**Introduction**

Extensive evidence demonstrates that glucocorticoid (GC) excess decreases osteoblast differentiation and functions, as well as increases apoptosis of osteoblast and osteocytes. This ultimately leads to osteoporosis characterized by the inhibition of bone formation, named glucocorticoid-induced osteoporosis (GIO)\(^1,2\). However, the underlying mechanisms of these deleterious effects caused by GC on skeletal tissue remain unclear, and there are also no ideal drugs and methods suitable for GIO.

Recently, epidemiological and genetic studies on both humans and rodents show that the accumulation of reactive oxygen species (ROS) and enhancement of oxidative stress (OS) play a pivotal role in the development of osteoporosis\(^3-5\). In skeletal tissue, excessive ROS generation directly promotes apoptosis of osteoblasts and osteocytes\(^6-8\), and ROS-elicited OS also contributes to increased osteoclast differentiation and function\(^9,10\). Furthermore, studies confirm that GC elicits increased ROS production and subsequent OS in skeletal tissue\(^11-13\). These studies indicate that excessive GC may significantly contribute to bone loss, partially due to oxidative stress, and antioxidant treatment may counteract GC-induced osteopenia and osteoporosis.

Salvianolic acid B (Sal B, or lithospermic acid B), the major aqueous bioactive component of *Salvia miltiorrhiza Bunge*, is widely used clinically in China as the main pharmacological ingredient of approved *Salvia miltiorrhiza* agents for cardio-
cerebral vascular diseases. As a polyphenolic acid compound with seven phenolic hydroxyl groups, Sal B is one of the strongest natural antioxidants\(^{[14,15]}\) and is metabolized in vivo into tanshinol, another aqueous bioactive ingredient of *Salvia miltiorrhiza* and a strong natural antioxidant containing two phenolic hydroxyl groups (Figure 1). Interestingly, our previous studies demonstrated that both Sal B and tanshinol promoted osteoblastic differentiation and functions in vitro, and the aqueous extracts of *Salvia miltiorrhiza*, of which the major components are Sal B and tanshinol, effectively prevented osteopenia in rats treated with long-term excessive prednisone\(^{[16–18]}\).

*Danio rerio* (zebrafish) is a powerful whole in vivo animal model for bone study and drug screening because of its many advantages\(^{[19,20]}\). Since 2006, when Fleming *et al*\(^{[21]}\) found that wild-type larval zebrafish exposed to prednisolone showed an obvious delay of skull mineralization based on alizarin red staining, zebrafish larvae have been widely used for the high-throughput screen for anti-osteoporotic candidates\(^{[22,23]}\). Tg (sp7:egfp) is a transgenic line zebrafish characterized by the osterix gene driven expression of green fluorescent protein (GFP) in osteoblast differentiation\(^{[24,25]}\). Because GFP-positive osteoblasts are visible in tg(sp7:egfp) larvae, the process of osteoblast differentiation and bone formation can be directly monitored via alteration of GFP fluorescence intensity using a fluorescence microscope.

Based on the above, we elucidated the preventive effects of Sal B on the inhibition of bone formation induced by excessive GC in both wild-type AB strain zebrafish larvae and bone transgenic juvenile zebrafish-tg(sp7:egfp) and further investigated the underlying mechanism in this study.

**Materials and methods**

**Zebrafish husbandry**

Zebrafish of the AB wild line and bone transgenic osterix:nlsGFP line, named tg(sp7:egfp)\(^{[26]}\), were used. The embryos of the AB line and tg(sp7:egfp) line zebrafish were, respectively, collected from the natural mating and spawning of wild-type AB line zebrafish and tg(sp7:egfp) transgenic zebrafish. At 36 h post fertilization, the embryos with green fluorescence, considered tg(sp7:egfp), were identified and selected under a fluorescence microscope. Both the embryos and larvae of the AB strain and tg(sp7:egfp) were reared in egg water (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.4 mmol/L CaCl\(_2\), 0.16 mmol/L MgSO\(_4\), and 10 ppm methylene blue) containing 30 ppm N-phenylthiourea and in egg water without N-phenylthiourea under standard conditions, respectively\(^{[27]}\).

