Acupuncture and Herbal Medicine

Exploring a novel class tryptophan hydroxylase 1 inhibitor derived from *Sambucus williamsii Hance* for the osteoporosis treatment

Yu-Xin Zhu[1], Zi-Ling Tang[2], Lu Lu[1], Zuo-Cheng Qiu[2], Dabo Pan[3], Yang Yu[3], Hui-Hui Xiao[1,4,5,*], Man-Sau Wong[1,4,5]

[1]State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation), The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, China; [2]Guangzhou Key Laboratory of Formula-Pattern of Traditional Chinese Medicine, School of Traditional Chinese Medicine, Jinan University, Guangzhou, China; [3]Guangdong Provincial Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, College of Pharmacy, Jinan University, Guangzhou, China; [4]Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Hong Kong, China; [5]Research Center for Chinese Medicine Innovation, The Hong Kong Polytechnic University, Hong Kong, China

Abstract

Objective: Gut-derived serotonin strongly inhibits bone formation by inhibiting osteoblast proliferation. Our previous study demonstrated that the lignan-rich fraction prepared from Sambucus willimasii Hance, a folk herbal medicine used to treat bone fractures and joint diseases in China, exerted bone-protective effects, and its actions were modulated by suppressing the synthesis of gut-derived serotonin *via* the inhibition of intestinal tryptophan hydroxylase 1 (TPH-1). However, there is no direct evidence for the action of lignans on TPH-1. This study aimed to verify the direct action of lignans on the TPH-1 and its influence on serotonin synthesis and bone properties.

Methods: Molecular docking and surface plasmon resonance were performed to determine the affinities of lignans to TPH-1. The cell viability and the protein activity and expression of TPH-1 were measured in RBL2H3 cells. The serum serotonin level and bone mineral density upon lignan treatment in ovariectomized mice were determined.

Result: The lignans showed high binding scores and binding affinities to TPH-1, inhibited the activity and protein expression of TPH-1, suppressed the serum serotonin levels in ovariectomized mice as well as promoted bone mineral density.

Conclusion: This is the first study to report that lignans are novel TPH-1 inhibitors and that these lignans could be potential agents for the management of serotonin-related diseases, including osteoporosis.

Keywords: Lignan, Osteoporosis, *Sambucus williamsii* Hance, Serotonin, TPH-1 inhibitor

Graphical abstract: http://links.lww.com/AHM/A93.

Introduction

Osteoporosis is a chronic metabolic skeletal disease associated with aging that leads to the deterioration of bone microstructure and an increase in fracture risk[1]. With aging of the global population, osteoporosis has become a serious disease affecting societies and families. Traditional Chinese medicine (TCM) has a long history of use in the prevention and treatment of bone and joint diseases. Many clinical studies and animal experiments have demonstrated the anti-osteoporotic activities of TCM[2], and the active components of TCM are potential candidates for osteoporosis management.

Sambucus williamsii Hance was first recorded in the Tang Materia Medica (*Tang Ben Cao*). It is a traditional fork medicine used to treat bone fractures and joint-associated diseases[3]. Our previous studies demonstrated the beneficial effects of the lignan-rich fraction of *S. williamsii* on estrogen deficiency-induced bone loss[4–6] and revealed that these actions were mediated by suppressing the synthesis of gut-derived serotonin through inhibition of intestinal tryptophan hydroxylase 1 (TPH-1)[7].

Serotonin (5-hydroxytryptamine [5-HT]), synthesized from the ingested tryptophan in food, is an important regulator of the gastrointestinal tract and other organ systems[8]. In the gut mucosal enterochromaffin (EC) cells, tryptophan is converted to 5-HT by the rate-limiting synthetic enzyme TPH-1[9]. Gut-derived 5-HT (GDS) accounts for more than 90% of the 5-HT in the body and is implicated in several age-related diseases, such as

*Corresponding author. Hui-Hui Xiao, E-mail: huihui.xiao@polyu.edu.hk.

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inflammation, immunosenescence, neurodegeneration, and osteoporosis[10–13]. GDS powerfully inhibits bone formation by blocking the proliferation of osteoblasts through the forkhead box protein O1 (FOXO1) signaling pathway but does not affect bone resorption. Daily oral administration of LP533401 (a small-molecule inhibitor of TPH-1) for up to 6 weeks reportedly promoted bone formation[14]. Several ursolic acid derivatives were synthesized using the docking technique, and their inhibitory effects on serotonin biosynthesis were evaluated in RBL2H3 cells and *Tph1*-expressing cells. Triterpene 9a was discovered to promote bone microarchitecture in ovariectomized rats[15]. These studies provide strong evidence that TPH-1 could be a novel target for the development of new anabolic agents for the treatment of osteoporosis.

Although our previous study verified the involvement of circulating serotonin and TPH-1 in the positive effects on bone health induced by the lignan-rich fraction from *S. williamsii*, there is no direct evidence for the modulating effects of lignans on serotonin synthesis. The present study aims to further investigate the effects of major lignans from *S. williamsii* on serotonin synthesis and identify new TPH-1 inhibitors. In this study, molecular docking and surface plasmon resonance (SPR) of lignans with TPH-1 were performed. Additionally, the TPH-1 protein activity, TPH-1 protein expression, and serum serotonin levels in mice upon treatment with lignans were determined.

Materials and methods

Molecular docking

The binding affinities of 14 lignans from *S. williamsii* and their intestinal metabolites to TPH-1 protein were predicted using the extra precision Glide module of Schrodinger 2018. First, the molecular crystal structure of the TPH-1 protein was retrieved from the Protein Data Bank with PDB ID code 1MLW. The structures of all lignans were drawn, and LigPrep was employed to preprocess them using the OPLS-2005 force field, resulting in the generation of corresponding low-energy conformers. The protonation state was assigned using Epik at the target pH value of 7.0 ± 2.0. During the initial phase of the molecular docking calculations, 500 poses were generated, from which the top 100 poses were selected for energy minimization through 1,000 conjugate gradient minimization steps.

