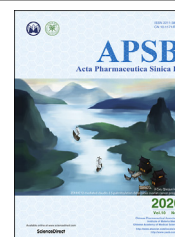




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ORIGINAL ARTICLE

Design, synthesis and pharmacological evaluation of 4-(3-chloro-4-(3-cyclopropylthioureido)-2-fluorophenoxy)-7-methoxyquinoline-6-carboxamide (WXFL-152): a novel triple angiokinase inhibitor for cancer therapy



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Abbreviations: ATCC, American Type Culture Collection; AUC, area under the plasma concentration–time curve; CE, collision energy; CL, systemic clearance; C_{max} , maximum plasma concentration; EC, vascular endothelial cell; ECM, endothelial cell medium; ERKs, extracellular signal-regulated kinases; FGF, fibroblast growth factor; FGFRs, fibroblast growth factor receptors; HBVPs, human brain vascular pericytes; HUVECs, human umbilical vein endothelial cells; IC_{50} , half maximal inhibitory concentration; IHC, immunohistochemistry; i.v., intravenous injection; LC–MS, liquid chromatography mass spectrometry; LLOQ, lower limit of quantification; MRM, multiple reaction monitoring; MeOH, methanol; NMR, nuclear magnetic resonance; PD, pharmacodynamics; PDB, protein data bank; PDGF, platelet-derived growth factor; PDGFRs, platelet-derived growth factor receptors; PDX, patient-derived tumor xenograft; PK, pharmacokinetics; PM, pericyte medium; *p.o.*, *per os*; QC, quality control; RE, values and relative error; RSD, relative standard deviation; RTKs, receptor tyrosine kinases; TGI, tumor growth inhibition rate; TLC, thin-layer chromatography; T_{max} , time the maximum concentration occurred; ULOQ, upper limit of quantitation; V_{dss} , volume of distribution at steady state; VEGF, vascular endothelial growth factor; VEGFRs, vascular endothelial growth factor receptors.

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KEY WORDS

Drug synthesis;
Tumor;
Anti-angiogenesis
therapy;
Multi-angiokine
inhibitor;
Pharmacokinetic

Abstract Angiokines, such as vascular endothelial-, fibroblast- and platelet-derived growth factor receptors (VEGFRs, FGFRs and PDGFRs) play crucial roles in tumor angiogenesis. Anti-angiogenesis therapy using multi-angiokine inhibitor has achieved great success in recent years. In this study, we presented the design, synthesis, target identification, molecular mechanism, pharmacodynamics (PD) and pharmacokinetics (PK) research of a novel triple-angiokine inhibitor WXFL-152. WXFL-152, identified from a series of 4-oxyquinoline derivatives based on a structure–activity relationship study, inhibited the proliferation of vascular endothelial cells (ECs) and pericytes by blocking the angiokine signals VEGF/VEGFR2, FGF/FGFRs and PDGF/PDGFR β simultaneously *in vitro*. Significant anticancer effects of WXFL-152 were confirmed in multiple preclinical tumor xenograft models, including a patient-derived tumor xenograft (PDX) model. Pharmacokinetic studies of WXFL-152 demonstrated high favourable bioavailability with single-dose and continuous multi-dose by oral administration in rats and beagles. In conclusion, WXFL-152, which is currently in phase Ib clinical trials, is a novel and effective triple-angiokine inhibitor with clear PD and PK in tumor therapy.

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1. Introduction

Vascular endothelial-, fibroblast- and platelet-derived growth factor receptors (VEGFRs, FGFRs and PDGFRs) are a class of receptor tyrosine kinases (RTKs), which contain extracellular domain of ligand binding sites, hydrophobic helicity of single transmembrane, and intracellular kinases domains^{1,2}. Angiokines, such as the intracellular kinases domains of VEGFRs, FGFRs and PDGFRs, which mainly promote angiogenesis, are proven to be clinical targets in cancer therapy^{3–6}. When the growth factors, such as VEGF, FGF and PDGF, bind the correspondingly extracellular domains of the RTKs, the angiokine signals will be activated and deliver growth signals intracellularly by transferring a phosphate group of ATP to the tyrosine residue. Overactivated angiokine signals contributed to the angiogenesis, tumorigenesis, metastasis and progression^{7,8}.

Tumor angiogenesis is a complicated process that involves multiple angiokine signals and angiogenesis-related cells (vascular endothelial cell, pericytes and smooth muscle cells)⁹. VEGF/VEGFR2 signals promote tumor angiogenesis by stimulating the growth of adjacent vascular endothelial cells (ECs) to form new vascular sprouts in tumor tissue^{10,11}. The PDGF-BB produced by ECs promotes the pericytes (PDGFR-positive) to enwrap and stabilize the tumor vasculature, and protect the new vascular sprouts by PDGF/PDGFR signalling¹². Abnormal FGF/FGFRs not only promote tumor development by directly driving cancer cells and endothelial cells proliferation, but also support tumor angiogenesis by synergizing with the VEGF and PDGF signalling pathways^{13–15}. Fibroblast growth factor 2, also known as bFGF, is an important pro-angiogenic factor in tumor angiogenesis^{16,17}. bFGF and VEGF synergistically increased vascular density and permeability¹⁸. The simultaneous overexpression of bFGF and PDGF-BB in mouse fibrosarcoma results in the formation of high density primary vascular plexus¹⁹. Additionally, bFGF can stimulate the expression of PDGFR- α and - β in activated endothelial cells, thus stimulating the tumor angiogenesis by promoting PDGF/PDGFR β signalling pathway²⁰. This

synergistic effect of multiple angiokine signals in tumor angiogenesis may limit the anti-tumor effect of single-target therapy²¹. Some drugs targeting VEGF or VEGFR2 have been approved for cancer therapy, such as bevacizumab (monoclonal antibody targets VEGF) and apatinib (VEGFR2 inhibitor)²². However, limited clinical benefits have been observed during anti-angiogenic therapy for cancer by blocking VEGF/VEGFR2 signals alone²³. The reasons are that alternative mechanism of tumor adaptation to VEGF/VEGFRs inhibition includes the induction of compensatory pro-angiogenic growth signals, such as FGF/FGFRs, and the protection effect of pericytes to ECs driven by the PDGF/PDGFRs signal^{24,25}. Anti-angiogenesis therapy targeting multiple specific angiokines, such as nintedanib (VEGFRs, FGFRs and PDGFRs inhibitor), provided great clinical benefits for cancer therapy^{26–28}.

In our previous study, we obtained the quinoline-thiourea derivative WXFL-255 from a series of 4-oxyquinoline derivatives as a potent angiokine inhibitor (targeting VEGFRs, FGFRs and PDGFRs)²⁹. To improve its selectivity for VEGFR2, FGFR1 and PDGFR β , further optimization on its four substituents (R₁–R₄) was performed. A novel and priority candidate WXFL-152 was identified based on the structure–activity relationship (SAR) study. The aim of the study was to clarify the design, synthesis, target identification, molecular mechanism, pharmacodynamics (PD) and pharmacokinetics (PK) of WXFL-152.