**Preparations of compound solution**

Dexamethasone (Dex, MP, USA) and Calcitriol Soft Capsule (rocalirol; Roche, Switzerland) were solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Japan) and stored at -20°C. The stock solution concentrations of Dex and rocalirol were 10 mmol/L and 6×10\(^{-5}\) μg/mL, respectively. Sal B (Tianjiangshilang, China; Sal B content 90%) was dissolved and diluted to 10 mmol/L in egg water before use.

**Compound treatment in 24-well plates**

Zebrafish larvae at 3 d post fertilization (dpf) were transferred into 24-well plates containing egg water. Compound treatment was performed in 24-well plates with 6 larvae per well. After being dissolved, the stock solution of each compound was diluted into egg water and added into 24-well plates. The final volumes of medium per well were adjusted to 1 mL. From 3 dpf to 9 dpf, half the medium in each well was replaced with fresh solution every 24 h. At 9 dpf, larvae were collected and fixed for bone tissue alizarin red staining or fluorescence imaging as described.

**Measurement of osteogenesis in intact zebrafish larvae exposed to Dex**

The AB wild-strain or tg(sp7:egfp) line zebrafish larvae were randomly divided into various treatment groups (n=12 larvae/2 wells per group). Larvae were exposed to different concentrations of Dex (5, 10, 15, and 20 μmol/L) from 3 dpf to 9 dpf, and 0.1% DMSO was the vehicle control treatment. At 9 dpf, zebrafish larvae were collected. The AB wild strain underwent alizarin red staining and bone mineralization quantitative analysis as described. The tg(sp7:egfp) line underwent fluorescence scanning and fluorescence picture quantitative analysis using laser scanning confocal microscope (LSCM) and Image-Pro Plus image analysis software version 6.0 (IPP 6.0), respectively, as described.

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**Figure 1.** Chemical structure of salvianolic acid B and tanshinol. Salvianolic acid B contains seven phenolic hydroxyl groups, tanshinol two phenolic hydroxyl groups. Three molecules of tanshinol and a molecule of caffeic acid constitute a molecule of salvianolic acid B.
Determination of osteogenesis in intact zebrafish larvae treated with Sal B
As described in the method as above, zebrafish larvae were treated with different concentrations of Sal B (0.2, 0.5, 1.0, and 2.0 μmol/L) from 3 dpf to 9 dpf, and egg water was used as the control treatment (n=12 larvae/2 wells per group). At the end of experiment, alizarin red staining and fluorescence scanning were performed.

Assay of bone formation in zebrafish larvae treated with Dex in the presence of Sal B
Based on the results of Dex on osteogenesis in intact zebrafish larvae, Dex at the dose of 10 μmol/L was used in subsequent experiments. Zebrafish larvae were divided into 9 groups (n=12 larvae/2 wells/group). Both the AB wild strain and tg(sp7:egfp) line zebrafish larvae were treated with 0.1% DMSO (vehicle control), 10 μmol/L Dex, 10 μmol/L Dex+Sal B (0.2, 0.5, 1.0, 2.0, 4.0, and 10 μmol/L), or 10 μmol/L Dex+6×10^4 μg/mL rocalirol (positive control) from 3 dpf to 9 dpf. The rocalirol 6×10^4 μg/mL group was the positive control group for Sal B in terms of attenuating Dex-induced inhibition of bone formation. At the end of the experiment, alizarin red staining and fluorescence scanning were performed.

Expression of genes related to osteogenesis and oxidative stress in zebrafish larvae
According to the experimental results of Sal B on bone formation in zebrafish treated with Dex, Sal B at the dose of 2 μmol/L was used in the following tests. The AB strain zebrafish larvae at 3 dpf were reared 30 per well in 6-well plates with egg water, divided into the following groups, and exposed to the corresponding compounds for 6 days (n=90 larvae/3 well per group): vehicle control (0.1% DMSO); 10 μmol/L Dex; and 10 μmol/L Dex+2 μmol/L Sal B. The total volume of medium per well was 5 mL, and half the medium of each well was changed every day. At 9 dpf, larvae were collected and subjected to qRT-PCR assay (n=10 larvae/group) and oxidative stress assay (n=80 larvae/group).