Surface plasmon resonance analysis

SPR experiments were performed using a Biacore 8k (GE Healthcare, Uppsala, Sweden). First, the CM5 chip (Cytiva, Uppsala, Sweden) was activated using 400 mM 1-ethyl3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 100 mM N-hydroxysuccinimide for 420 seconds at 10 µL/min. The target protein TPH-1 (SinoBiological, Beijing, China) was diluted to 50 µg/mL using 10 mM sodium acetate (GE Healthcare) at pH 4.0 and covalently immobilized on the chip surface for 600 seconds. The immobilized chip was blocked with 1 M ethanolamine for 420 seconds at a flow rate of 10 µL/min. The compound was diluted in running buffer containing phosphate buffered saline (PBS) (GE Healthcare), 1 mM Dithiothreitol (Genebase, Uppsala, Sweden), 0.5% P20 (GE Healthcare), and 5% dimethylsulfoxide (DMSO) at concentrations of 3.125, 6.25, 12.5, 2.5, and 5 µM, respectively, and incubated with the immobilized protein for 180 seconds at 30 µL/min. All data were analyzed using kinetic models with Biacore 8k Evaluation Software.

Cell culture and MTS assay

RBL2H3 cells were purchased from Procell (Wuhan, China) and cultured routinely in the medium and under the conditions described in our previous study[7]. Cells were seeded at a density of 1×10^4 and 5×10^4 cells per well in 96-well plates and 24-well plates, respectively. Cells were treated with vehicle, LP533401 (LP, 10^{-6} M), or lignan (10^{-8} to 10^{-5} M) for 48 h. LP is a reported intestinal TPH-1 inhibitor and was used as a positive control in the current study[16]. The [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (MTS) assay (Promega, Madison, MI, USA) was used to measure cell viability, as previously described[17].

TPH-1 protein level in vitro

RBL2H3 cells were inoculated into a 24-well plate, allowed to adhere to the wall for 4 h, and then treated with vehicle, LP533401, or lignan (L12, 10^{-8} to 5×10^{-5} M) for 48 h. Cell lysates were collected, and the total TPH protein level was measured according to the manufacturer's instructions (Rat Tryptophan Hydroxylase [TPH] enzyme linked immunosorbent assay ELISA Kit, CUSABIO, Wuhan, China).

Western blotting

RBL 2H3 cells were treated with lignans at 10^{-7} and 10^{-6} M for 48 h. Proteins were extracted from the treated cells, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane as previously described[17]. The proteins TPH-1 (1:1,000, Immunoway, Suzhou, China) and β-actin (1:1,000, Beyotime Biotechnology, Shanghai, China) were analyzed. The secondary antibody, HRP-conjugated goat anti-rabbit (1:2,000, Abcam, Cambridge, UK), was incubated with the bands and then detected *via* incubation with the enhanced chemiluminescence (ECL) substrate (Pierce qb Perbio, Rockford, IL, USA). Finally, the proteins were visualized through the ChemiDoc XRS + system using Quantity One software (Bio-rad, Hercules, CA, USA).

TPH-1 activity assay

RBL2H3 cells were inoculated into a six-well plate at 5×10^4/well and incubated for 4 h. The cells were then treated with vehicle, LP (10^{-6} M), or lignan (10^{-7} M) for 48 h. After a 6-minute trypsin digestion of the treated cells, the process was halted using 1 mL of medium. Subsequently, the cells were centrifuged at 1,000 rpm for 5 min, collected, and tested in accordance with the

manufacturer's instruction (HEPENGBIO, Shanghai, China). The absorbance of the samples was measured at 736 nm using a UV spectrophotometer (Biotech, Winooski, VT, USA). The TPH-1 activity was expressed as units of activity (U/mg) by normalizing it with the total protein determined through the Bradford protein assay.

Animal study

C57 BL/6J mice (14-week old) were employed to confirm the effects of selected TPH-1 inhibitors on serotonin levels *in vivo*. The mice (n = 40) were housed in a controlled specific pathogen-free environment (12-hour daylight cycle, (23 ± 2)°C and 40%–60% humidity) with water *ad libitum*. After 5 d of adaptation, the mice were sham-operated or bilaterally ovariectomized and then randomly divided into four groups (n = 10): (1) sham, sham-operated, vehicle; (2) OVX, ovariectomized, vehicle; (3) L2, ovariectomized + L2 (50 mg/kg); and (4) L12, ovariectomized + L12 (50 mg/kg). The lignan dosages were determined according to previous studies[14,18,19]. Following a 1-week recovery period, the mice were subjected to oral administration for 8 weeks. The serum, uterus, and femur were collected for subsequent analyses.

During the entire study period, the animals had free access to water. To avoid the potential interfering effects of soybean oil in normal chow on the bone, the animals were pair-fed with a phytoestrogen-free diet (D00031602; Research Diet, New Brunswick, NJ, USA). All animal procedures were approved by the Ethics Committee of the Hong Kong Polytechnic University Animal Subjects (ASEC Case: 180402).

Micro-computed tomography analysis of bone

The distal femurs of all mice were scanned using micro-computed tomography (micro-CT; μCT40; Scanco Medical, Zurich, Switzerland). All data were acquired at 70 kVp and 114 mA, with a resolution of 21 μm and voxel sizes of 10.5 μm^3. Scanning was initiated at the metaphyseal growth plates, and the volume of interest (VOI) was delineated based on 50 consecutive slices. Bone mineral density (BMD) was evaluated using VOI images.

Determination of serum serotonin level using ultra performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-TQD-MS/MS) analysis

The analysis of serotonin, kynurenine (Kyn), their respective isotopologs (D_4-serotonin, D4-L-Kyn), and internal standard (IS, 5-Methoxytryptamine) was performed on an ACQUITYTM UPLC system (Waters Corp., Milford, CT, USA) coupled to a Xevo TQ-XS mass spectrometer (Waters Corp.), as described in our previous study[7].

Statistical analysis

Statistical analyses were conducted with GraphPad PRISM 8 software package. Data are presented as mean ± standard error of the mean (SEM) and were analyzed using unpaired Student t test and one-way analysis of variance (ANOVA) with Tukey *post hoc* test for *in vitro* and *in vivo* experiments, respectively. Statistical significance was set as $P < 0.05$.

Results

Molecular docking of lignans to TPH-1

Docking studies were carried out for TPH-1 protein against 14 lignans either isolated from *S. williamsii*[20] or the intestinal metabolites of these lignans[21]. Detailed information on these lignans and docking scores, which reveals a direct reflection of the molecular interactions between TPH-1 and the ligand, are presented in Table 1. The docking score values were negative, with a higher negative value indicating a higher binding affinity of the ligand to the TPH-1 protein. The binding affinity positively correlated with the inhibitory effects of the ligand on TPH-1. The positive control LP533401 (LP) showed a score of –3.19, whereas the 13 tested lignans showed lower scores than that of LP. The binding models of all compounds in the X-ray structure of TPH-1 protein are shown in Figure 1. The results showed that the O and H atoms of the hydroxyl group and the O atom of the tetrahydrofuran ring in lignans formed hydrogen bonds with the carboxyl and H atoms of the amino acid residues in the TPH-1. In addition, the benzene ring of lignans formed strong π–π interactions with amino acid residues of the TPH-1.