2. Results and discussions

2.1. Chemistry and SAR study

The compound WXFL-255, 4-(3-chloro-4-(3-cyclopropylthioureydo)phenoxy)-7-methoxyquinoline-6-carboxamide, is a potential triple-angiokine inhibitor (IC₅₀ values are 9.4 (VEGFR2), 188.0 (FGFR1), 143.0 (PDGFR β), 90.2 (HUVEC) nmol/L, respectively) obtained in our previous study as shown in Fig. 1A and Scheme 1²⁹. In this study, WXFL-255, as the lead compound, was optimized in its four major groups (R₁–R₄) based on the consideration of lipophilicity, lipid water distribution coefficient

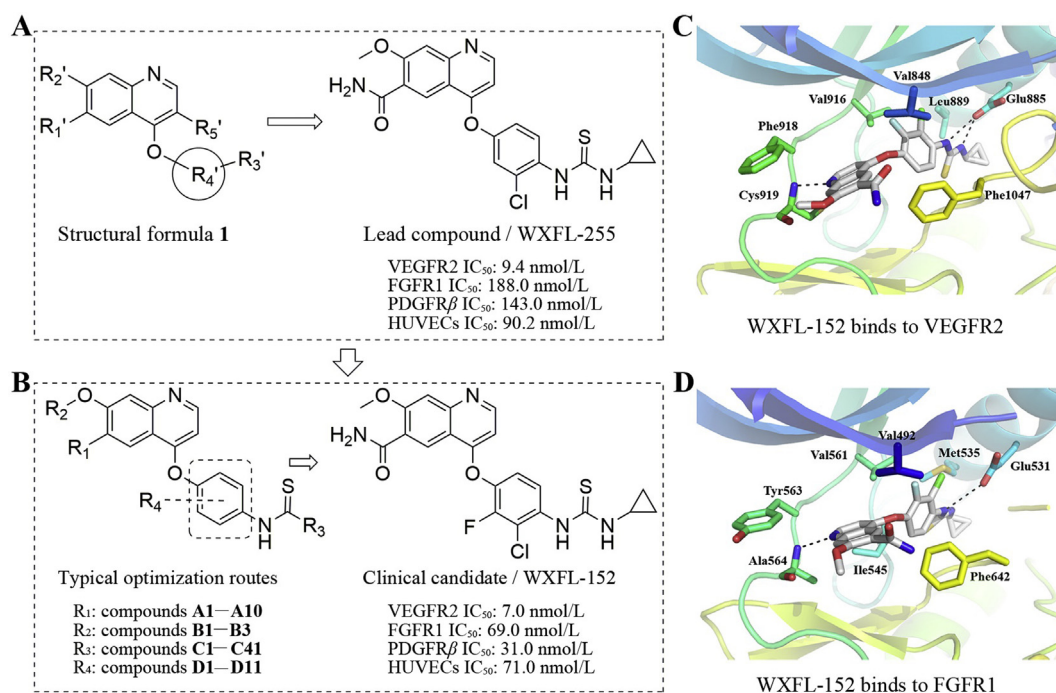


Figure 1 Typical optimization routes of the clinical candidate compound **D4** (WXFL-152). (A) WXFL-255 as the lead compound was obtained from a series of 4-oxyquinoline derivatives. (B) WXFL-152 was identified based on the four major groups optimization of the lead compound (R₁–R₄). (C) and (D) Molecular docking simulation of WXFL-152 with VEGFR2 and FGFR1.

under physiological conditions, apparent volume of distribution and the affinity toward the targets, as shown in Fig. 1B.

Partially typical compounds (**A1**–**A10**) with R₁ substituted groups, including various amide, ester, heterocycle and cyan, were showed in Table 1, and their synthetic routes as shown in Scheme 2. Based on a kinase inhibition assay for VEGFR2, FGFR1 and PDGFRβ, amide-substituted in R₁ of the quinoline ring was determined to be superior to the other alternatives. Especially the *N,N*-disubstituted amide derivatives significantly decreased the inhibitory activity of the three kinases. Interestingly, amide-substituted compounds **A1** (IC₅₀ = 67.6 nmol/L), **A5** (IC₅₀ = 101.5 nmol/L) and ester-substituted compound **A2** (IC₅₀ = 72.5 nmol/L) even surpassed the lead compound (IC₅₀ = 143.0 nmol/L) in the inhibition of PDGFRβ. However, the compounds showed no superiority in the inhibition of VEGFR2 and FGFR1. The ester-substituted compound **A2** was effective at inhibiting VEGFR2 and PDGFRβ, but failed to inhibit FGFR1 (IC₅₀ values are 28.1 (VEGFR2), 2394.9 (FGFR1) and 72.5 (PDGFRβ) nmol/L, respectively).

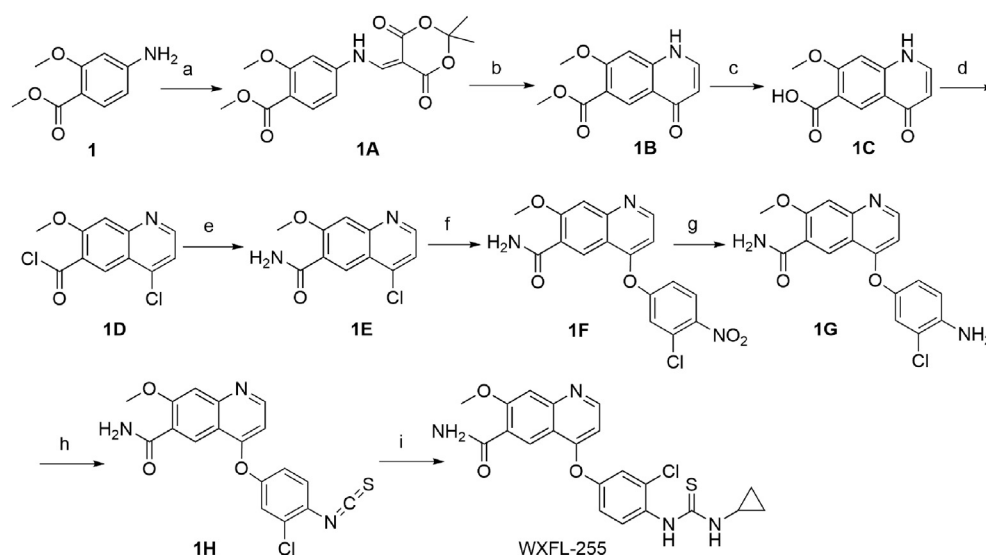
Alkyl-, alkoxy-, and heterocycle-substituted groups in R₂ were synthesized and tested for kinase inhibition. The typical compounds **B1**–**B3** were shown in Table 2 and the synthetic routes as shown in Scheme 3. The results indicated that bulky group decreased the inhibition of VEGFR2, FGFR1 and PDGFRβ significantly. However, extending the straight chain in R₂ increased the inhibition of PDGFRβ, such as the compound **B2**. Unfortunately, the activity against VEGFR2 and FGFR1 of compound **B2** was significantly decreased.