Alizarin red staining of bone tissue and quantitative analysis of bone mineralization
At 9 dpf, the AB strain zebrafish were fixed in 4% paraformaldehyde solution for 2 h. The protocol for bone alizarin red staining was adapted from that described in previous experiments. After rinsing with PBS for 10 min and subsequently stained with 0.1% alizarin red (MP, USA) in 0.5% KOH overnight. After washes in ultrapure water, larvae were bleached in 1.5% H2O2-1% KOH for 30 min. Next, destaining was performed with different proportions of mixtures of 0.5% KOH-glycerol (3:1, 1:1, 1:3), and zebrafish samples were stored in 100% glycerol at 4°C.

Oxidative stress analysis
The levels of ROS and malonic dialdehyde (MDA) and the activity of superoxide dismutase (SOD), glutathione peroxi-

| mRNA     | Forward sequence (5'–3') | Reverse sequence (5'–3') |
|----------|---------------------------|-------------------------|
| runx2a   | GACTCCGACCTCAGGAAACATT   | CGTCCCGTCAAGGAAATCT     |
| sp7      | AAGAAACCTGTCGCAAAGCTG    | GAGGCTTTACGCCTACACCTT   |
| ALP      | CAGTGGGAGTCCGCAACAA      | CCACAGGCTGGGCAATAGCA    |
| OC       | TGCTCTCTATCATGAGGACA     | CCTCGGAGCTGAATGGGACAGT  |
| β-Actin  | CAACAGGGGAAAGATGACAGAT   | CAGCTGGGATGGCAACAGT     |

Oxidative stress analysis
The levels of ROS and malonic dialdehyde (MDA) and the activity of superoxide dismutase (SOD), glutathione peroxi-
dase (GSH) and hydrogen peroxidase (catalase, CAT), respectively, reflect the oxidation and antioxidant levels of the body. Briefly, zebrafish larvae (n=80 larvae/group) were washed twice using ultrapure water and then homogenized in 500 μL of ultrapure water with a glass homogenizer. The homogenate was centrifuged, and the supernatant was collected and used for tests. ROS was measured using an oxidation-sensitive fluorescent probe dye (DCFH-DA) chemical reagent kit (Beyotime Institute of Biotechnology, China), and the other indices of oxidative stress above were determined using chemical colorimetry according to the reagent manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analysis
Data were analyzed using SPSS version 16.0 (SPSS Inc, Chicago, IL, USA). If the data were normally distributed and had an equivalency of variances, one-way analysis of variance (ANOVA) with Fisher’s PLSD test was used to test the statistical differences among various treatments. Otherwise, Dunn’s method for post hoc test was used to perform pairwise comparisons of treatment groups. If probabilities are less than 0.05 (P<0.05), statistical differences are considered significant. Data for all parameters are presented as the mean±SD or the mean±SEM.

Results
Dex inhibits osteoblast differentiation and reduces mineralized matrix in intact larval zebrafish skull
To clarify the influence of glucocorticoid on bone formation in an earlier vertebrate model, such as zebrafish, larval zebrafish were exposed to different concentrations of Dex. Compared to the vehicle control, there was a significant decrease in the area and IOD of skull green fluorescence in tg(sp7:egfp) zebrafish larvae treated with Dex at concentrations ranging from 5 to 20 μmol/L (P<0.05), and they showed a dose-dependent decrease with increasing concentrations of Dex (Figure 2). The osterix gene (sp7) is a pivotal and necessary regulatory factor during osteoblast differentiation. Tg(sp7:egfp) is a transgenic line zebrafish characterized by osterix gene driven expression of green fluorescent protein (GFP) during osteoblast differentiation. The osteoblasts of tg(sp7:egfp) are selectively marked with GFP, and the area and IOD of green fluorescence directly reflects the osteoblast differentiation and amount. Dex at concentrations from 5 to 20 μmol/L also decreased the area and IOD of the skull mineralized matrix stained with alizarin red in the AB strain larval zebrafish in a dose-dependent manner (P<0.05) (Figure 3). These results suggest that Dex caused a dose-dependent decrease of mineralized matrix and osteoblast differentiation in zebrafish larvae. However, Dex at concentrations of 15 and 20 μmol/L also markedly reduced the

