Identification of mundoserone by zebrafish *in vivo* screening as a natural product with anti-angiogenic activity

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Abstract. The present study aimed to screen natural products with anti-angiogenic potential from the Natural Products Collection of MicroSource. The anti-angiogenic activity of 240 natural products was assessed using the zebrafish line Tg(fli1a: EGFP)^{y1}. At 24 h post-fertilization, the embryos were treated with the library compounds for 24 h and, the morphology of the intersegmental vessels (ISVs) was then assessed using a fluorescence microscope, followed by counting of ISVs and calculation of the inhibition ratio. The expression of angiogenesis-associated genes was determined by quantitative polymerase chain reaction. The results indicated that mundoserone inhibited ISV formation in zebrafish embryos in a dose-dependent manner, with a significant anti-angiogenic activity observed at a concentration of 10 μM, leading to an ISV inhibition ratio of 73.6±1.3%. Mundoserone significantly reduced the expression of slit guidance ligand 3 (SLIT3), roundabout guidance receptor 1 (ROBO1) and -2, fibroblast growth factor receptor (FGFR)2 and -3, as well as protein tyrosine phosphatase, receptor type B (PTP-RB), but increased the expression of NOTCH1A. Accordingly, mundoserone may be an effective angiogenic inhibitor, which acts via downregulation of SLIT/ROBO1 and FGFR/PTP-RB, and upregulation of NOTCH1A signaling.

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Abbreviations: ISVs, intersegmental vessels; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; NPs, natural products; EGFP, enhanced green fluorescent protein; hpf, hours post-fertilization; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: angiogenesis, inhibitor, natural products, mundoserone, slit guidance ligand 3/roundabout guidance receptor, fibroblast growth factor

Introduction

Angiogenesis is defined as the formation of new blood vessels from the pre-existing vascular system through endothelial proliferation and migration due to abnormal expression of several pro-angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), their receptors and genes of associated signaling pathways (e.g., phosphoinositide-3 kinase/Akt, mitogen-activated protein kinase and Notch) (1-4). Accumulating evidence suggests that angiogenesis is an essential process for the initiation and development of numerous diseases, including cancer (5), psoriasis (6), rheumatoid arthritis (7), retinopathy (8) and endometriosis (9). Therefore, targeted inhibition of angiogenesis may potentially be beneficial for the treatment of the above diseases, which has been demonstrated in clinical trials on VEGF inhibitors (e.g. dovitinib, sunitinib, sorafenib or bevacizumab) (10,11). However, high proportional rates of adverse events caused by the currently available drugs limits their wide application and acceptability (10,11). The aforementioned experimental results indicate the requirement of more effective and safe angiogenesis inhibitors.

Natural products (NPs) are biologically active secondary metabolites produced by plants, animals and microorganisms. NPs have long served as an important source for drug discovery due to their potentially low toxicity. It is estimated that >2/3 of Food and Drug Administration-approved drugs are NPs or their derivatives (12). Thus, screening of NPs has been proposed as an important approach to obtain effective and safe angiogenesis inhibitors. Although previous studies have reported the anti-angiogenic effects of certain NPs, including genistein (13), camptothecin (14), kaempferol (15), ferulic acid (16) and quercetin (17), studies that identify anti-angiogenic NPs by screening of a drug library remain rare.

The goal of the present study was to screen NPs with anti-angiogenic activity from the Natural Products Collection of MicroSource Discovery Systems (http://www.msdiscovery.com) (18) using the zebrafish embryo *in vivo* model. This model allows for direct visualization of the vascular system via endothelium-specific enhanced green fluorescent protein (EGFP) expression in the Tg(fli1a: EGFP)^{y1} zebrafish line, which ensures rapid evaluation of the responses of live embryos to drugs (19). In the present study, mundoserone

was identified to have potent anti-angiogenic activity in zebrafish embryos and this effect was dose-dependent. The present results may provide a theoretical basis for the future clinical use of this compound to treat diseases associated with excessive angiogenesis.