A series of novel candidates **C1**–**C41** with amino-substituted groups including –NH₂, –NH–R' (R' were alkyl, benzene series, heterocycle and cyclane) and a nitrogen heterocyclic ring in the R₃ position were designed, synthesized and tested as shown in Table 3 and Scheme 4. Since the R₃ group was located in the

hydrophobic pockets of the VEGFR2, FGFR1 and PDGFRβ, the different size of the three kinase cavities determined the selection of the R₃ group. The kinase inhibition results showed that cyclopropane amine-substituted in R₃ demonstrated the best inhibition of VEGFR2 (IC₅₀ = 9.4 nmol/L), and dimethylamino-substituted compound **C2** in R₃ had the best inhibitory effect on FGFR1 (IC₅₀ = 63.0 nmol/L). The 2-thioxoimidazolidin-4-one-substituent compound **C41** in the thiourea-structure exhibited the best inhibition of PDGFRβ (IC₅₀ = 12.2 nmol/L) but poor inhibition of FGFR1.

Compounds **D1**–**D11**, which contained substitutes on the benzene and benzoheterocycle in the R₄ position, were obtained and tested as shown in Table 4 and Scheme 5. The R₄ substituted phenyl group was inserted into another hydrophobic pocket near the hinge region of the kinases, so the aryl heterocycles substitution of the nonpolar phenyl weakened the binding force to the pocket, and decreased the inhibition of VEGFR2, FGFR1 and PDGFRβ, such as **D7**–**D10**. On the contrary, appropriate substituents connected to the phenyl could increase the van de Waals of the compounds with the kinase hydrophobic pockets, and improve the activity against the kinases. The compounds with the benzene ring substituted by two groups in the R₄ position tended to inhibit VEGFR2, FGFR1 and PDGFRβ simultaneously. Interestingly, the compound **D4** (WXFL-152) containing the 1-chloro-2-fluorobenzene group in R₄ was identified as the priority candidate for further testing *in vitro* and *in vivo* based on the cytotoxicity on HUVECs induced by VEGFA-165 (Table 5).

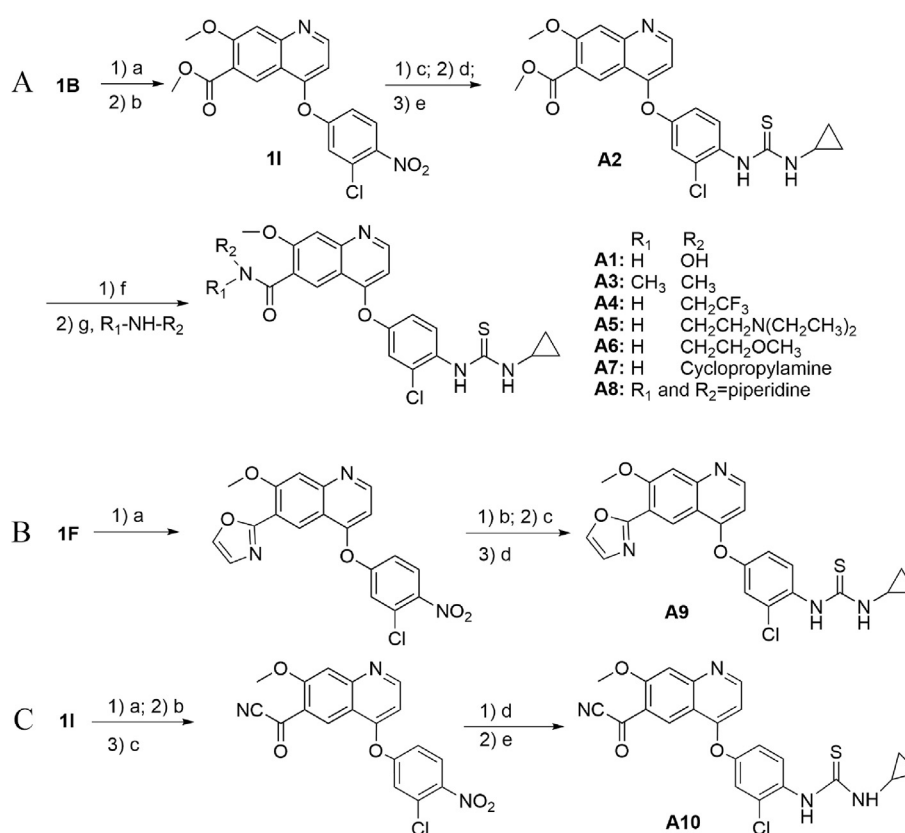
The method for synthesis of WXFL-152 was demonstrated in Scheme 6. The Cl of quinoline was replaced by a phenoxy to obtain compound **b**. WXFL-152 was obtained by the condensation of compound **b** and amine with 1,1'-thiocarbonyldi-2(1*H*)-pyridone. Then, WXFL-152 reacted with methane



Scheme 1 Synthesis of the lead compound WXFL-255²⁹. Reagents and conditions: (a) **1A**, 2,2-dimethyl-1,3-dioxane-4,6-dione, trimethyl orthoformate, 90 °C, 1 h; (b) **1B**, diphenyl ether, 220 °C, 1 h, methyl *tert*-butyl ether, 140 °C, 12 h; (c) **1C**, NaOH, water and methanol, 30 °C, 2 h; (d) **1D**, *N,N*-dimethylformamide, 1,2-dichloroethane, thionyl chloride, 110 °C, 2 h; (e) **1E**, dichloromethane, aqueous ammonia, 25 °C, 0.5 h; (f) **1F**, 3-chloro-4-nitro-phenol, 140 °C, 12 h; (g) **1G**, Fe, HOAc, 80 °C, 16 h; (h) **1H**, 1,1'-thiocarbonyldi-2(1*H*)-pyridone, dioxane, 15 °C, 1 h, refluxing, 12 h; (i) lead compound WXFL-255, cyclic propylamine, 25 °C, 16 h.

Table 1 VEGFR2/FGFR1/PDGFR β inhibition of the typical compounds **A1**–**A10** obtained by R₁ group optimization.

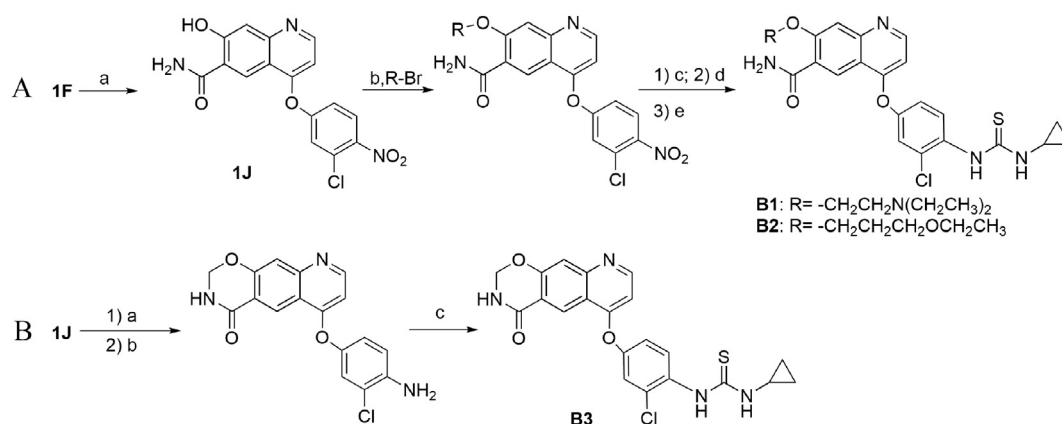
Compd.	Structure or R ₁	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFR β
Lead compd.		9.4	188.0	143.0
A1		20.1	293.7	67.6
A2		28.1	2394.9	72.5
A3		503.4	8130.1	414.3
A4		79.5	4436.8	316.1
A5		156.4	8262.5	101.5
A6		119.9	4235.1	240.6
A7		116.3	6804.1	210.2
A8		1668.4	>10,000	1050.6
A9		101.3	2644.5	135.6
A10		172.4	1903.3	504.0