Affinities of lignans with TPH-1

To determine whether lignans could bind directly to the TPH-1, real-time binding analysis *via* SPR was performed using Biacore 8K. The affinities of different types of lignans, including dibenzylbutanes (L2), arylnaphthalenes (L5), 8-*O*-4′ neolignans (L9 and L11), and eupomatenoid benzofurans (L12), to TPH-1 were tested. The binding curves and values of the lignans to the TPH-1 are shown in Figure 2 and Table 2. The tested compounds showed dose-dependent binding to the TPH-1, suggesting a good affinity between them. The calculated dissociation constant (KD) of LP were 34.8 μM and those of the lignans were 5.33, 0.26, 16, 17.3, and 1.58 μM for L2, L5, L9, L11, and L12, respectively, which indicated that all tested lignans had stronger affinities than that of LP to the TPH-1. The affinity of LP was calculated *via* a general kinetic analysis model owing to its slow binding and dissociation with TPH-1, whereas the lignans were measured *via* a steady-state analysis model because of their fast binding and dissociation with TPH-1.

Influence of lignans on RBL 2H3 cells viability

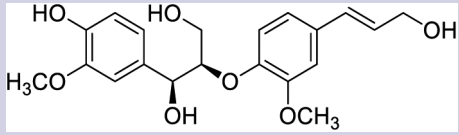
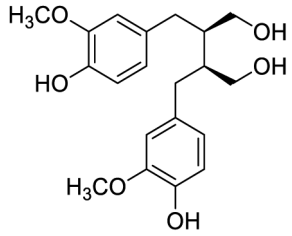
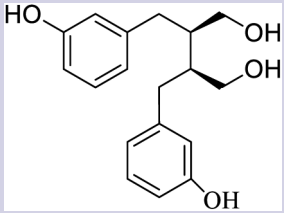
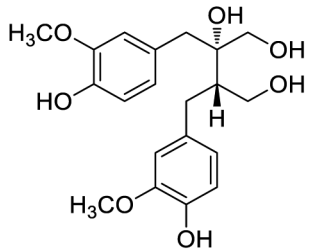
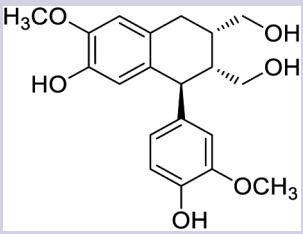
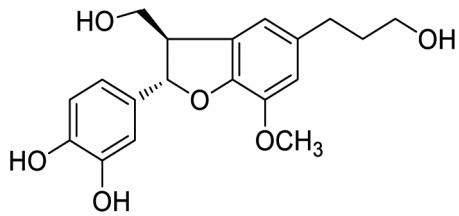
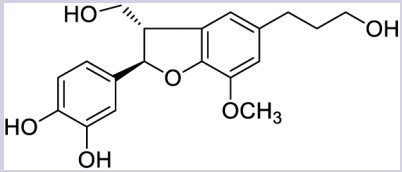
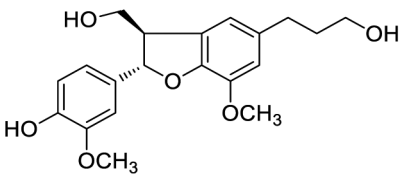
The viability of eight lignans, L2, L5, L9, L10, L11, L12, L13, and L14, was evaluated in RBL2H3 cells using the MTS assay to examine whether the inhibitory effects of lignans on TPH-1 expression were caused by their cytotoxic effects. As shown in Figure 3A, none of the lignans except the metabolite enterolactone (L13) exerted cytotoxic effects on RBL2H3 cells at the concentration from 10^{-8} to 10^{-5} M.

Inhibitory activities of lignans on TPH-1 in RBL2H3 cells

The TPH protein levels of the eight lignans were determined *via* ELISA at concentration from 10^{-8} to 10^{-5} M.

Table 1

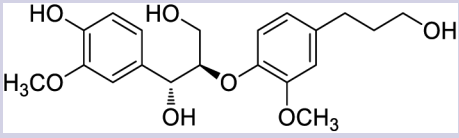
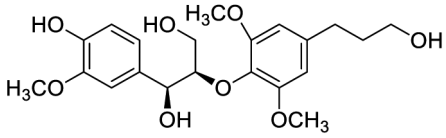
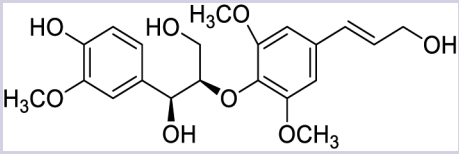
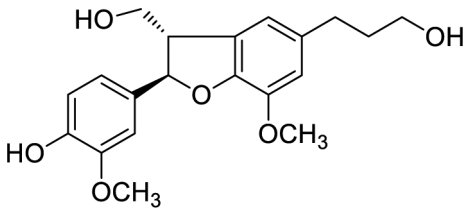
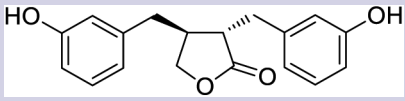
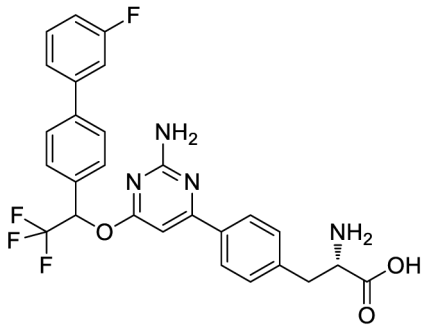
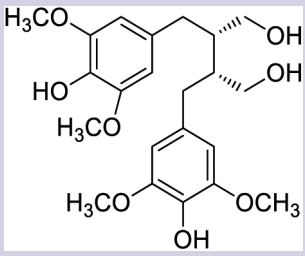
The structures of compounds and their docking scores