Materials and methods

Zebrafish care and maintenance. The zebrafish line Tg(fli1a: EGFP)^{y1} with transgenic endothelial cells expressing EGFP was provided by the Shanghai Research Center for Model Organisms (Shanghai, China). The zebrafish were maintained in a constant flow water system at a temperature of 28.5˚C under a 14-h light/10-h dark cycle. All protocols were in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care (20) and approved by the Institutional Animal Care and Use Committee of Shanghai Ninth People's Hospital (Shanghai, China).

Embryo collection and drug treatment. Zebrafish embryos were generated by natural pair-wise mating (5-6 pairs for the generation of 200-300 embryos) and raised at 28.5˚C in deionized water. At 24 h post-fertilization (hpf), embryos were distributed into 12-well plates (30 embryos/well) in 0.2% Instant Ocean Salt (Aquarium Systems, Inc., Mentor, OH, USA) in deionized water and treated with 5 μM PTK787 [a VEGF receptor (VEGFR) antagonist for generation of a positive control group; Selleck Chemicals, Houston, TX, USA; dissolved in 0.4% v/v dimethyl sulfoxide (DMSO)], 0.1% DMSO (negative control) and 10 μM natural product (Natural Products Collection, Microsource, Gaylordsville, CT, USA) for 24 h to screen anti-angiogenic drugs (Fig. 1), of which mundoserone was selected. Subsequently, other embryos were added to 12-well plates (30 embryos per well) and exposed to 2.5, 5 and 10 μM of mundoserone for 24 h to observe the concentration-dependent anti-angiogenic effects of mundoserone. All of the experiments were repeated three times.

Angiogenesis assessment. After 48 hpf, 10 embryos in each well were anesthetized with 0.016% MS-222 (tricaine methanesulfonate; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and the morphology of the intersegmental vessels (ISVs), which normally connect the dorsal longitudinal anastomotic vessels (DLAVs) to the dorsal aorta (DA), was visually assessed using a Nikon SMZ 1500 Fluorescence microscope (Nikon Corp., Tokyo, Japan) equipped with a digital camera. Quantitative image analyses were performed using image based morphometric analysis software (NIS-Elements D3.1; Nikon Corp.) and Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA, USA). A total of 10 embryos per group were evaluated by two blinded observers in 3 independent experiments. Drug effects were calculated according to the following formula: % Inhibition=(1-$ISV_{concentration\ of\ compound}/ISV_{vehicle}$) x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The effects of mundoserone on the expression of angiogenesis-associated genes were determined by RT-qPCR. Total RNA was extracted from 30 embryos that had been treated with DMSO or 10 μM mundoserone for 24 h using TRIzol reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was reverse transcribed using the PrimeScript RT reagent kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc., Otsu, Japan). Quantification of the gene expression was performed in triplicate using Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with detection on the Realplex system (Eppendorf, Hamburg, Germany). Gene expression was relatively quantified based on the comparative threshold cycle method ($2^{-\Delta\Delta Cq}$) (21) using β-actin as an endogenous control gene. Primer sequences are listed in Table I.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Statistical analysis and graphical representation of the data were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance of differences in the number of ISVs among the negative control and positive control groups as well as those treated with different doses of mundoserone was assessed using analysis of variance followed by Tukey's post-hoc test for multiple comparisons. The difference in gene expression between the control and mundoserone groups was analyzed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mundoserone inhibits the formation of the ISVs in zebrafish. Transgenic zebrafish with fluorescent blood vessels were used to screen angiogenic inhibitors from 240 compounds commercially available from the Microsource NP library. Of the compounds in the library, mundoserone, was identified to have anti-angiogenic activity, for which it has not been previously reported, to the best of our knowledge. Exposure of zebrafish to mundoserone inhibited the development of the ISVs and DLAV in comparison with the vehicle and positive control (PTK787) (Fig. 1).