Scheme 2 Synthesis of A route (compounds **A1–A10**). Reagents and conditions: (A)-(a) thionyl chloride, trimethyl orthoformate, 90 °C, 1 h; (b) 3-chloro-4-nitro-phenol, chlorobenzene, 140 °C, 15 h; (c) Fe, NH₄Cl, 90 °C, 2 h; (d) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, 25 °C, 0.5 h, 120 °C, 12 h; (e) cyclopropylamine, 25 °C, 2 h; (f) LiOH, 25 °C, 2 h; (g) amine, 25 °C, 15 h; (B)-(a) dimethyl 1,2-vinylene carbonate, 190 °C, 0.5 h; (b) Fe, NH₄Cl, 80 °C, 1 h; (c) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, dioxane, 65 °C, 1 h; (d) cyclic propylamine, 25 °C, 1 h; (C)-(a) LiOH, THF, 20 °C, 4 h; (b) NH₂Cl, HATU, DIPEA, DCM, 20 °C, 12 h; (c) thionyl chloride, 80 °C, 3 h; (d) Fe, NH₄Cl, 90 °C, 2 h; (e) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, 10 °C, 10 min, 110 °C, 2 h, cyclic propylamine, 20 °C, 2 h.

Table 2 VEGFR2/FGFR1/PDGFR β inhibition of the typical compounds **B1–B3** obtained by R₂ group optimization.

Compd.	Structure or R ₂	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFR β
Lead compd.		9.4	188.0	143.0
B1		335.2	9032.0	388.9
B2		26.7	216.4	56.9
B3		226.8	777.1	409.8

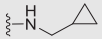
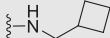
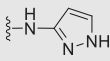
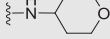
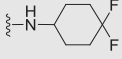
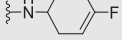
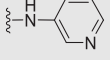
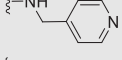
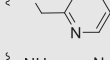
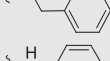
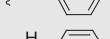
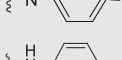
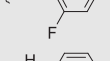
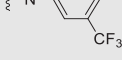
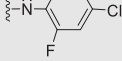
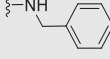
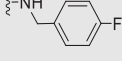
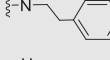
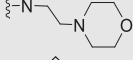


Scheme 3 Synthesis of B route (compounds **B1–B3**). Reagents and conditions: (A)-(a) BBr₃, 25 °C, 16 h; (b) KCO₄, bromide, DMF, 80 °C, 16 h; (c) Zn, AcOH, 80 °C, 16 h; (d) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, 80 °C, 16 h; (e) cyclopropylamine, 20 °C, 16 h; (B)-(a) trioxymethylene, H₂SO₄, 70 °C, 2.5 h; (b) Raney Ni, MeOH, 25 °C, 0.5 h; (c) 1,1'-thiocarbonyldi-2(1*H*)-pyridone, 70 °C, 1 h, 25 °C, cyclopropylamine, 1 h.

Table 3 VEGFR2/FGFR1/PDGFR β inhibition of the typical compounds **C1–C41** obtained by R₃ group optimization.

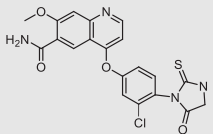
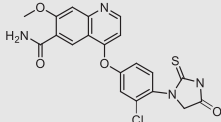
Compd.	Structure or R ₃	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFR β
Lead compd.		9.4	188.0	143.0
C1		32.7	436.4	77.2
C2		78.6	63.0	311.1
C3		10.0	129.9	84.3
C4		22.6	378.4	139.9
C5		30.1	645.7	78.5
C6		110.3	634.7	89.1
C7		55.1	415.9	80.1
C8		59.7	1216.3	87.3
C9		244.7	4358.0	696.7
C10		2353.5	6757.7	62.8
C11		208.5	1934.4	254.6
C12		52.2	765.7	19.7
C13		216.9	1583.1	508.6
C14		234.4	1420.8	28.1

Table 3 (continued)

Compd.	Structure or R ₃	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFRβ
C15		16.6	406.2	31.1
C16		20.5	1103.5	161.4
C17		72.7	1831.4	347.8
C18		339.0	5073.1	178.4
C19		1649.1	7616.3	167.3
C20		1103.5	7478.2	399.5
C21		107.5	2671.4	54.2
C22		195.8	1729.0	62.8
C23		372.6	2748.4	73.3
C24		127.6	2933.7	37.0
C25		74.1	6100.4	193.7
C26		104.6	906.7	24.7
C27		138.8	2798.5	37.3
C28		117.6	3541.8	563.7
C29		307.5	9154.3	2040.9
C30		675.8	4898.2	361.0
C31		206.8	3608.5	85.9
C32		255.3	3377.2	186.4
C33		397.9	3754.0	41.7
C34		63.6	70.4	63.1
C35		36.1	135.8	184.2
C36		25.8	226.8	867.9
C37		79.6	475.7	974.5
C38		194.0	457.3	323.7
C39		472.2	>10,000	221.9

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Table 3 (continued)

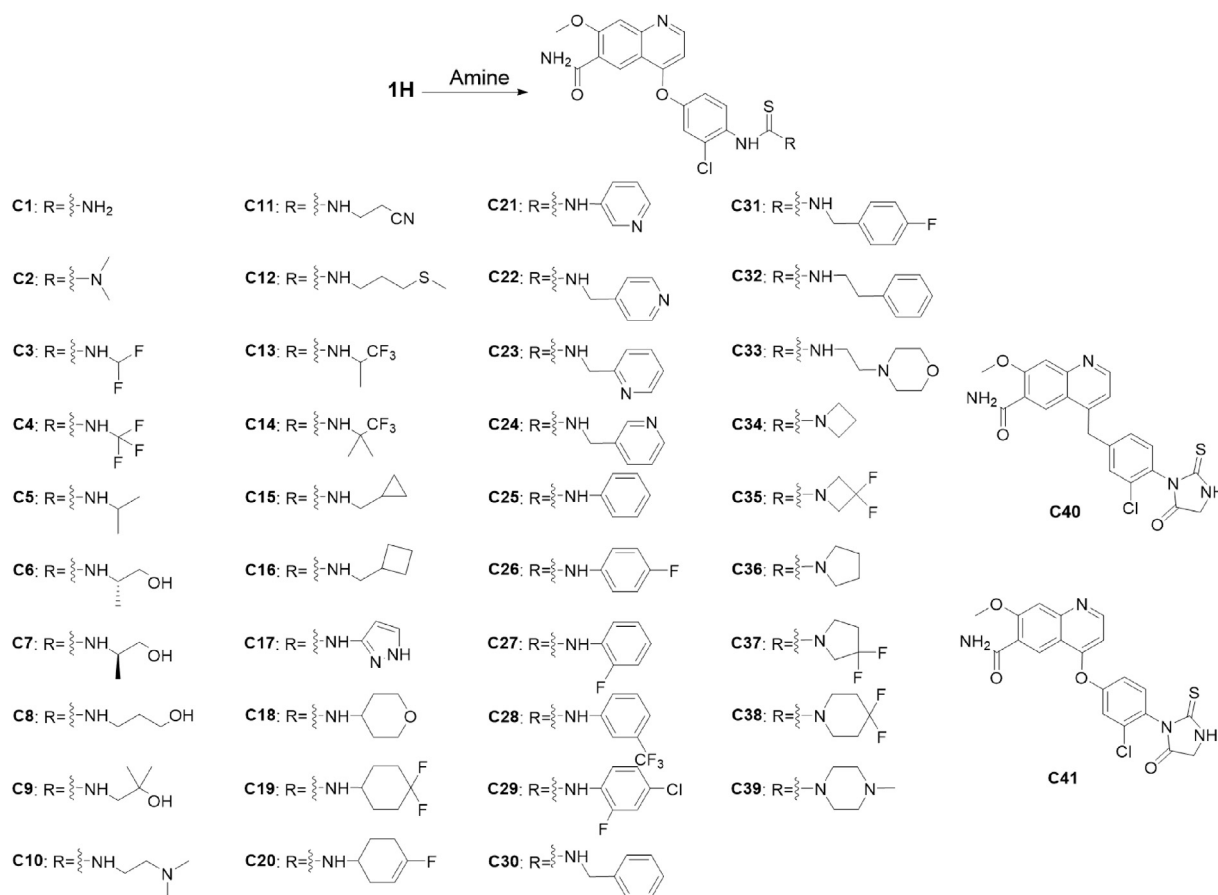
Compd.	Structure or R ₃	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFR β
C40		68.1	1258.7	156.5
C41		51.7	1565.8	12.2