	Structure	Name	Docking score (kcal/mol)
L1		*erythro*-1-(4-Hydroxy-3-methoxyphenyl)-2-[4-[(E)-3-hydroxy-1-propenyl]-2-methoxyphenoxy]-1,3-propanediol	−8.74
L2		Secoisolariciresinol	−8.33
L3		Enterodiol	−7.09
L4		Alashinol G	−6.93
L5		(1S,2R,3S)-1,2,3,4-Tetrahydro-7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol	−6.78
L6		4-[(2S,3R)-2,3-Dihydro-3-(hydroxymethyl)-5-(3-hydroxypropyl)-7-methoxy-2-benzofuranyl]-1,2-benzenedio	−6.55
L7			−5.97
L8		(2S,3R)-Dihydrodehydroconiferyl alcohol	−5.64

(Continued)

Table 1

(Continued)

	Structure	Name	Docking score (kcal/mol)
L9		threo-guaiacylglycerol-β-O-4'-dihydroconiferyl alcohol	−4.95
L10		*erythro* -1-(4-Hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol	−4.73
L11		*erythro* -1-(4-Hydroxy-3-methoxyphenyl)-2-[4-[(1E)-3-hydroxy-1-propen-1-yl]-2,6-dimethoxyphenoxy]-1,3-propanediol	−4.03
L12		Di-hydrodehydrodiconiferyl alcohol	−3.76
L13		Enterolactone	−3.28
LP		LP533401	−3.19
L14		5,5'-Dimethoxy-secoisolariciresinol	−2.90

All lignans, except L11, had significant inhibitory effects on TPH levels at a certain dose (Figure 3B). Western blot analysis (Figure 4A) showed that, except for L13, all the tested lignans significantly downregulated TPH-1 protein expression at 10^{-7} and 10^{-6} M.

Suppressive actions of lignans on TPH-1 activity

Because TPH-1 is a synthetic enzyme, its activity is related to its function. Therefore, the activity of TPH-1 upon lignan treatment in RBL2H3 cells was determined using a commercial kit. As shown in Figure 4B, compared with the control, all tested lignans suppressed the activity of the TPH-1 at 10^{-7} M, which was similar to the actions of the positive control LP.

Inhibitory effects of lignans on 5-HT synthesis in vivo, and the negative correlation of serum 5-HT level with BMD

As a rate-limiting enzyme in the synthesis of serotonin, the activity of the TPH-1 is directly related to the synthesis of serotonin. To further confirm the inhibitory effects

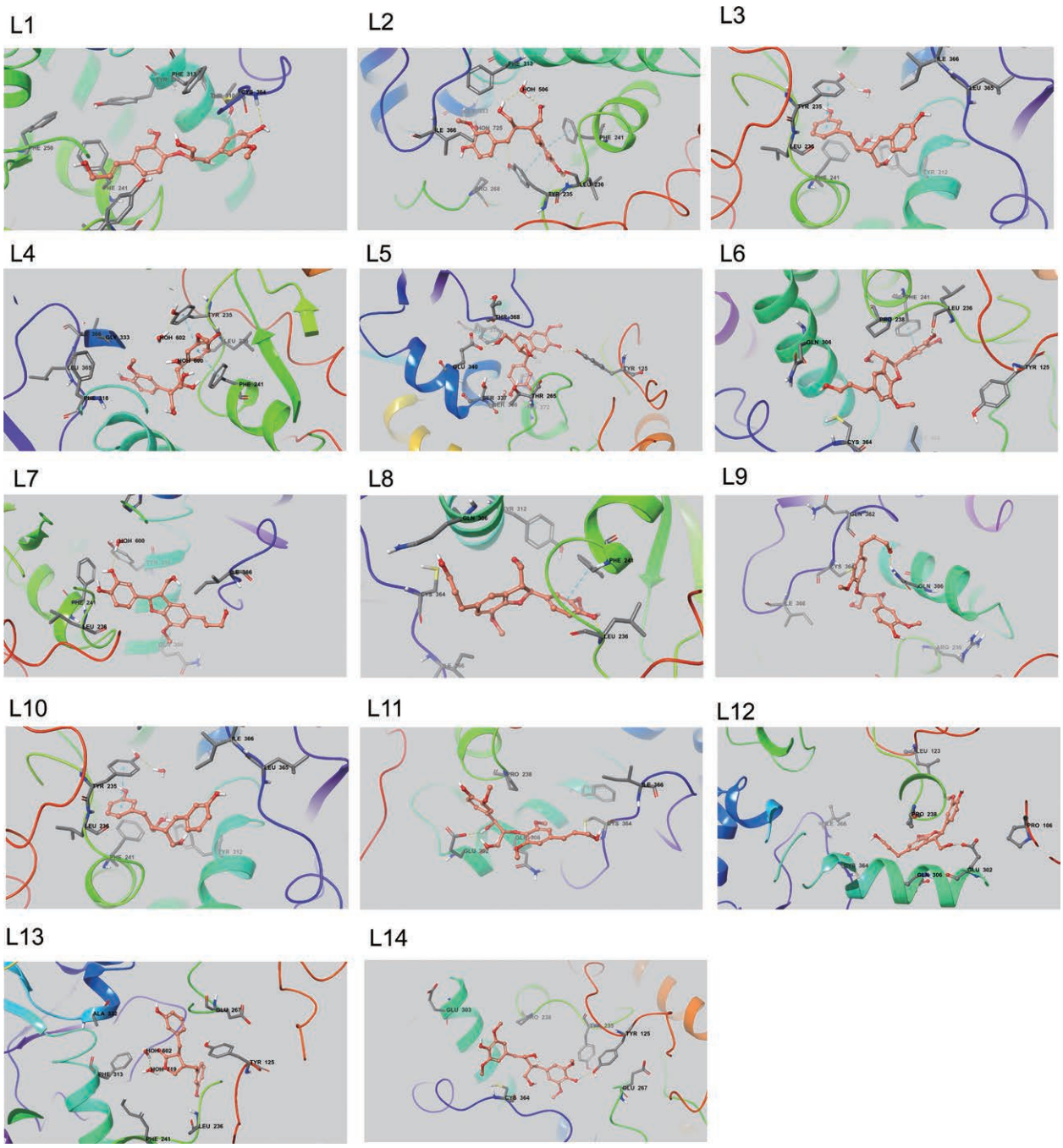


Figure 1. Binding model of lignans into the X-ray structure of TPH-1. Dashed yellow and blue lines indicate H-bonds and π–π interactions, respectively. TPH-1: Tryptophan hydroxylase 1.

of lignans on the TPH-1, serum serotonin levels in OVX C57BL/6J mice in response to oral treatment with two lignans, secoisolariciresinol (L2) and dihydrodehydrodiconiferyl alcohol (L12), were determined. As shown in Figure 5A, ovariectomy significantly induced uterine atrophy in mice compared to the sham group, suggesting that the ovariectomy was successful. None of the treatments increased the uterine index of the OVX mice, indicating the safety of lignans in the uterus. As shown in Figure 5B, 9 weeks after surgery, ovariectomy significantly increased serum serotonin levels, whereas upon treatment with L2 and L12 at 50 mg/kg for 8 weeks, serum serotonin levels were significantly suppressed in OVX mice. These results revealed that, as TPH-1 inhibitors, the lignans indeed inhibit TPH-1 function *in vivo*. Lignan treatment did not influence serum Kyn levels in OVX mice (Figure 5C).