Figure 2. Dex treatment leads to decreased osteoblast differentiation of skull in tg(sp7:egfp) larval zebrafish. (A) Green fluorescence images of skull dorsal aspect using LSCM in tg (sp7:egfp) larval zebrafish at 9 dpf exposed to 0, 5, 10, 15, and 20 μmol/L Dex for 6 d; (B) The influence of Dex on skull green fluorescence area analyzed by IPP6.0 in 9-dpf tg(sp7:egfp) larval zebrafish and (C) The influence of Dex on skull green fluorescence IOD analyzed by IPP6.0 in 9 dpf tg(sp7:egfp) larval zebrafish. Notes: LSCM (laser scanning confocal microscopy); IPP6.0 (Image-Pro Plus image analysis software version 6.0); IOD (integral optical density); Veh (vehicle control, 0.1% dimethyl sulphoxide); Dex5 (Dex 5 μmol/L); Dex10 (Dex 10 μmol/L); Dex15 (Dex 15 μmol/L); Dex20 (Dex 20 μmol/L). Data are given as mean±SD. n≥9. *P<0.05 vs vehicle control.
swimming activity of the fish and even led to death in larval zebrafish (data not shown). Based on the above results, Dex at 10 μmol/L was used in the following experiment.

Sal B stimulates osteoblast differentiation and increases mineralization of the skull of larval zebrafish

To assess the benefit of Sal B on bone formation, zebrafish larvae were exposed to different concentrations of Sal B. As shown in Figure 4, Sal B significantly increased the area and IOD of the green fluorescence of the skull at concentrations of 0.5 to 2.0 μmol/L in tg(sp7:egfp) zebrafish larvae compared to the control ($P<0.05$). However, different concentrations of Sal B, from 0.5 to 2.0 μmol/L, increased the area and IOD of green fluorescence to the same extent. Sal B (0.5, 1, and 2.0 μmol/L) also increased the area of the skull stained with alizarin red and the staining intensity in zebrafish larvae compared to the control ($P<0.05$). Furthermore, the effects of the different concentrations on increasing the area and IOD of the skull stained with alizarin red were identical (Figure 5). No effect in zebrafish larvae was observed with a lower concentration of Sal B at 0.2 μmol/L. These results indicate that during the early development of zebrafish skull, Sal B can promote bone formation by stimulating osteoblast differentiation and bone mineralization.

Sal B attenuates Dex-induced inhibition of osteoblast differentiation and mineralization in the skull of zebrafish larvae

Because Sal B can promote bone formation during early development of the zebrafish skull, we determined whether Sal B may also attenuate the deleterious effect of GC on bone formation in larval zebrafish. From 3 dpf to 9 dpf, zebrafish larvae were exposed to 10 μmol/L Dex alone or a mixture of 10 μmol/L Dex and different concentrations of Sal B ranging from 0.2 to 10 μmol/L. As shown in Figure 6, Sal B at concentrations from 0.5 to 10 μmol/L attenuated the decrease in the area and IOD of alizarin red staining in the skull of larval zebrafish trigged by 10 μmol/L Dex ($P<0.05$), particularly at 2 μmol/L. Treatment with Sal B at concentrations from 0.5 to 10 μmol/L also rescued 10 μmol/L Dex-induced reduction of the green fluorescence area and IOD in tg(sp7:egfp) zebrafish larvae ($P<0.05$). The strongest rescue effect of Sal B occurred for 2 μmol/L (Figure 7), which is consistent with the results of the alizarin red-stained zebrafish larvae. As a positive control, rocalirol also hampered the decrease in the area and IOD of alizarin red staining and green fluorescence caused by GC. Sal B at 2 μmol/L and rocalirol show the same effect of attenuating the Dex-induced inhibition of osteoblast differentiation and bone mineralization in larval zebrafish. These results suggest that Sal B rescues Dex-induced inhibition of bone forma-
in zebrafish larvae by stimulating osteoblast differentiation and bone mineralization. Based on the above data, Sal B at 2 μmol/L was used in the following study.