sulfonic acid (MsOH) to yield WXFL-152 mesylate as described in our previous patent³⁰. The detailed synthesis methods, including the reagents and conditions of the other compounds mentioned in this study, were described in our previous patent²⁹. The mesylate form of WXFL-152 was used for further biological tests, including PD and PK.

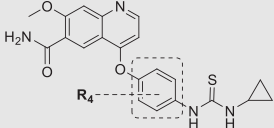
2.2. Molecular modelling of WXFL-152

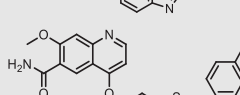
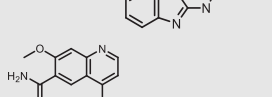
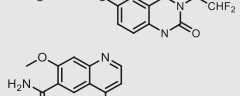
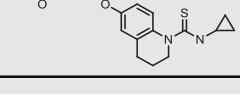
Molecular docking simulations of WXFL-152 with VEGFR2 and FGFR1 were performed using AutoDock Vina. The co-crystal

structure of VEGFR2 complexed with sorafenib (protein data bank, PDB ID: 4ASD) was used as a template. As shown in Fig. 1C, the quinoline ring and the thiourea of WXFL-152 bound to Cys919 (the hinge region amino acid) and Glu885 on VEGFR2 in the form of hydrogen bonds, thus preventing ATP from binding to the ATP binding site of VEGFR2. In the co-crystal structure molecular modelling of FGFR1 with ponatinib (PDB ID: 4V04), the quinoline ring and the thiourea group of WXFL-152 bound to Ala564 and Glu531 on FGFR1 via forming hydrogen bonds to prevent ATP from binding to the ATP binding site of FGFR1 (Fig. 1D).



Scheme 4 Synthesis of C route (compounds **C1–C43**). Reagents and conditions: amines react with **1H** to form thiourea under the similar reaction conditions of the last step of lead compound WXFL-255, see Supporting Information.

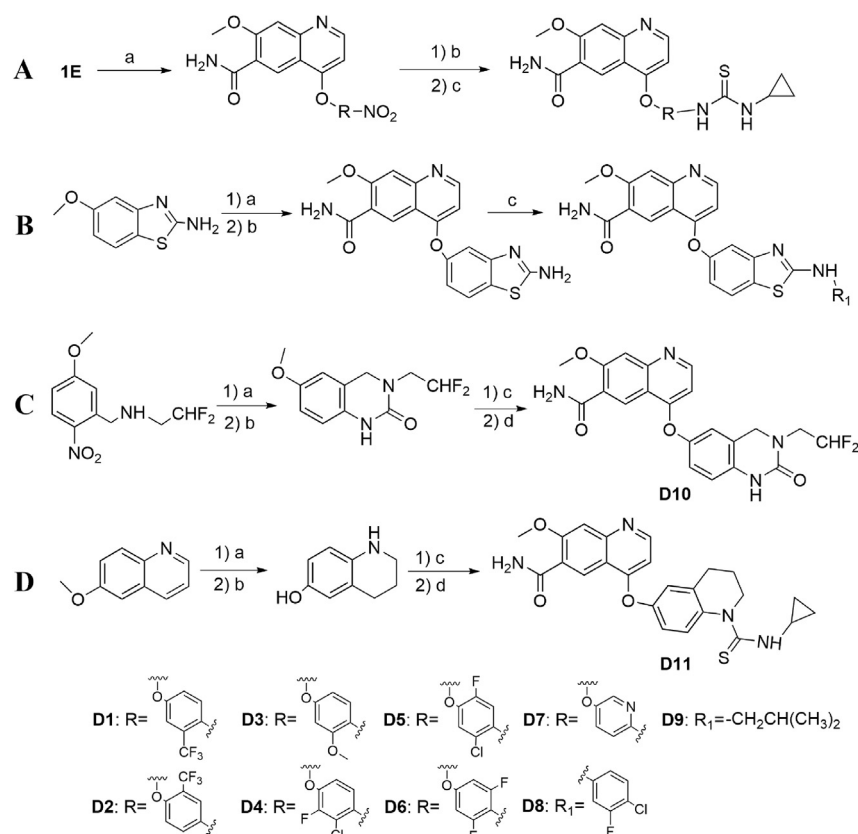
Table 4 VEGFR2/FGFR1/PDGFR β inhibition of the typical compounds **D1–D11** obtained by R₄ group optimization.


Compd.	Structure or R ₄	IC ₅₀ (nmol/L)		
		VEGFR2	FGFR1	PDGFR β
Lead compd.		9.4	188.0	143.0
D1		16.2	242.4	406.1
D2		2043.5	4923.5	956.3
D3		231.0	3144.0	54.0
D4/WXFL-152		7.0	69.0	31.0
D5		25.2	713.0	68.8
D6		64.0	541.0	162.0
D7		8803.8	>10,000	3807.4
D8		4802.4	3683.8	175.8
D9		>10,000	>10,000	>10,000
D10		375.9	6042.3	3183.4
D11		75.7	3704.6	857.4

2.3. Kinase inhibition assay of WXFL-152

Invitrogen Z'-LYTE[®] Kinase detection technology was used to analyse the optimized compounds and profile the kinase inhibition of WXFL-152. As shown in Table 6, the target kinases of WXFL-152 were VEGFR types 1, 2 and 3 (IC₅₀s: 30.9, 7.0 and 8.5 nmol/L, respectively); PDGFR α and PDGFR β (IC₅₀s: 135.0 and 31.0 nmol/L); and FGFR types 1, 2, 3 and 4 (IC₅₀s: 69.0, 15.5, 132.0 and 131.0 nmol/L, respectively). WXFL-152 also inhibited

MAP4K4, MINK, TNIK, AUR2, ABL and LCK, with IC₅₀ values of 79.0, 55.0, 118.0, 262.0, 520.0 and 252.0 nmol/L, respectively. WXFL-152 showed almost no inhibitory activity against 16 other selected human protein kinases. These data demonstrated that WXFL-152 is a potent triple inhibitor targeting VEGFRs, PDGFRs and FGFRs. In addition, we tested and compared the kinases inhibition activity of WXFL-152 with that of nintedanib. Angiokinases, such as VEGFRs, FGFRs and PDGFRs, are not only a pro-angiogenic factors, but also a pro-fibrotic factors³¹.