Considering the inhibitory effects of gut-derived serotonin on bone formation, the relationship between BMD and serum serotonin levels upon treatment with lignans was further determined in this study. BMD was measured using micro-CT scanning and is shown in Figure 5D. L2 and L12 treatment with 50 mg/kg significantly increased femoral BMD in ovariectomized mice. Linear regression analysis of BMD and serum 5-HT (Figure 5E) showed a negative correlation with $R^2 = 0.1670$ ($P = 0.016$), which supported the idea that the beneficial effects of lignans on bone were related to gut-derived serotonin.

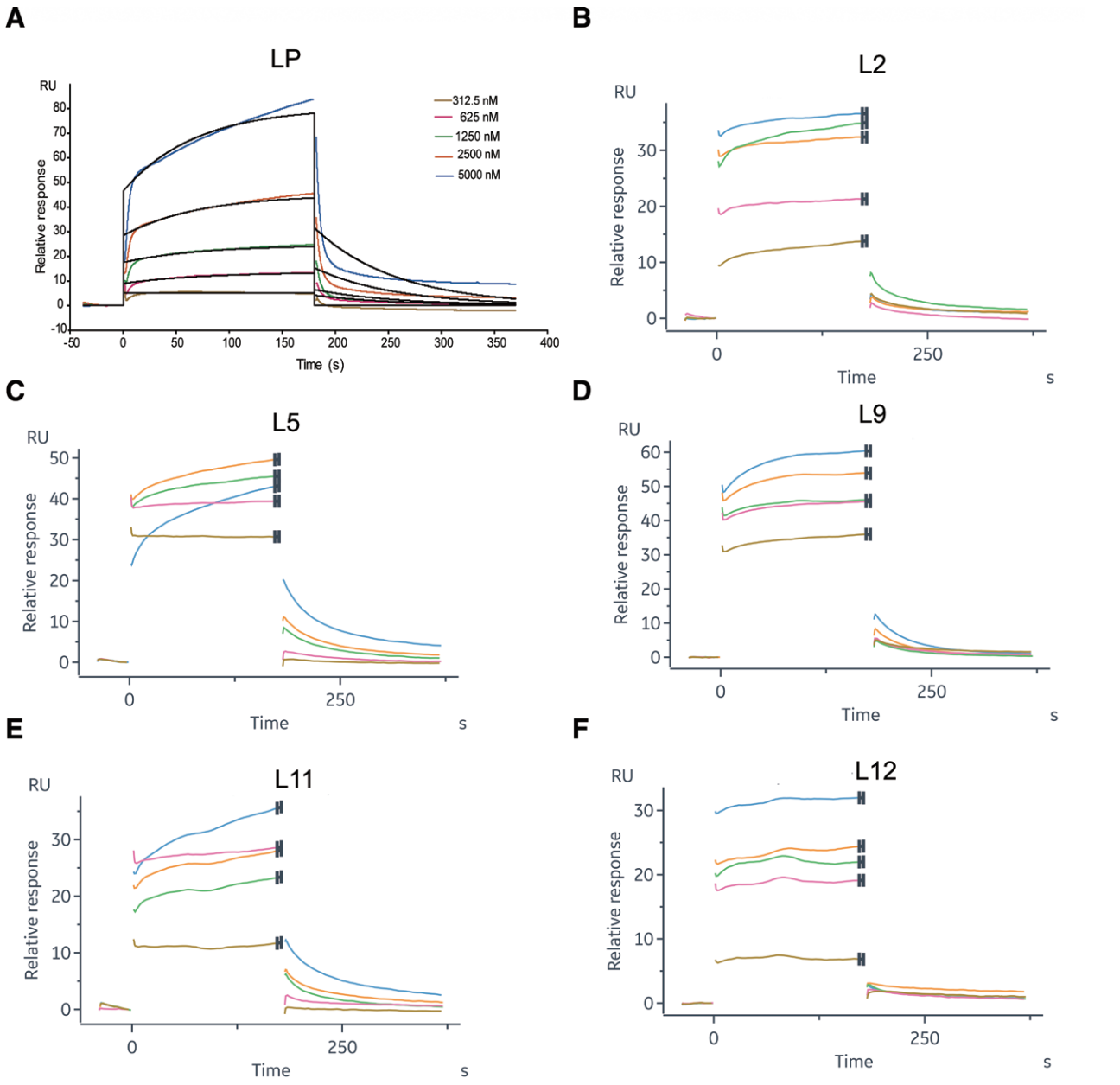


Figure 2. Binding curve of lignans to TPH-1. A series of concentrations (312.5–50,000 nM) of lignans was applied to TPH-1 for kinetic analysis to determine the affinity between lignan and TPH-1. (A) LP; (B) L2; (C) L5; (D) L9; (E) L11; (F) L12. TPH-1: Tryptophan hydroxylase 1.

Discussion

Sambucus williamsii Hance is an herbal folk medicine that has been clinically applied to bone fractures and joint diseases in China for thousands of years. The anti-osteoporotic effects of *S. williamsii*[4,6,22] have been demonstrated in our previous studies. Furthermore, we also found that the bone-protective effects of *S. williamsii* are related to tryptophan metabolism[5] and mediated by suppressing the synthesis of gut-derived serotonin *via* inhibition of intestinal TPH-1. The present study focuses on investigating the direct effects of lignans derived from *S. williamsii* or the intestinal metabolites of these lignans on the TPH-1. We found that lignans exhibited high binding affinities for the TPH-1 protein, inhibited the activity and expression of TPH-1 in RBL2H3 cells, and suppressed serotonin levels in ovariectomized mice. We confirmed that the BMD is negatively correlated with serum serotonin, which indicates that the decreased serum serotonin level after treatment with lignans accounts for the protective effects of lignans on the bone.