Expression of osteoblast-specific genes is down-regulated by Dex in zebrafish and is rescued by Sal B
To further confirm the protective effect of Sal B on osteogenesis under conditions of GC treatment, a quantitative real-time PCR assay was performed to ascertain alterations in the expression of osteoblast-specific genes in larval zebrafish, including runx2a, osteocalcin (OC), alkaline phosphatase (ALP) and osterix (sp7). As shown in Figure 8, compared to the corresponding control, the mRNA expression of runx2a, OC, ALP and sp7 was significantly reduced in zebrafish larvae treated with 10 μmol/L Dex. Conversely, Sal B rescued the Dex-induced down-regulation of osteoblast-specific genes. These results further demonstrate that Sal B rescues the Dex-induced inhibition of osteogenesis by up-regulating the expression of osteoblast-specific genes.

Sal B attenuates Dex-induced oxidative stress in zebrafish
As shown in Figure 9, compared to the vehicle control, the level of ROS determined using an oxidation-sensitive fluorescent probe dye, DCFH-DA, was significantly increased in 10 μmol/L Dex-treated larvae, based on the increase of the malonic dialdehyde (MDA) level (Figure 9A and 9B). Moreover, in larval zebrafish exposed to Dex, the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH) and hydrogen peroxidase (CAT) showed obvious decreases. Furthermore, compared to the 10 μmol/L Dex-treatment, 2 μmol/L Sal B decreased the levels of ROS and MDA. Furthermore, the changes of antioxidants, including SOD, GSH and CAT were not significantly different (Figure 9C, 9D, and 9E). These results indicate that Dex stimulates the generation of ROS and enhances oxidative stress, which can be reduced by Sal B in zebrafish larvae.

Discussion
It has been reported that zebrafish is highly conserved in terms of bone architecture and bone metabolism regulation compared to humans[30, 31]. Evidence shows that the zebrafish skull develops from 3 dpf to 9 dpf by endochondral and intramembranous ossification, which is similar to the osteogenesis mechanism of humans[32]. The early-stage zebrafish larvae skull, which contains both osteoblasts and osteoclasts, is a complete system of bone formation and bone resorption[33, 34]. Osteoblast differentiation and proliferation lead to bone formation, and osteoclastogenesis occurs at the same time, highly similar to that in mammals. Our results showed that the skull of 9 dpf AB zebrafish larvae was stained red with alizarin red, indicating that mineralized skull had been formed in 9 dpf larval zebrafish. Our results are consistent with the litera-
ture. Alizarin red staining in tiny larva is a fine and complicated procedure, and it only reflects bone mineralization. In 
tg(sp7:egfp), osteoblasts are specifically marked by GFP, which
make osteoblasts visible\cite{24–26}. Osteoblast differentiation and
bone formation in 
tg(sp7:egfp) can be directly monitored. Our
results also showed that the skull with green fluorescence in

tg(sp7:egfp) can be directly observed using LSCM. Therefore,
we selected 3–9 dpf wild-type AB strain and 
tg(sp7:egfp) zebrafish larvae to evaluate the protective actions of Sal B on
osteogenesis in this study.

Dex is a common GC and is a recognized model drug for
inducing GIO in mammals, such as rats and mice\cite{35, 36}. Our
data demonstrated that Dex caused a dose-dependent decrease
in osteoblast differentiation and bone matrix mineralization in
zebrafish larvae. \textit{Runx2a}, \textit{osteocalcin}, \textit{ALP} and \textit{osterix} are
the main osteoblast-specific genes related to bone formation,
which regulate osteoblastic differentiation and function\cite{37}.
Our study showed that Dex also down-regulated the mRNA
expression of these osteoblast-specific genes in zebrafish lar-
vae, which further confirmed that excessive Dex decreased
osteogenesis by inhibiting osteoblast differentiation, mineral-
ization and maturation. Excessive Dex can evoke osteoporosis
by depressing osteoblastic bone formation in humans and
higher vertebrates. Excess GC also inhibits osteoblast differ-
etiation and bone nodule formation in bone marrow stroma
stem cells\cite{10}, consistent with our previous studies\cite{16}. Further-
more, previous reports indicate that GC induces the inhibition
of osteogenesis in zebrafish larvae\cite{21}, consistent with our pres-
ent results.