Scheme 5 Synthesis of D route (compounds **D1–D11**). Reagents and conditions: (A) the synthesis methods are similar to the synthesis of lead compound (from **1E** to lead compound); (B)-(a) HBr, reflux, 4 h; (b) compound **1E**, 100 °C, 0.5 h; (c) aldehyde, STAB, 100 °C, 12 h; (C)-(a) Renny Ni/H₂, 20 °C, 1 h; (b) CDI, DMF, 70 °C, 16 h; (c) BBr₃, DMF, 78 °C, 0.5 h; (d) compound **1E**, Cs₂CO₃, 100 °C, 1.5 h; (D)-(a) PtO₂, MeOH, 45 °C, 24 h; (b) BBr₃, CHCl₃, 15 °C, 2 h; (c) compound **1E**, Cs₂CO₃, NMP, 120 °C, 2 h; (d) cyclopropylthiocyanate, THF, 25 °C, 12 h.

Nintedanib, which targets VEGFRs, FGFRs and PDGFRs simultaneously, has achieved great clinical success in tumor therapy and idiopathic pulmonary fibrosis (IPF). The lead compound WXFL-255 has the similar targets with nintedanib. Thus, nintedanib was chosen as the control compound in our study. The results showed that WXFL-152 and nintedanib demonstrated similar inhibition effect on VEGFR2 (*i.e.*, IC₅₀ value of WXFL-152 *vs.* nintedanib: 7.0 ± 2.0 *vs.* 8.8 ± 3.6 nmol/L), FGFR1 (IC₅₀ value of WXFL-152 *vs.* nintedanib: 69.0 ± 30.0 *vs.* 93.9 ± 37.0 nmol/L) and PDGFRβ (IC₅₀ value of WXFL-152 *vs.* nintedanib: 31.0 ± 7.0 *vs.* 35.4 ± 14.0 nmol/L).

2.4. WXFL-152 inhibited HUVECs proliferation, tube formation, migration and invasion

VEGF/VEGFR2 is a key signal to induce the proliferation, migration, and tube formation of endothelial cell in tumor angiogenesis. To analyse the proliferation inhibition of WXFL-152 *in vitro*, VEGF-induced HUVECs proliferation treated with WXFL-152 was evaluated using a CellTiter-Glo luminescent cell viability assay. The results indicated that WXFL-152 significantly inhibited the proliferation of HUVECs (IC₅₀ 71.0 ± 5.0 nmol/L) induced by 20 ng/mL hVEGF-165. Tube formation of ECs is important for angiogenesis. WXFL-152 significantly suppressed the tube formation of HUVECs induced by 50 ng/mL hVEGF-165 at doses of 0.5 and 5.0 μmol/L (WXFL-152 treated groups *vs.* hVEGF-165 control group, *P* < 0.01). The inhibition effects at 2,

4 and 6 h were shown in Fig. 2A (representative images of tube formation of HUVECs) and B (the numbers of HUVECs tubes in the different groups and times). Wound healing assays were performed to investigate the effects of WXFL-152 on cell migration. The migration of HUVECs was appreciably inhibited by treatment with 5.0 μmol/L WXFL-152 compared with the hVEGF-165 treated control group (5.0 μmol/L, *P* < 0.05; 50.0 μmol/L, *P* < 0.001). Representative images of wound closure were shown in Fig. 2C, and the wound closure percentages in the different groups were shown in Fig. 2E. A transwell invasion assay was used to evaluate the invasion ability of HUVECs driven by 50 ng/mL hVEGF-165 under exposure to WXFL-152. The results showed that WXFL-152 significantly inhibited the invasion of HUVECs in a dose-dependent manner compared with the hVEGF-165 treated control group (0.5 μmol/L, *P* < 0.01; 5.0 and 50.0 μmol/L, *P* < 0.001). Typical images were shown in Fig. 2D, and the invasive cell number percentages were shown in Fig. 2F. In conclusion, these results confirmed that WXFL-152 was an effective bioactive inhibitor that blocked VEGF/VEGFR2 signals in cellular level.

2.5. WXFL-152 inhibited HBVPs proliferation

In tumor angiogenesis, ECs-secreted PDGF-BB promotes the pericytes proliferation to protect the new vascular sprouts by PDGF/PDGFR signal. To analyse the proliferation inhibition of WXFL-152 in pericytes, cell model of PDGF-BB-induced HBVPs

Table 5 Cytotoxicity of optimized compounds on HUVECs (induced by 20 ng/mL VEGFA-165), HBVPs (induced by 20 ng/mL PDGF-BB) and inhibition of optimized compounds on VEGFR2/FGFR1/PDGFR β .

Compd.	IC ₅₀				
	VEGFR2 (nmol/L)	FGFR1 (nmol/L)	PDGFR β (nmol/L)	HUVECs (nmol/L)	HBVPs (μ mol/L)
Lead compd.	9.4	188.0	143.0	90.2	/
D4/WXFL-152	7.0	69.0	31.0	71.0	1.94
A1	20.1	293.7	67.6	228.8	/
B2	26.7	216.4	56.9	145.9	/
C2	78.6	63	311.1	>10,000.0	/
C3	10.0	129.9	84.3	31.0	/
C4	22.6	378.4	139.9	30.9	/
C16	20.5	1103.50	161.4	20.7	/
C25	74.1	6100.40	193.7	84.1	/
Nintedanib	8.8	93.9	35.4	41.0	1.67

(PDGFR-positive) proliferation and cell counting kit-8 (CCK-8) assay were performed. The results indicated that WXFL-152 significantly inhibited the proliferation of HBVPs (IC₅₀ value of WXFL-152 vs. nintedanib: 1.94 ± 0.14 vs. 1.67 ± 0.11 μ mol/L) induced by 20 ng/mL hPDGF-BB as shown in Table 5. These results indicated that WXFL-152 could not only inhibit ECs proliferation induced by hVEGF-165, but also inhibit pericytes proliferation induced by PDGF-BB in tumor angiogenesis.