Although serotonin is a neurotransmitter, it has both central and peripheral functions, such as the regulation of mood, appetite, food consumption, gastrointestinal sensation, motility, sex, sleep, and pain[23]. Different forms of the rate-limiting synthetic enzymes of 5-HT, TPH-1 and TPH-2, are expressed in EC cells of the intestinal mucosa and neurons of the brainstem raphe nuclei, respectively. EC cells synthesize and store the largest pool of 5-HT, which is distinct from centrally synthesized 5-HT, because the positive charge of 5-HT at normal physiological pH prevents it from freely passing through the blood-brain barrier[8]. Upon synthesis by TPH-1 in EC cells, serotonin is released to nearby fibers, immune cells, and epithelial cells as a paracrine

signaling molecule and is then absorbed by epithelial cells, while the rest of the serotonin is transported into the circulatory system by platelets. Several serotonin receptors are expressed on different cell types. Circulating serotonin inhibits osteoblast proliferation by binding to 5-HT_{1B} receptors in preosteoblasts, leading to bone resorption[24].

Depression is linked to osteoporosis owing to the incidence increase of bone fractures and osteoporosis in patients taking serotonin reuptake inhibitors (SSRIs)[25–27]. SSRIs increase the circulation of serotonin synthesized in the gut by inhibiting serotonin-specific reuptake transporters. In addition, the administration of TPH-1 inhibitors, such as LP533401 and ursolic acid derivatives, to young and ovariectomized rodent models decreased circulating serotonin levels and improved bone formation[14,15,28]. These clinical and preclinical studies indicate that serotonin is critical for bone anabolism. Our results confirmed that serum 5-HT levels were negatively correlated with BMD ($R^2 = 0.1670$, $P = 0.016$; Figure 5E), further supporting the fact that inhibiting gut-derived serotonin could be an anabolic approach for the treatment of osteoporosis.

Karsenty et al.[14] first demonstrated the success of using LP533401, a synthesized small-molecule inhibitor of TPH-1, to treat osteoporosis in ovariectomized rodents after once-daily oral administration for a duration up to 6 weeks. Their work was followed by

Table 2

The structures of compounds and their docking scores

Compound	Affinity model	KD (M)	Immobilized ligand
LP	General kinetics model	3.48e-5	50 μg/mL TPH-1 pH 4.0
L2	Steady-state affinity	5.33e-6	50 μg/mL TPH-1 pH 4.0
L5	Steady-state affinity	2.63e-7	50 μg/mL TPH-1 pH 4.0
L9	Steady-state affinity	1.60e-5	50 μg/mL TPH-1 pH 4.0
L11	Steady-state affinity	1.73e-5	50 μg/mL TPH-1 pH 4.0
L12	Steady-state affinity	1.58e-6	50 μg/mL TPH-1 pH 4.0

KD: Dissociation constant; TPH-1: Tryptophan hydroxylase 1.

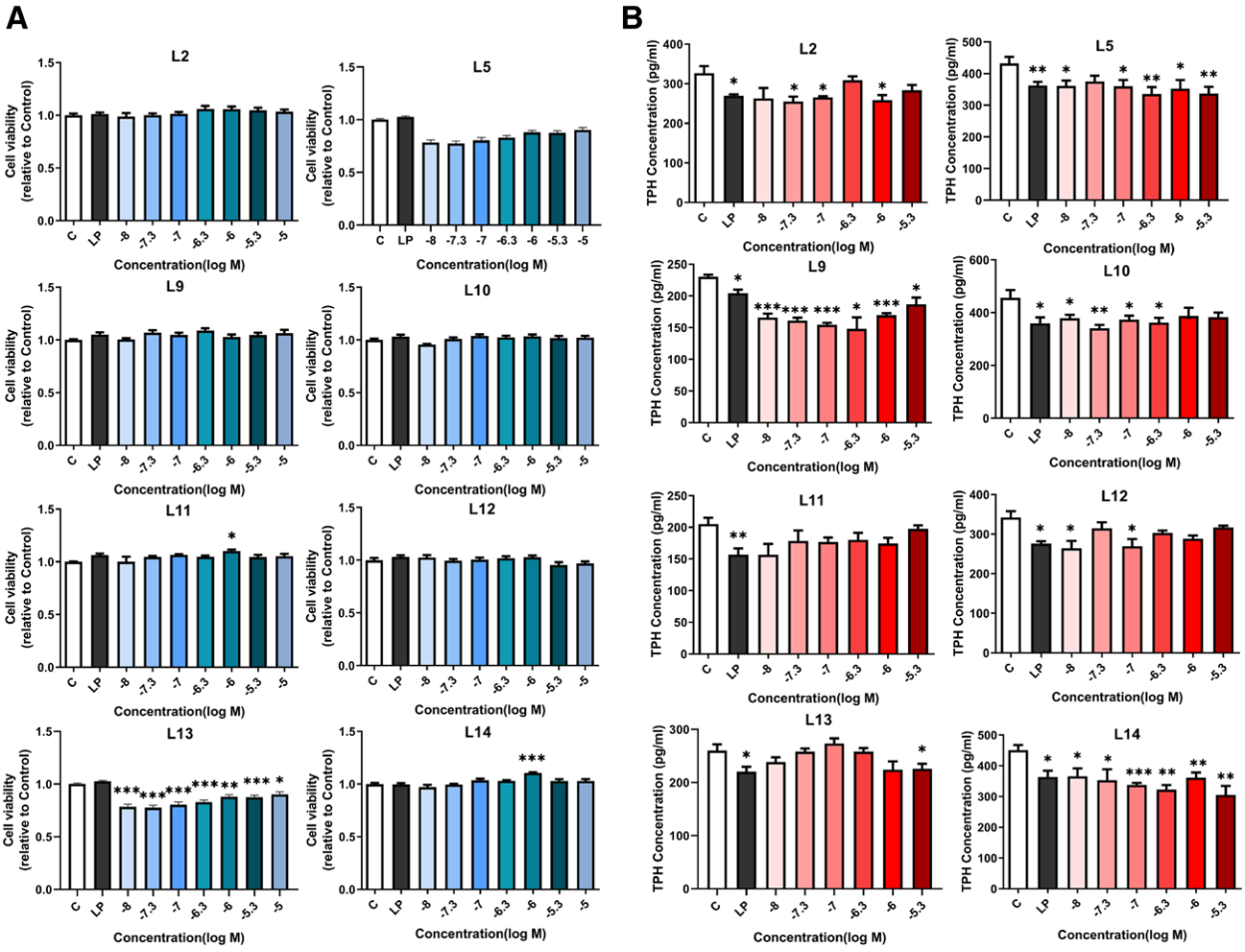


Figure 3. Cell viability (A) and TPH protein levels (B) upon treatment with lignans on RBL2H3 cells. Cells were treated with vehicle, LP533401 (LP, 10^{-6} M, a positive control), and lignans (10^{-8}–10^{-5} M) for 48 h. Cell viability rate was assessed using the MTS assay. TPH protein levels were determined with the ELISA kit. The results obtained are expressed as mean ± SEM ($n = 8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control using the unpaired Student t test. ELISA: Enzyme linked immunosorbent assay; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; SEM: Standard error of the mean; TPH-1: Tryptophan hydroxylase 1.