Furthermore, the dose-response effect of mundoserone on the ISVs of zebrafish was also investigated. As presented in Fig. 2, the ISVs were generally intact, but appeared thinner in zebrafish treated with 2.5 μM mundoserone for 24 h. In addition to becoming thinner, certain ISVs were incompletely formed in zebrafish treated with 5 μM mundoserone for 24 h. Of note, most of the ISVs in zebrafish were incomplete and DLAV development was inhibited by 10 μM mundoserone at 48 hpf. These results suggested that the anti-angiogenic effect of mundoserone was dose-dependent, which was further indicated by the quantitative results: The number of ISVs remaining in zebrafish following exposure to 5 (21.4±0.5) or 10 μM (7.3±1.2) mundoserone was significantly lower compared with the negative control (27.6±0.5). Similarly, the ISV inhibition ratio was higher in zebrafish following exposure to 10 μM mundoserone (73.6±1.3%) compared with the control (0±0.6%), 2.5 (0±0.6%) or 5 μM (22.5±0.6%) mundoserone groups.

Mundoserone exerts anti-angiogenesis effects via the downregulation of slit guidance ligand 3 (SLIT3)/roundabout guidance receptor (ROBO)1 and FGF receptor (FGFR)/protein tyrosine phosphatase, receptor type B (PTP-RB), and the upregulation of NOTCH1A. To investigate the potential

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
DLL4	AGGCCTGGCACTCACCTTACTC	CACCCCAGCCCTCTTTACAGTT
NOTCH1A	GCCGCAGATGCAGGGCAATGAAGT	GAGGGCAGGCAGGGCTGGTAGAGG
HEY2	CGGCTTCCGGGAGTGTCTGACT	TCCCCACGGTCGGTATGGTTTA
EFNB2A	TTGGGGCCTGGAGTTCTTCAGA	TCTTGGGCGTGGCTAATGTGCT
SLIT2	GAGCGACTGGACCTGAATG	GTAGATCCTGAAATGCCCCTC
SLIT3	GCGAGTGTTTCCAAGACCTG	GATTTCATTGTCGTTCAGCCG
ROBO1	AGGAGTCACATACAGGCTAGAG	GTCTGAGATCTGCTGGGAAATG
ROBO2	GAGGTGTGGATGTGGACTATG	CTACAATCCGAGGTGGAGAATC
ROBO4	GAGATCAGTCCCAAACCACAG	CCCACAGATATAGCCCAACG
FGFR2	CCCCGACAACCGCACGCTCGTA	TAGCCGCCCATGCGATCCTCCTGT
FGFR3	TCCCCGTATCCAGGTATCC	TCTGAACGTGGGTCTTTGTG
COX2	CCTTCCGGCCATCATTCTTATT	CCGCAGATTTCAGAGCATTGTC
PTP-RB	TTGGGCAGCATGCGGAATACTGAG	TTACCAGGCTGCCATGAAACATCC
VEGFAA	CTCCATCTGTCTGCTGTAAAGG	GGGATACTCCTGGATGATGTCTA
VEGFR-2	CCTGAGACCATCTTTGACCG	GTTCCCTCTTTAAGTCGCCTG
VEGFR-1	GTATTTGAACAGCACGGGTTTAG	CGGCTTCTTGATATGCGTTTG
PIK3R2	CCCGGAAACTGCTCCCCCTAATCT	AGCGGGAGGAGTCGGCTCTTGTT
β-actin	TCCGGTATGTGCAAAGCCGG	CCACATCTGCTGGAAGGTGG

FGFR, fibroblast growth factor receptor; SLIT3, slit guidance ligand 3; ROBO, roundabout guidance receptor; PTP-RB, protein tyrosine phosphatase, receptor type B; DLL, deltalike ligand 4; COX, cyclooxygenase; VEGFR, vascular endothelial growth factor receptor; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; HEY2, hes-related family basic helix-loop-helix transcription factor with YRPW motif 2; EFNB2, ephrin B2.