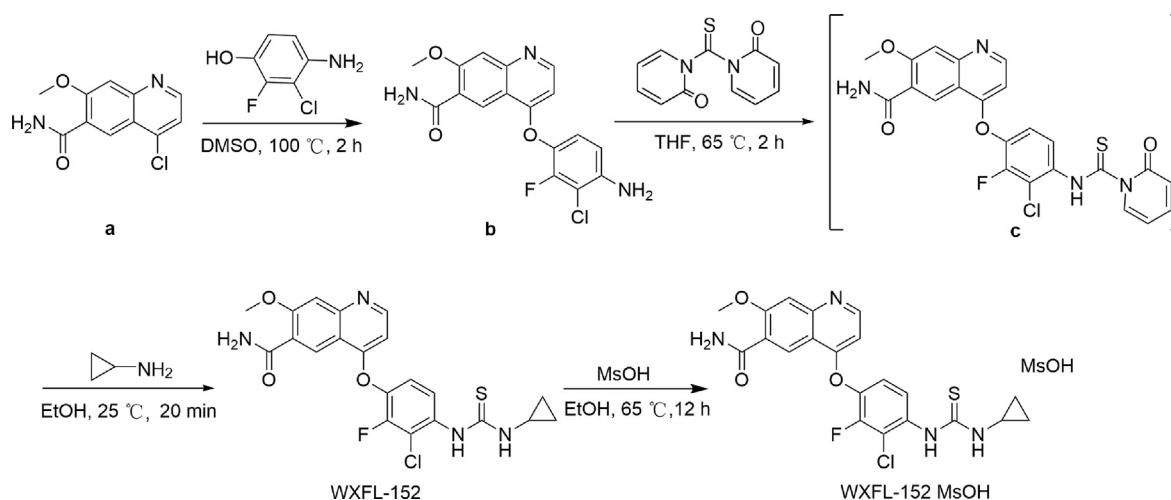
2.6. WXFL-152 inhibited the VEGF/VEGFR2/ERK, FGF/FGFRs/ERK and PDGF/PDGFR β /ERK signalling pathway

Tumor angiogenesis stimulated by multiple angiokinase signals such as VEGF/VEGFRs, FGF/FGFRs and PDGF/PDGFRs in ECs and pericytes plays a crucial role in tumorigenesis, metastasis and progression. Blocking these signals in ECs and pericytes can inhibit tumor angiogenesis and suppress tumor growth effectively³². The kinase inhibition assays showed that WXFL-152 significantly inhibited human VEGFR2, FGFRs and PDGFR β . To reveal the further molecular mechanism of WXFL-152, Western blot assays were used to evaluate the effects on VEGF/VEGFR2, FGF/FGFRs and PDGF-BB/PDGFR β and their downstream signalling. As shown in Fig. 3A and B, 5.0 μ mol/L WXFL-152 significantly suppressed the phosphorylation of VEGFR2 and ERK induced by 50 ng/mL hVEGF 165 in HUVECs ($P < 0.01$).

The PDGFR β phosphorylation leads to the activation of various downstream signalling substrates, and plays important roles in cell proliferation (pericytes, vascular smooth muscle cells and fibroblasts) and blood vessel formation. As shown in Fig. 3C and D, the phosphorylation of PDGFR β and ERK induced by 50 ng/mL

Table 6 *In vitro* kinase inhibition profile of WXFL-152.

Kinase	IC ₅₀ (nmol/L)	Kinase	IC ₅₀ (nmol/L)
VEGFR1	30.9	EphA2	>3333
VEGFR2	7.0	GSK3 β	>10,000
VEGRR3	8.5	MAP4K4	79.0
FGFR1	69.0	JAK2	>10,000
FGFR2	15.5	JNK1	>10,000
FGFR3	132.0	LCK	252.0
FGFR4	131.0	MAPKAPK2	>10,000
PDGFR α	135.0	MINK	55.0
PDGFR β	31.0	MST4	>10,000
ABL	520.0	p38 α	>3333
AUR2	262.0	PDK1	>10,000
CAMK2 α	>10,000	PKA	>10,000
CDK2	>10,000	SRC	2648.0
CHK1	>10,000	TAOK2	>10,000
CK1 α	>10,000	TNIK	118.0
DYRK3	>10,000	/	/

**Scheme 6** The synthesis route of WXFL-152 and its mesylate.

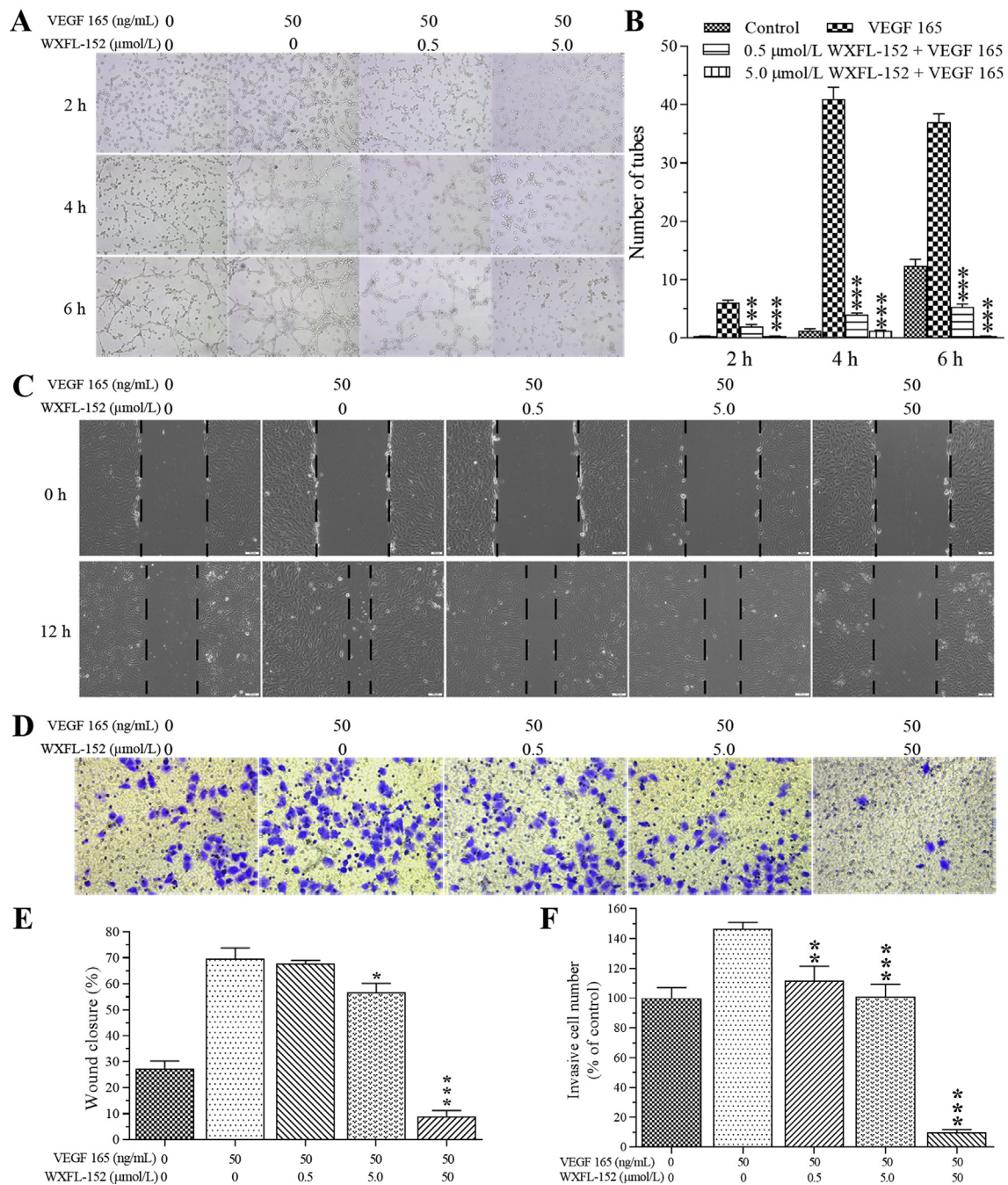


Figure 2 WXFL-152 inhibited HUVECs' tube formation, migration and invasion. (A) Representative images of tube formation of HUVECs in different groups. (B) The numbers of HUVECs tubes in different groups. (C) Representative images of wound closure. (D) Representative images of invasive cells in the transwell invasion assay. (E) The wound closure percentages of different groups in the wound healing assay. (F) The invasive cell number percentages of different groups in the transwell invasion assay (* $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$). Data are represented as mean $\pm$ SEM ($n = 3$).