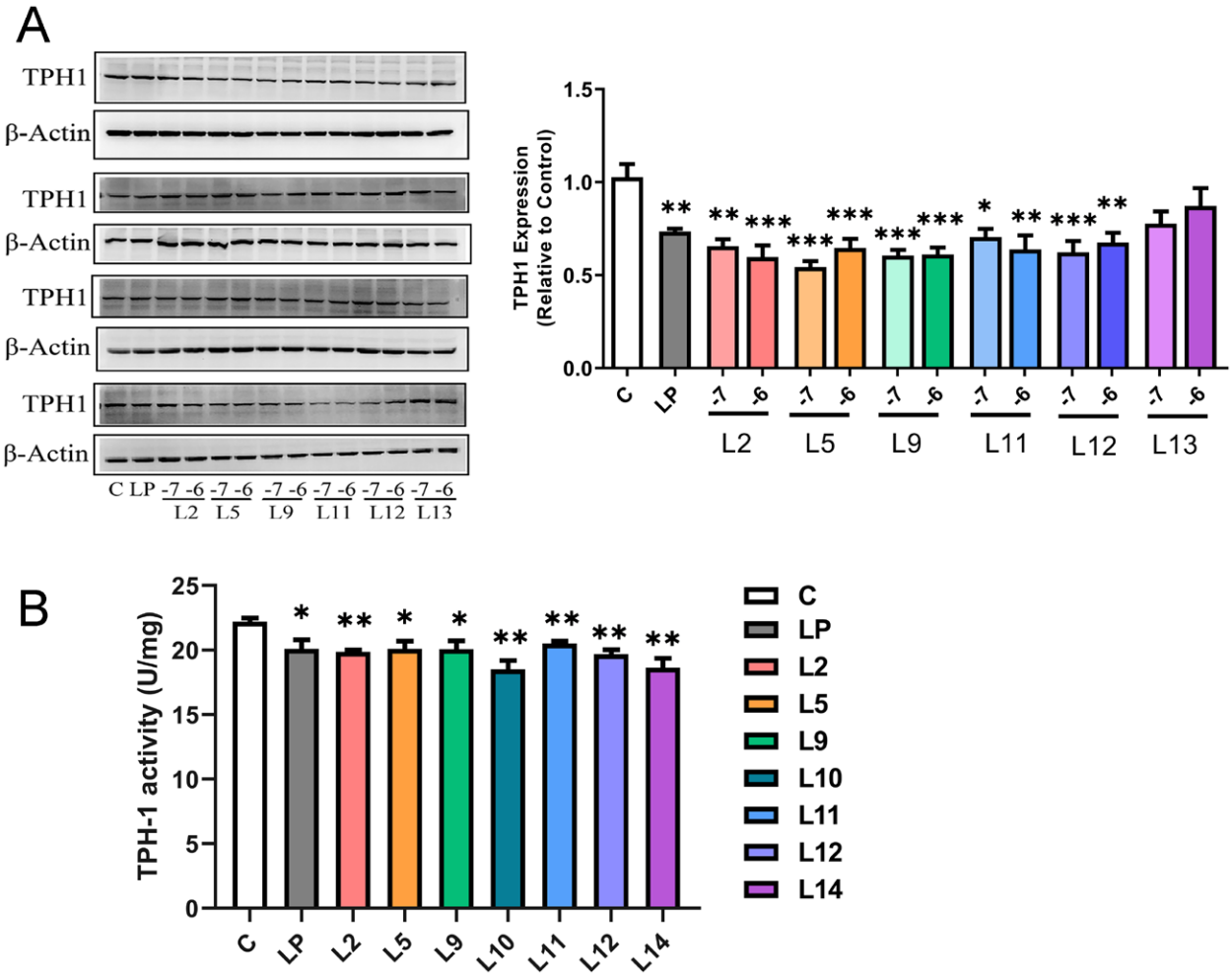


Figure 4. Expression (A) and activity (B) of TPH-1 following treatment with lignans on RBL2H3 cells. Cells were treated with vehicle, LP533401 (LP, 10^{-6} M, a positive control), and lignans (10^{-7}–10^{-6} M) for 48h. TPH-1 protein expression was measured *via* immunoblotting. TPH-1 activity was tested utilizing the colorimetric quantitation kit. The results are expressed as mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control using the unpaired Student *t* test. SEM: Standard error of the mean; TPH-1: Tryptophan hydroxylase 1.

a study that evaluated 27 ursolic acid derivatives synthesized using the docking technique in RBL2H3 cells and ovariectomized rats[15]. The latter study identified a derivative, 9a, that demonstrated the ability to suppress mRNA and protein expression of TPH-1, reducing serotonin levels in both serum and gut, without affecting the content in the brain. Similarly, Ji and Zhao[29] showed that ursolic acid, 18 β-glycyrrhetinic acid, phytolaccic acid, 3-acetoxy-11-carbonyl-β-boswellic acid, asiatic acid, triptolide, and betulinic acid inhibited serotonin biosynthesis in RBL2H3 cells, while 18 β-glycyrrhetinic acid further decreased serotonin level in serum and intestine of OVX rats. In addition, Chen et al.[30] reported that the triterpene of poricoic acid can inhibit renal fibrosis by modulating TPH-1 expression. Currently, all the discovered natural TPH-1 inhibitors are triterpenes. In the present study, we identified and verified a new class of natural inhibitors of TPH-1. Our study estimated the affinities of four types of lignans including eupomatenoid benzofurans (L6, L7, L8, L12), arylnaphthalenes (L5), dibenzylbutanes (L2, L4, L14), and 8-*O*-4′ neolignans (L1, L9, L10, L11) as well as two intestinal metabolites enterodiol (L3) and enterolactone (L13) to TPH-1 and revealed that all the tested lignans showed good binding scores. In addition, five lignans tested using SPR had good binding affinities to the TPH-1, and the activity and protein expression of TPH-1 in RBL2H3 cells were significantly suppressed by these lignans. Moreover, the *in vivo* study further supported that lignans L2 and L12 significantly decreased serum 5-HT levels in OVX mice. These results strongly suggest that lignans are potential TPH-1 inhibitors.

