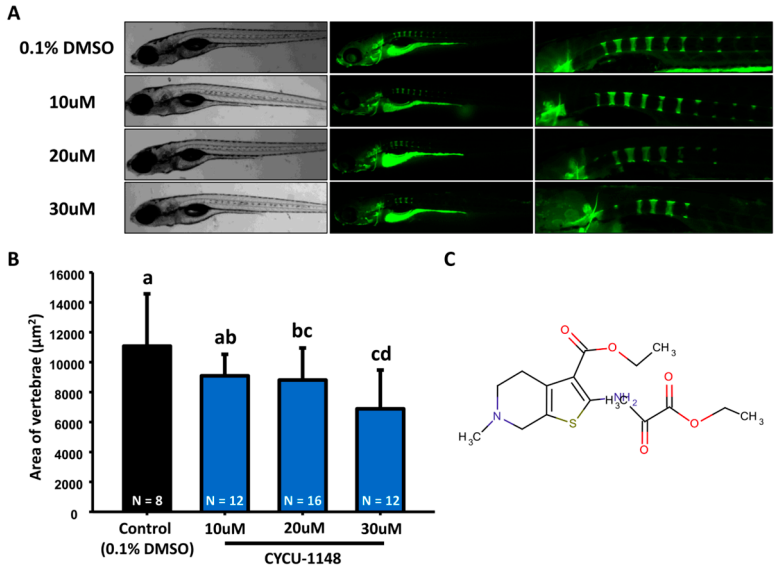

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Figure 6. Decrease of mineralization in BML-267ester-treated zebrafish. (A) Different concentration of BML-267ester (CYCU-1147, 10, 20, and 30 μM) were treated on 3 dpf zebrafish. Calcein staining on mock and BML-267ester-treated embryos at 7 dpf. (B) Quantification of mineralization degree detecting the fluorescence intensity at the area of centrum form ring in the notochord. (C) Structure of BML-267ester. (mean ± SD; tested by one-way ANOVA; N = fish number).

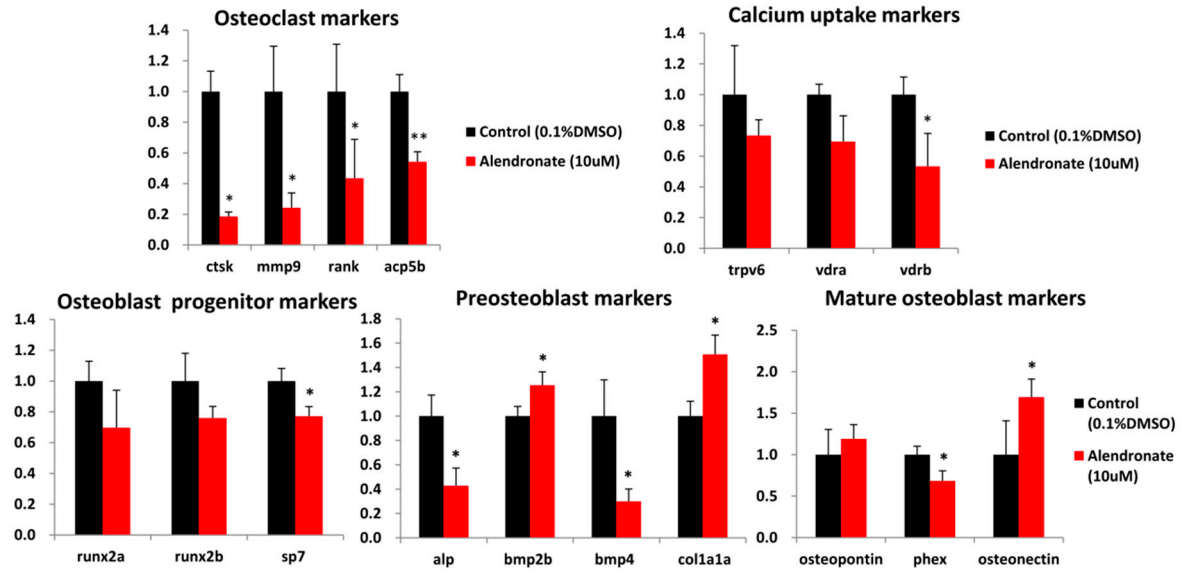

Figure 7. Relative expression level of osteogenesis-related genes in Alendronate-treated zebrafish. Total RNA was extracted from 10 fish embryos aged at 3 dpf and marker genes were tested. (mean ± SD; * $p < 0.05$; ** $p < 0.001$; tested by student's T test with 1% DMSO control group)