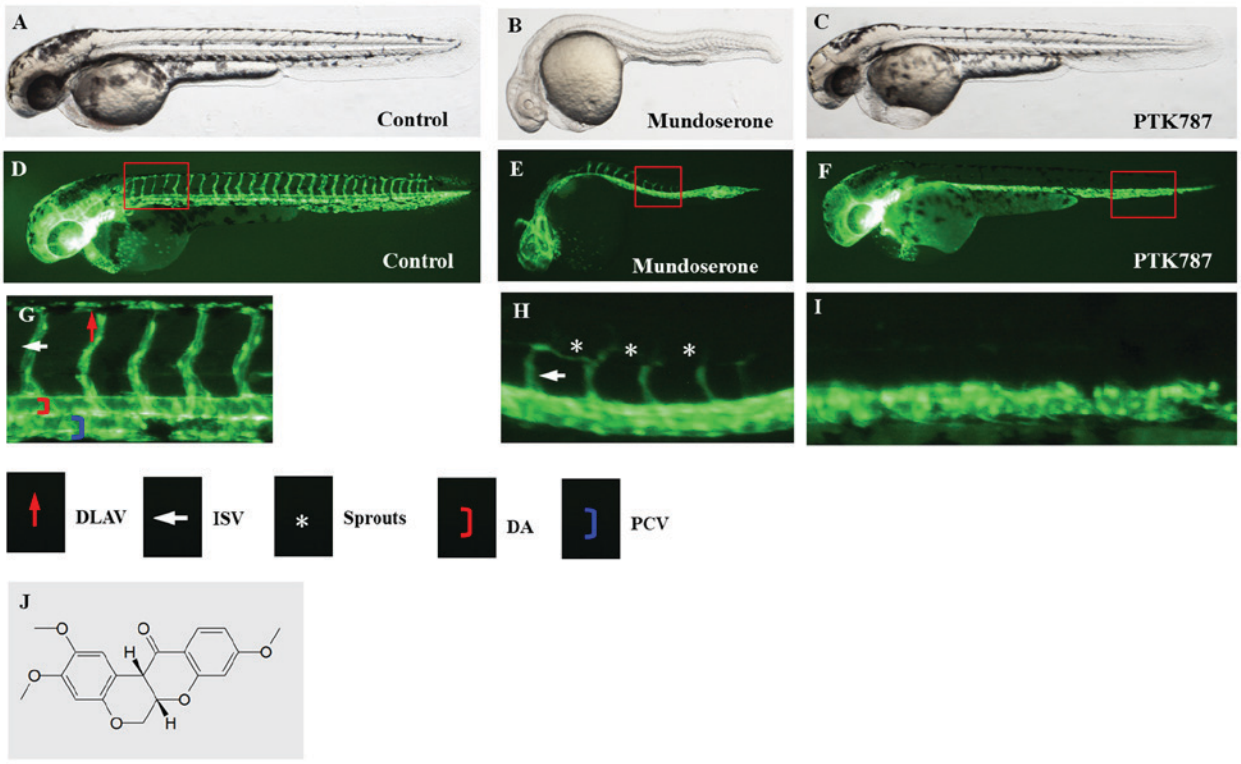

Figure 1. Among the natural products screened in Tg(fli1a: EGFP)^{y1} zebrafish, mundoserone was identified as an anti-angiogenic compound. Representative (A-C) bright-field and (D-F) fluorescent images of zebrafish embryos at 24 h post-fertilization treated with 0.1% dimethyl sulfoxide (control), 10 μM mundoserone or 5 μM PTK787 (positive control) for 24 h (magnification, x40). (G-I) Magnification of images (D-F) (magnification, x112.5). Compared with those in the control group, embryos treated with mundoserone presented with a lower number of incomplete ISVs and only occasional sprouts (asterisks) of the DA were observed. (J) Chemical structure of mundoserone. DLAV, dorsal longitudinal anastomotic vessels; ISVs, intersegmental vessels; DA, dorsal aorta; PCV, posterior cardinal vein.