Sal B is the main pharmacologically active ingredient of
approved drugs for cardio-cerebral vascular diseases. Our
previous study\cite{16, 18} showed that Sal B \textit{in vitro} promoted bone
marrow stromal cell differentiation into osteoblasts, increased
osteoblast activity and decreased GC associated adipogenic
differentiation. Moreover, an aqueous extract of Radix \textit{Salviae
miltiorrhizae}, of which the main ingredient is Sal B, prevented
GC-induced bone loss\cite{16}. Our data from this study showed
that Sal B increased bone formation at concentrations ranging
from 0.2 to 2 μmol/L in a dose-dependent manner by promot-
ing osteoblastic differentiation and bone matrix mineralization
in zebrafish larvae, consistent with our previous findings \textit{in
vitro}\cite{16, 18}. Importantly, Sal B ranging from 0.5 to 10.0 μmol/L
attenuated the decrease of osteoblast differentiation and bone
matrix mineralization induced by Dex in a dose-dependent
manner, particularly at 2 μmol/L. Furthermore, Sal B also
counteracted the down-regulation of osteoblast-specific gene
expression triggered by Dex. Taken together, these results sug-
gest that Sal B promotes osteogenesis at lower concentrations,
and it may also prevent GC-induced inhibition of osteogenesis
at relatively high concentrations in zebrafish larvae by stimu-
lating osteoblast differentiation and bone mineralization. This
finding is consistent with our previous report \textit{in vitro}\cite{16, 18}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Salvianolic acid B increases skull mineralized matrix in intact AB strain larval zebrafish. (A) Images of skull dorsal aspect of alizarin red
stained AB strain larval zebrafish at 9 dpf treated with 0, 0.2, 0.5, 1, and 2 μmol/L Sal B for 6 d; (B) the influence of Sal B on skull mineralization area
analyzed by IPP6.0 in 9-dpf AB strain larval zebrafish and (C) the influence of Sal B on skull mineralization IOD analyzed by IPP6.0 in 9-dpf AB strain
larval zebrafish. Notes: IPP6.0 (Image-Pro Plus image analysis software version 6.0); IOD (integral optical density); Con (control, egg water); S0.2 (Sal B 0.2
μmol/L); S0.5 (Sal B 0.5 μmol/L); S1 (Sal B 1 μmol/L); S2 (Sal B 2 μmol/L). Data are given as mean±SD. n≥9. *P<0.05 vs vehicle control.}
\end{figure}
Rocalirol, an effective bone-forming agent in clinical practice, is often used as a positive control anti-osteoporosis drug\textsuperscript{[38]}. As is shown in our results (Figure 6), rocalirol at a concentration of $6\times10^{-6}$ µg/mL increased bone mineralization and osteoblast differentiation in zebrafish larvae exposed to Dex, and the degree of increase in bone formation is similar to that of 2 µmol/L Sal B. In addition, we found that rocalirol at concentrations from $2\times10^{-6}$–$10\times10^{-6}$ µg/mL showed a dose-dependent trend toward increased bone formation in larval zebrafish exposed to Dex, particularly at $10\times10^{-6}$. However, the larvae treated with rocalirol at concentrations of $8\times10^{-6}$–$10\times10^{-6}$ µg/mL had reduced swimming activity, malformation and death (data not shown). Interestingly, the larvae exposed to 10 µmol/L Dex plus Sal B at concentrations ranging from 0.2 to 10 µmol/L showed no decrease in swimming activity, malformation and death (data not shown). Together, these data suggest that Sal B may have protective effects on bone loss triggered by GC.

To clarify the mechanism by which Sal B attenuates GC-induced osteogenesis inhibition, the levels of ROS and MDA were used as indicators of oxidation, and the activities of SOD, GSH and CAT were used as indicators of antioxidant levels in zebrafish exposed to 10 µmol/L Dex in the presence or absence of 2 µmol/L Sal B. Our study showed that Dex led to excessive ROS generation and increased MDA, whereas it inhibited the activity of SOD, GSH, and CAT in larval zebrafish, consistent with reports that GC is an oxidant stimulus that triggers ROS generation and activates OS in bone\textsuperscript{[13]}. Sal B hampered the increase in ROS generation and MDA levels; however, it did not significantly increase the activities of these antioxidant enzymes. These results indicate that Sal B, as a natural potent antioxidant, directly clears ROS through its own antioxidant activity rather than by up-regulating endogenous antioxidant enzymes in zebrafish.