Table 1 Primers for realtime-PCR

Gene name	Symbol	Forward primer	Reverse primer
		Osteoclast markers	
cathepsin K	*ctsk*	GGACTCAATCACTATCACT	AGAACAAGACATCTAAGACA
matrix metallopeptidase 9	*mmp9*	TCGGCCTACCAAGCGACTT	TCATGTGAATCAATGGGCACTC
receptor activator of nuclear factor kappa-B ligand	*rank*	GCACGGTTATTGTTGTTA	TATTCAGAGGTGGTGTTAT
acid phosphatase 5b	*acp5b*	GCTGCTGCTAACAAACAAT	GACCAACCACGATGACAA
		Calcium uptake markers	
transient receptor potential cation channel subfamily V member 6	*trpv6*	GATCGCAATGACATAATG	CTCCATCACTCTTAGAAG
vitamin D receptor a	*vdra*	CTTCAGACTCATTCAACCAT	GATACATCATCAGCAGATTACT
vitamin D receptor b	*vdrb*	CTCATCAGACTCCTTCAG	TACATCATCAGCAGGTTAC
		Osteoblast progenitor markers	
runt-related transcription factor 2a	*runx2a*	GACGGTGGTGACGGTAATGG	TGCGGTGGGTTCGTGAATA
runt-related transcription factor 2b	*runx2b*	CGGCTCCTACCAGTTCTCCA	CCATCTCCCTCCACTCCTCC
sp7 transcription factor 7	*sp7*	GGCTATGCTAACTGCGACCTG	GCTTTCATTGCGTCCGTTTT
		Preosteoblast markers	
alkaline phosphatase	*alp*	CAAGAACTCAACAAGAAC	TGAGCATTGGTGTTATAC
bone morphogenetic protein 2b	*bmp2b*	CGGCTCCTACCAGTTCTCCA	CCATCTCCCTCCACTCCTCC

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bone morphogenetic protein 4	*bmp4*		
collagen type II, alpha 1 (cartilage collagen)	*cola1a*	CTGTGCCAATCCCATCATTTC	ATATCGCCTGGTTCTCCTTTC
		Mature osteoblast markers	
osteopontin	*opn*	GCCTCCATCATCATCGTA	AATCACCAAGCACCAGTA
phosphate regulating endopeptidase homolog, X-linked)	*phex*	GAGAATGAATGGATGGATGA	TTGATGTCTTCGTTAATATAGGT
osteonectin	*on*	ACTAACAACAAGACCTAC	TCCGATGTAATCTATGTG

3. Discussion

Generally, during preclinical drug mining, candidate validation and toxicity analysis is performed at early-stages with *in vitro* cell-based approaches. They are expected to represent essential aspects during *in vivo* pharmacology and toxicology in mammalian model [28,29]. The current drug development process is costly and inefficient [14,30]. Therefore, the translational role of zebrafish in high-throughput drug screening has rapidly developed into a popular platform for various disease [31]. As the aging population increasing, the number of people with osteoporosis-related fractures may increase exponentially. This increases overall suffering and health care costs in the aging population. So far, few compounds promoting bone formation have been developed. Therefore, it is important to develop a relatively simple animal platform to screen and evaluate drug activity.

Zebrafish, like mammals, consists of cartilage and bones. In the process of embryonic development, the formation of bones is mainly through two processes: intramembranous ossification and endochondral ossification [32]. The process of intramembranous ossification begins with the formation of interstitial stem cells in the cartilage, then the bone forms in the cartilage, and finally the cartilage is replaced by the bone. The flat bones that compose most of the skull are formed by intramembranous ossification. The ossification process does not undergo the formation of cartilage. Rather, interstitial stem cells directly differentiate into osteoblasts, secret extracellular matrix for mineralization, and finally form bones [33].

Bone formation process are conservative in mammalian and fish system, which both intramembranous and endochondral ossification take place in the craniofacial skeleton [34,35]. Moreover, zebrafish homologs of human *runx2* gene have been shown to be expressed during skeletal development. The molecular mechanisms underlying the bone physiology are similar between two species [36]. However, there are differences in which zebrafish bone development vary from those of rodents. The initial ossification events in the axial skeleton appeared at late larval stages, from 7 to 9 dpf, which were initially through an acellular rather than an endochondral mechanism [34]. Spinal cord development in zebrafish is also different from the mammals, in that the spinal cord of mammalian embryo was formed by intramembranous ossification during early development, while in zebrafish it was formed through endochondral ossification, without the formation of cartilage skeleton. As early as seven-day post fertilization, part of the notochord began to mineralize, and formed the vertebrae directly. In addition, regional differences in the response of the zebrafish skeleton to BMP have been documented [37,38]. The ossification processes within craniofacial region are similar to those in mammals. Osteoblasts and osteocytes both existed in larval zebrafish [34]. Therefore, larval zebrafish bones contained the sufficient and necessary cells for both bone formation and resorption activity. The translucent zebrafish embryos also allow for direct observation of the mineralization process *in vivo*.