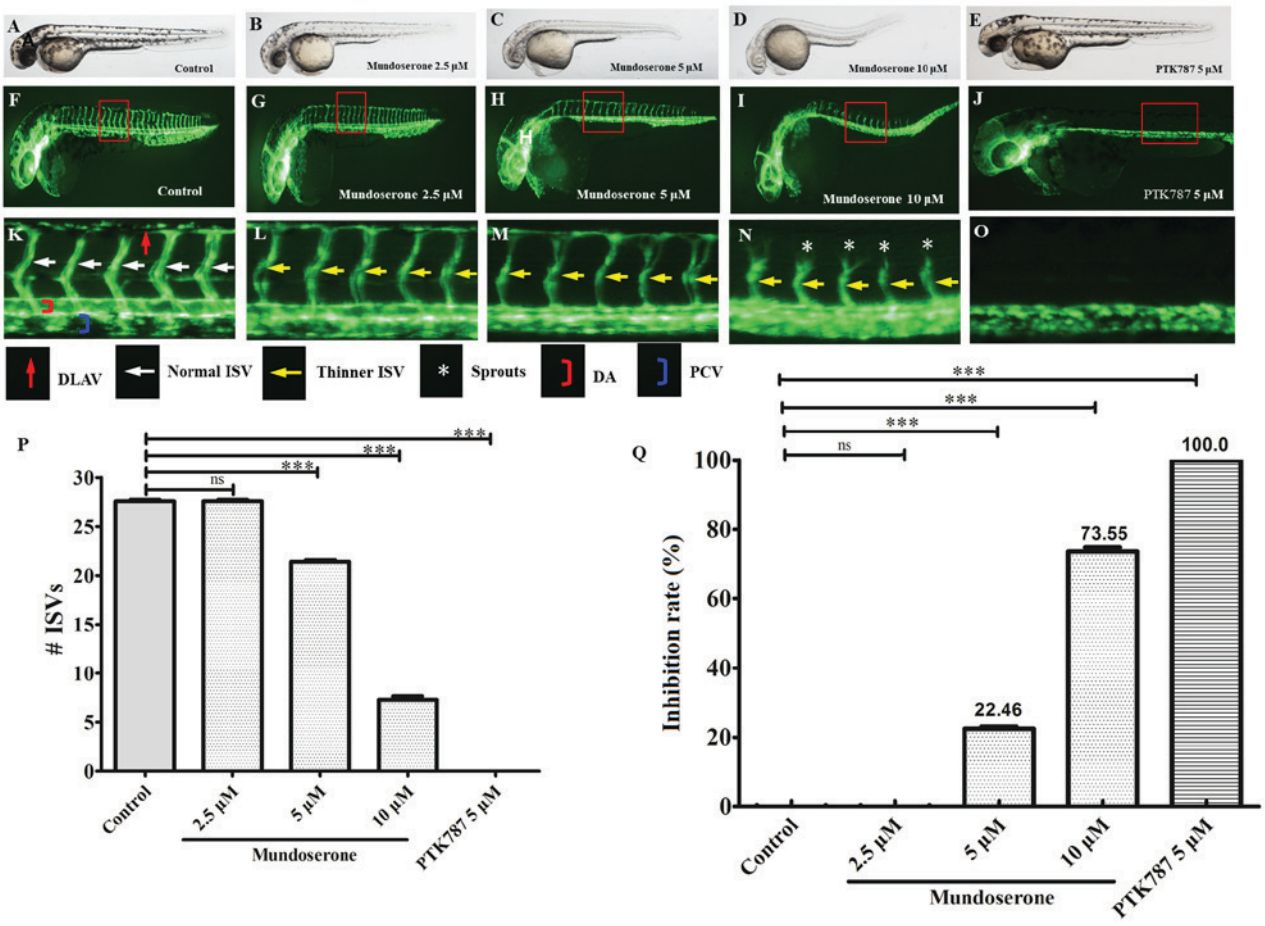

Figure 2. Mundoserone inhibits the trunk angiogenesis of zebrafish in a dose-dependent manner. Representative (A-E) bright field and (F-J) fluorescent images of zebrafish embryos at 24 h post-fertilization treated with 0.1% dimethyl sulfoxide (control), mundoserone (2.5, 5 or 10 μM) or 5 μM PTK787 (positive control) for 24 h (magnification, x40). (K-O) Magnification of images F-J (magnification, x112.5). Compared with the control, embryos exposed to mundoserone exhibited a lower number of incomplete ISVs and only occasional sprouts (asterisks) of the DA were observed. Quantification of (P) the number of complete ISVs and (Q) inhibition rate in mundoserone-treated embryos. Values are expressed as the mean ± standard error of the mean (n=10). ***P<0.001. ns, not significant; DLAV, dorsal longitudinal anastomotic vessels; ISVs, intersegmental vessels; DA, dorsal aorta; PCV, posterior cardinal vein.

mechanisms of the anti-angiogenesis effects of mundoserone, RT-qPCR was performed to analyze the expression of genes involved in angiogenesis-associated signaling pathways in embryos treated with 10 μM mundoserone. The results indicated that mundoserone significantly reduced the expression of SLIT3, ROBO1, ROBO2, FGFR2, FGFR3 and PTP-RB, but increased the expression of NOTCH1A. No significant difference was observed in the expression of cyclooxygenase 2, SLIT2, ROBO4, deltalike ligand 4 (DLL4), hes-related family basic helix-loop-helix transcription factor with YRPW motif 2 and ephrin B2A between the mundoserone treatment and negative control groups (Fig. 3). As an unexpected result, mundoserone promoted the expression of VEGFAA, VEGFR-2 and VEGFR-1.

Discussion