Both Sal B and tanshinol are strong natural antioxidants due to their phenolic hydroxyl groups\textsuperscript{[14, 15]}, and Sal B can be transformed into tanshinol \textit{in vivo}. Our previous study demonstrates that tanshinol not only stimulates osteoblast
differentiation and function\textsuperscript{[17]} but also scavenges ROS triggered by H\textsubscript{2}O\textsubscript{2} and attenuates OS and OS-triggered inhibition of osteoblast differentiation by regulating \emph{wnt/foxO3a/PPAR\gamma/\beta-catenin} signaling in C\textsubscript{2}C\textsubscript{12} cells\textsuperscript{[39]}. Our previous finding showed that Sal B has beneficial effects on osteoblast growth, differentiation and mineralization \textit{in vitro} by activating the expression of \textit{Runx2} and \textit{\beta-catenin} mRNA and decreasing the expression of \textit{Dickkopf 1} and \textit{PPAR\gamma} mRNA\textsuperscript{[39]}. We found that Sal B counteracts the Dex-induced decreases in bone formation and osteoblast-specific gene expression in zebrafish larvae. In summary, our data reveal that Sal B attenuates GC-induced inhibition of osteogenesis, partially by removing ROS and reducing oxidative stress caused by GC.

In conclusion, this study demonstrated that Dex inhibited osteoblastic differentiation and bone mineralization, downregulated osteoblastic-specific genes and increased ROS generation and the subsequent activation of OS in zebrafish larvae. Sal B attenuates these deleterious effects of Dex on bone formation in zebrafish larvae in part by scavenging excessive ROS and decreasing the oxidative stress elicited by Dex. Our findings support the further investigation of Sal B on bone formation and indicate that Sal B may have protective effects against bone disorders, particularly GC-induced bone loss.

Acknowledgements
The authors are thankful to Dr Chung-der HSIAO’s group from Chung Yuan Christian University, Taiwan, China for use of the \textit{tg(sp7:egfp)} zebrafish. This research was supported by grants from the National Natural Science Foundation of China (81273518 and 31370824), the Science and Technology Planning Project of Guangdong Province (No 2012B060300027) and the Scientific Research Subject of Traditional Chinese Medicine Bureau of Guangdong Province (No 20152152).

Author contribution
Liao CUI, Shi-ying LUO, and Jing-jing ZHANG designed the research; Shi-ying LUO, Jing-feng CHEN, and Zhi-guo ZHONG performed the research; Xiao-hua LV and Ya-jun YANG contributed new analytical tools and reagents; Shi-ying LUO and Jing-feng CHEN analyzed data; and Shi-ying LUO, Liao CUI, Jing-jing ZHANG, and Ya-jun YANG wrote the paper.
Figure 8. Sal B up-regulates the expressions of osteoblast-specific genes in larval zebrafish treated with Dex. Quantitative real-time PCR was performed to determine mRNA levels of (A) alp, (B) OC, (C) runx2a and (D) sp7 genes. Note: Veh (vehicle control, 0.1% dimethyl sulphoxide); Dex (Dex 10 μmol/L); D+S (Dex+Sal B 2 μmol/L). Dates are given as mean±SEM of three independent experiments. *P<0.05 vs vehicle control. #P<0.05 vs Dex treatment.

Figure 9. Sal B suppresses Dex-triggered oxidative stress in zebrafish larvae. (A) The level of ROS; (B) The level of MDA; (C) The activities of CAT; (D) The activities of SOD; (E) The activities of GSH. Note: Veh (vehicle control, 0.1% dimethyl sulphoxide); (Dex (Dex 10 μmol/L); D+S (Dex+Sal B 2 μmol/L). Data are given as mean±SEM of three independent experiments. *P<0.05 vs vehicle control. #P<0.05 vs Dex treatment.

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