High-throughput *in vivo* screening has been established for bone anabolic compounds with zebrafish [18]. The application of zebrafish model has been successfully demonstrated for some clinically well-known drugs, such as vitamin D3 (cholecalciferol), calcitriol, parathyroid hormone Teripalatide (Teriparatide), etc., which promote bone mineralization. In our study, we have developed a high-throughput screening of anti-osteoporosis drug in zebrafish. We used calcein as an indicator dye for bone mineralization to screen for a large number of small compounds in zebrafish. As a pilot screening, we evaluate the small molecular compounds in our library that affect bone

mineralization. Initially, we evaluated alkaline-phosphatase-inhibitor-like compounds in our chemical library. We identified 3 compounds increased embryonic mineralization, while 6 compounds were inhibitory to bone mineralization (Fig. 1, Fig. 3). Of these three compounds, our finding was consistent with previous studies that alendronate is an activator for bone development [20,21]. 6 compounds were found to be inhibitory to bone mineralization.

Using alendronate as a validation compound for our system, we demonstrated that phenotypic endpoints can corroborate with omics data to characterize the mechanism of the candidate drug(s) in a conceptual framework of cause-and-effect, with verifications from known molecular interactions and phenotypic anchoring. We examine several marker genes that are associated with bone development for the activities of these genes: osteoclast-associated markers, calcium absorption-related markers, osteoblast progenitor markers, preosteoblast markers, and mature osteoblast markers, by real time-PCR to confirm the affected osteogenesis processes in alendronate treated embryos. The results suggested to us that osteoclast-related pathway was generally inhibited. On the other hand, *bmp2b and col1a1a* were up-regulated, which were consistent with increased bone formation seen in the clinical settings [26]. In addition, *osteonectin* was significantly upregulated, which has been reported to link to bone mineralization [27]. In this study, we have provided *in vivo* evidences to show alendronate triggered bone formation through inhibiting on osteoclast-associated signaling, and activate bone mineralization through *bmp2b-osteonectin* pathway. We demonstrated that in our zebrafish model, mechanistic insights into the candidate drug(s) can be elucidated by using knowledge-based data mining and algorithms on the high-content omics data to discover perturbed molecular pathways and biological processes.

Taken together, our results demonstrated that our zebrafish system is a potential drug-screening and mechanism-analysis platform for bone mineralization. It has the advantage of *in vitro* cell-based assays allowed quick screening and be affordable and reliable for identifying drug candidates and the advantages of whole animal experimental design that afford reliable predictive capacity for understanding drug candidate performance *in vivo*. Our zebrafish model not only provides a simple high throughput screening for bone anabolic and catabolic compounds. and allows for a better mechanistic understanding of the mode of drug action at molecular level.

4. Materials and Methods

Zebrafish maintenance

Fish were maintained as described in the zebrafish book [19]. Wild-type fish used were the AB strain. Fish were well fed with dry food and *Artemia salina* once a day. The night before breeding, males and females were separated into different tanks at a ratio of 1 male to 2 females. At the beginning of next light cycle, the males and females were allowed to mate in the same tank. Embryos were collected and cultured in petri dishes containing E3 water (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4) at 28°C under 14 hr on/10 hr off light cycle.

Small molecular library

The CYCU-1120~1152 chemical library (Chung Yuan Christian University, Department of Bioscience Technology) was purchased from Enzo Ltd (SCREEN-WELL® Kinase Inhibitor library, BML-2832) and used as the source of small molecules for the experiments. The active compounds in the library and dorsomorphin (Sigma, P5499) were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted with E3 water for later experiment.

Calcein labeling and calculation of bone formation

Calcein staining is a fluorescent dye used for detecting calcium as an index of mineralization. Zebrafish embryos were immersed in 1% calcein solution (Sigma-Aldrich, C0875) for 3-10 mins according to embryo size and washed with E3 water three times to remove unbound dye. Quantification of bone calcification was performed with fluorescence microscopy within spinal region of zebrafish.

Small molecular screening

Synchronized embryos were collected and arrayed by pipette. Twenty embryos per well were in a 12-well plate. Compound were dissolved in E3 water, and a serial dilution of compounds was added at 0-2 hpf. The toxicological effects of zebrafish were observed under the microscope within the next 7 days. During the screening period, dead fish were removed frequently and replaced the diluted compound at 72-hr time point to keep the culture medium clean.

Quantitative Real-time PCR

Total messenger RNA from zebrafish embryos aged at 3 dpf were harvested with the RNAZol®RT (Invitrogen), and quantified with NanoDrop (Thermo Scientific). RevertAid first cDNA synthesis kit (K1622, Fermentas) was used to synthesize first-strand cDNA from total zebrafish RNA according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad iCycler using β-actin as control, and data were analyzed using the ΔΔCt method. The primer sequence used to perform quantitative real-time PCR was listed in Table 1.

Image processing

Embryos and larvae were paralyzed with 0.16% tricaine methane sulfonate in E3 water. Water was dispensed and the paralyzed embryo and larvae were moved into a plate containing 4% methyl cellulose. Images were captured with florescence microscopy (SMZ1500, Nikon) and quantified with Image J (version 1.44, https://imagej.nih.gov/ij/).

Statistical Analysis

The data were expressed as mean ± SD and tested by Student's-*t* test or one-way ANOVA. The significance level was set at 5% ($p < 0.05$).

Conflicts of Interest: The authors declare no conflict of interest.

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