To the best of our knowledge, the present study was the first to identify mundoserone as a potential anti-angiogenic drug, which dose-dependently suppressed the formation of ISVs in a zebrafish embryo model. This inhibitory effect may be exerted by blocking of the SLIT/ROBO and FGFR pathways, as well as activation of NOTCH1A signaling.

Although studies investigating the role of mundoserone are rare (22), mundoserone is a structural analog of rotenone, and extensive studies have demonstrated that rotenone inhibits cell proliferation and migration. For instance, Srivastava and Panda (23) reported that rotenone inhibited the proliferation of HeLa and MCF-7 cells by perturbing microtubule assembly dynamics, with half maximal inhibitory concentrations of 0.2±0.1 and 0.4±0.1 μM, respectively. Ishido and Suzuki (24) indicated that exposure to rotenone inhibited the migration, decreased the proliferation and increased the apoptotic rate of mesencephalic neural stem cells in a dose-dependent manner. In addition, administration of rotenone for 6 h was reported to result in a significant dissipation of the mitochondrial membrane potential in microvascular endothelial cells, indicating the disruption of the mitochondrial respiratory chain and induction of apoptosis (25). These results imply that application of mundoserone or rotenone may possibly prevent excessive angiogenesis, and this hypothesis was also demonstrated in the present study, as a 73.55% ISV inhibition ratio was achieved with mundoserone.

Although VEGFs and FGF are considered as indispensable angiogenic factors for angiogenesis (1-4), the results of the present study suggested that mundoserone did not exert any anti-angiogenic activity by inhibiting VEGFs and VEGFRs,