Conclusions

The present study revealed that lignans exhibited high binding scores and high binding affinities to the TPH-1, inhibited the activity of the TPH-1, suppressed TPH-1 expression *in vitro*, and suppressed serum serotonin levels in ovariectomized mice *in vivo*. In addition, we confirmed that BMD was negatively correlated with serum serotonin levels. A limitation of the present study was the lack of a multiple-dose treatment of lignans in the mice study. Further in-depth *in vivo* mechanistic studies of lignans at various doses are warranted. This is the first study to report that lignans are novel

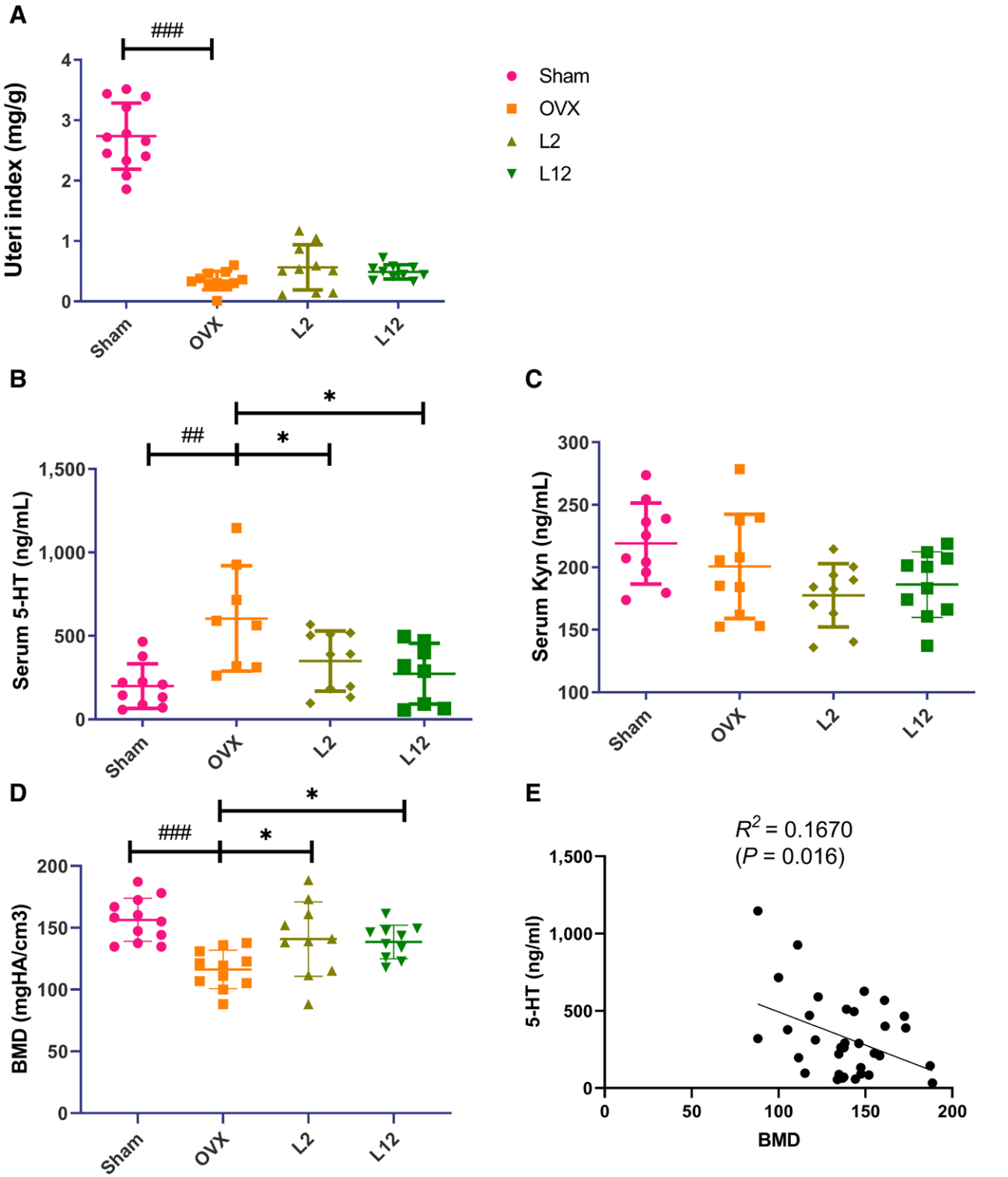


Figure 5. Inhibitory effects of L2 and L12 on serotonin biosynthesis *in vivo*. (A) Uteri index, calculated by the ratio of uterus weight and body weight at the end of treatment; (B) 5-HT level; (C) serum Kyn level; (D) bone mineral density; (E) the correlation between 5-HT and bone mineral density. The mice were sham operated or bilateral ovariectomized and treated with L2 (50 mg/mL) or L12 (50 mg/mL) for 8 wk. Data are presented as the mean ± SEM (n = 8–10). Compared to the Sham group, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$. Compared to the OVX group, $^{*}P < 0.05$. Linear regression analysis was used to calculate the Pearson R^2. Statistical significance was set at $P < 0.05$. 5-HT: Serum serotonin; BMD: Bone mineral density; Kyn: Kynurenine; SEM: Standard error of the mean.

TPH-1 inhibitors and could be potential agents for the management of serotonin-related diseases, including osteoporosis.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Yu-Xin Zhu and Lu Lu conducted the research and investigation process, specifically performed the *in vitro* and *in vivo* experiments, respectively, and Yu-Xin Zhu and Zi-Ling Tang wrote the manuscript. Dabo Pan and Yang Yu performed the docking experiments and analyzed the results. Zi-Ling Tang and Zuo-Cheng Qiu carried out the SPR analysis and did the analysis. Hui-Hui Xiao supervised the whole research activities. Man-Sau Wong and Hui-Hui Xiao acquired the funding and edited the manuscript.

Ethical approval of studies and informed consent

All animal procedures were ethically approved by the Ethics Committee of the Hong Kong Polytechnic University Animal Subjects (ASEC Case: 180402).

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Data availability

All data generated or analyzed during this study are included in this published article.

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