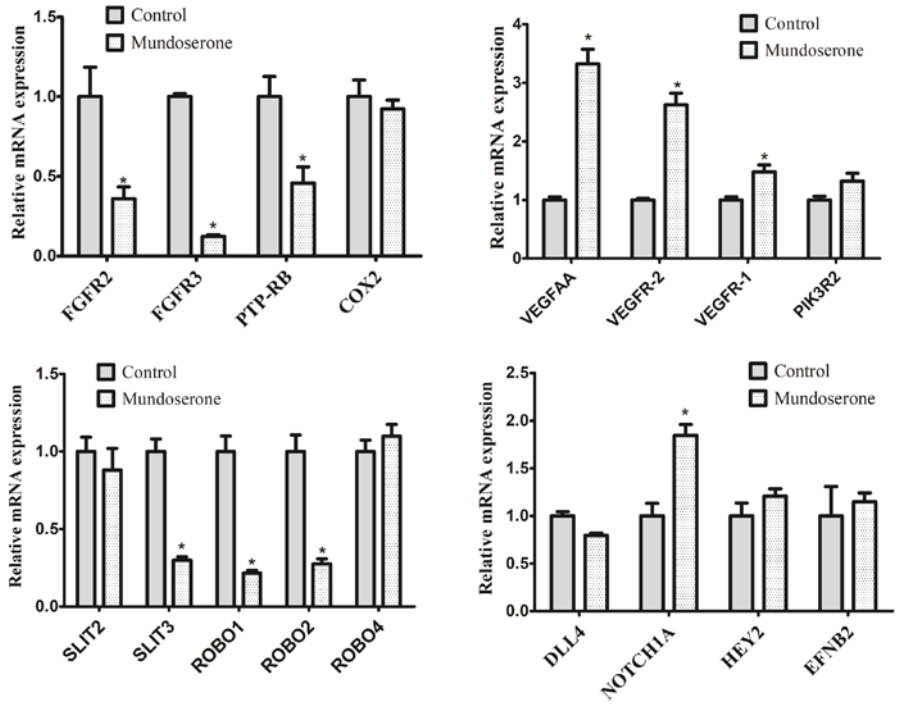

Figure 3. Expression of angiogenesis-associated genes in zebrafish embryos after treatment with 10 μM mundoserone. Values are expressed as the mean ± standard error of the mean (n=4). *P<0.05 vs. Control. FGFR, fibroblast growth factor receptor; SLIT3, slit guidance ligand 3; ROBO, roundabout guidance receptor; PTP-RB, protein tyrosine phosphatase, receptor type B; DLL, deltalike ligand 4; COX, cyclooxygenase; VEGFR, vascular endothelial growth factor receptor; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; HEY2, hes related family bHLH transcription factor with YRPW motif 2; EFNB2, ephrin B2.

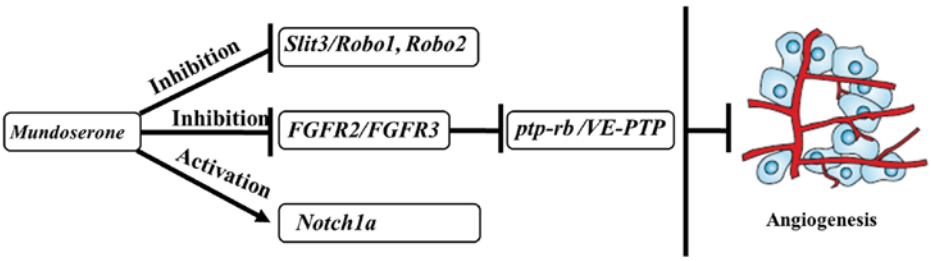

Figure 4. Schematic model illustrating the mechanism of action of mundoserone on angiogenesis. FGFR, fibroblast growth factor receptor; SLIT3, slit guidance ligand 3; ROBO, roundabout guidance receptor; PTP-RB, protein tyrosine phosphatase, receptor type B; VE-PTP, vascular endothelial cell-specific phosphotyrosine phosphatase.

but only FGFR2 and -3. This may be a potential reason of the failure of blockage of angiogenesis by only using VEGF inhibitors in certain clinical patients. VEGF-independent angiogenic pathways should be considered when resistance to VEGF-targeted therapies is encountered (16). Furthermore, *in vitro* studies demonstrated that suppression of FGF signaling reduced the expression levels of PTP non-receptor type 11 (PTPN11) and then disrupted the PTPN11/VE-cadherin interaction, leading to the loss of endothelial junction integrity (26) and terminating endothelial cell interface elongation, ultimately preventing angiogenesis (27). As expected, PTP-RB expression was also lower after mundoserone treatment, indirectly inferring that mundoserone inhibits angiogenesis via the FGFR2/FGFR3/PTP-RB interaction.

In addition to VEGFs and FGF, SLIT/ROBO signaling has also been reported to have a significant role in angiogenesis (28,29). SLIT2 stimulation induced the expression of ROBO1 in lymphatic endothelial cells and mediation of the migration and tube formation, which was reversed by using the SLIT/ROBO-specific antibodies (30). Further *in vivo* experiments confirmed that overexpression of SLIT2 significantly enhanced tumor lymphangiogenesis and subsequently promoted mesenteric lymph node metastasis of pancreatic islet tumors (30). The mRNA and protein levels of SLIT3, ROBO1 and ROBB4 were reported to be significantly increased in human umbilical vein endothelial cells after hypoxia, which is a common cause for preeclampsia (31). SLIT2 was demonstrated to induce ocular neovascular diseases by promoting sprouting angiogenesis in retinas through ROBO1 and ROBO2 (32). Thus, blockade of SLIT/ROBO signaling may be therapeutically exploited to inhibit angiogenesis. As anticipated, mundoserone significantly reduced the expression of SLIT3, as well as ROBO1 and -2 in zebrafish embryos in the present study.

Although the role is controversial, studies have suggested that activation of the Notch signaling pathway may be a potential approach of inhibiting angiogenesis and preventing tumor

metastasis (33,34). For instance, Banerjee *et al* (33) reported that inhibition of NOTCH via a soluble NOTCH1 decoy, which acts as antagonists of DLL and protein jagged ligands, caused a marked increase in liver metastasis of neuroblastoma and breast cancer. This result was also confirmed in transgenic mice with heterozygous loss of NOTCH1 (33). A study by Lee *et al* (34) further illustrated that NOTCH activation suppresses retinal angiogenesis by reducing the expression of transcription factor sex-determining region Y box 17, which may be inhibited by DLL4 blockade. By constructing a transgenic mouse model with Cre-conditional expression of the constitutively active intracellular domain of NOTCH1, Liu *et al* (35) demonstrated that NOTCH signaling may retard basic FGF-induced angiogenesis and ovarian follicle development. In line with these studies, the present results also suggested that mundoserone treatment increased the expression of NOTCH1A and thus inhibited angiogenesis in zebrafish.

Of note, the present study had certain limitations. First, the study only preliminarily screened mundoserone as a natural product with anti-angiogenic activity using a zebrafish embryo model. The efficiency of mundoserone in inhibiting angiogenesis in humans and the optimal concentration require further validation in a series of mammalian models (i.e., mice, monkey) and phase-III clinical trials. Second, further *in vitro* studies are necessary to confirm the effects of mundoserone on the proliferation and migration of microvascular endothelial cells and the expression of associated pathway proteins (SLIT, ROBO1, FGFR, PTP-RB and NOTCH1A) (15,16). Third, the safety of mundoserone should be further evaluated by *in vivo* and *in vitro* experiments in the future because growth retardation of the embryos was observed with mundoserone treatment. Fourth, the VEGF-independent, but FGF-dependent mechanisms of mundoserone should also be validated. In addition, the unexpected upregulation of VEGFAA, VEGF-1 and VEGF-2 may also be a result of the stress response, which counteracts the effects of mundoserone on angiogenesis. Thus, mundoserone and other anti-VEGF drugs should be combined for anti-angiogenesis treatment (36). Fifth, other angiogenesis-associated mechanisms should be examined in the future, including matrix metalloproteinases (37,38).

In conclusion, the present study identified a novel compound, mundoserone, which may be an effective angiogenic inhibitor, which acts via downregulation of the SLIT/ROBO1 and FGFR/PTP-RB pathways, as well as upregulation of NOTCH1A signaling (Fig. 4). Further *in vitro* studies are necessary to confirm the role of mundoserone on the proliferation and migration of microvascular endothelial cells via the above signaling pathways.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KC, CW and YF designed the study and interpreted the data. JG, ZH and YW collected the data. LG and HZ performed the statistical analyses. KC, JG and ZH prepared the figures. All authors wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Shanghai Ninth People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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