PDGF-BB in HBVPs were significantly blocked by 0.5 and 5.0 μmol/L WXFL-152, respectively. The phosphorylation of FGFR1–4 and ERK induced by 50 ng/mL bFGF in HUVECs was significantly inhibited by 5.0 μmol/L WXFL-152, respectively ($P < 0.01$, as shown in Fig. 3E and F). The above-mentioned results suggested that the anti-angiogenic effect of WXFL-152 inhibited the proliferation, migration, invasion, and tube formation of endothelial cells and pericytes respectively by targeting

VEGFR2 and FGFRs on the endothelial cell membrane and PDGFRβ on the pericytes, as shown in Fig. 3G.

2.7. WXFL-152 inhibited tumor-induced angiogenesis in a transgenic zebrafish model

To visually display the anti-tumor mechanism and effectiveness of WXFL-152 *in vivo*, a xenograft tumor model in the Tg

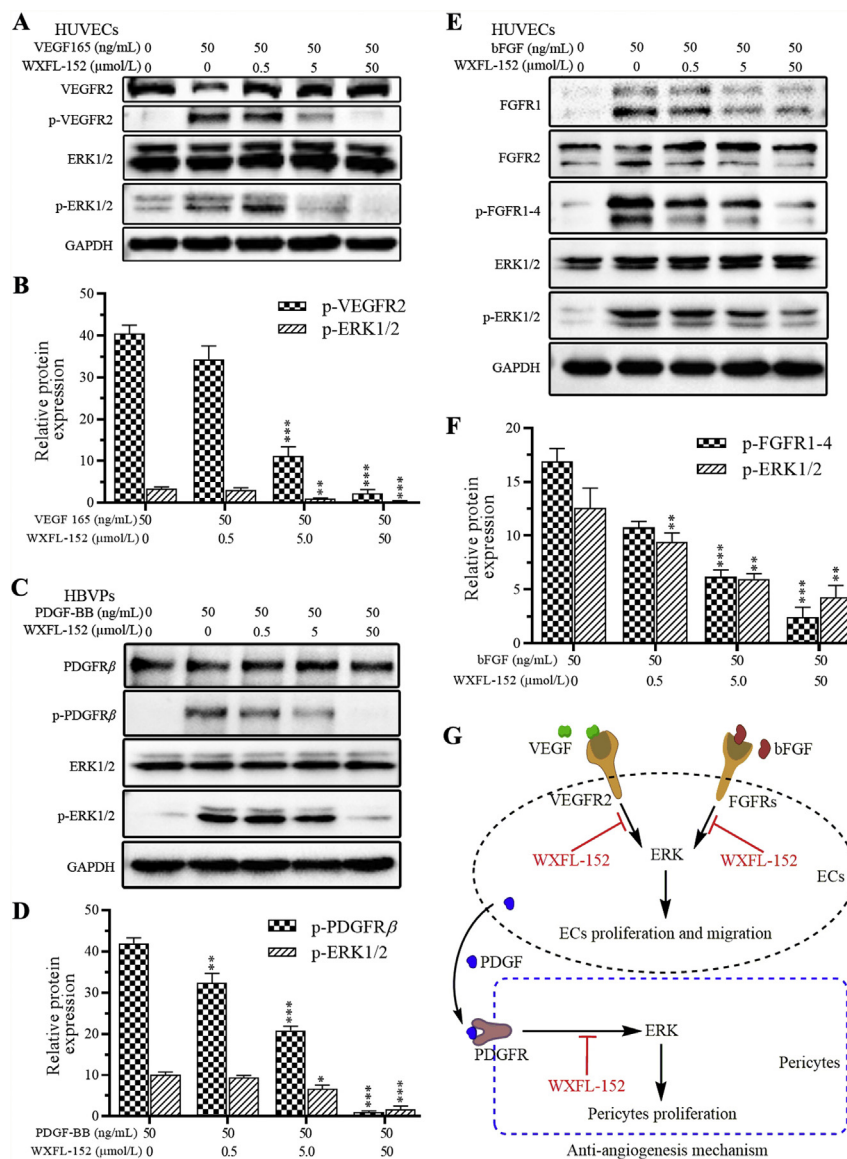


Figure 3 WXFL-152 inhibited the VEGFR2, FGFRs and PDGFR β /ERK signalling pathways. (A) Western blot images of VEGFR2/ERK proteins and their phosphorylation induced by 50 ng/mL VEGF-165 in HUVECs. (B) Statistical analysis of (A). (C) PDGFR β and ERK1/2 protein and their phosphorylation induced by 50 ng/mL PDGF-BB in HBVPs. (D) Statistical analysis of (C). (E) Western blot images of FGFRs/ERK proteins and their phosphorylation forms induced by 50 ng/mL bFGF in HUVECs. (F) Statistical analysis of (E). (G) The diagram displayed the anti-angiogenesis mechanism of WXFL-152. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are represented as mean $\pm$ SEM ($n = 3$).

(flk1:eGFP; Gata1:dsRed) double transgenic zebrafish model was established as shown in Fig. 4A. In this model, the volume and neovascularization in microtumors could be observed by high-resolution fluorescence microscope using time-lapse imaging strategy. Red fluorescence-labelled CT26 colon adenocarcinoma cells were microinjected into zebrafish embryos. After 7 days, endothelial cells of zebrafish in the control group had filled the inner space of the xenografted tumor and established a primary vascular network as the tumor grew. Moreover, few tumors exhibiting angiogenesis and small tumors were observed in the group treated with 0.5 and 2.0 $\mu\text{mol/L}$ WXFL-152. Typical images of tumor cells (red) and tumor angiogenesis (green) in transgenic zebrafishes were shown in Fig. 4B, and the numbers of tumor

vessels were counted as shown in Fig. 4C. These results not only confirmed the anti-tumor effect of WXFL-152 but also visually demonstrated neovascularization during tumor growth *in vivo*.

2.8. WXFL-152 significantly inhibited tumors in the subcutaneous xenograft models and patient-derived tumor xenograft model

To further evaluate the anti-tumor efficacy of WXFL-152 *in vivo*, two non-small cell lung cancer xenograft models and a liver cancer PDX model were established in Balb/c nude mice. After 21 days of treatment with WXFL-152, the tumors were significantly inhibited by all the dose treatment groups, as shown in Fig. 4D and Table 7.

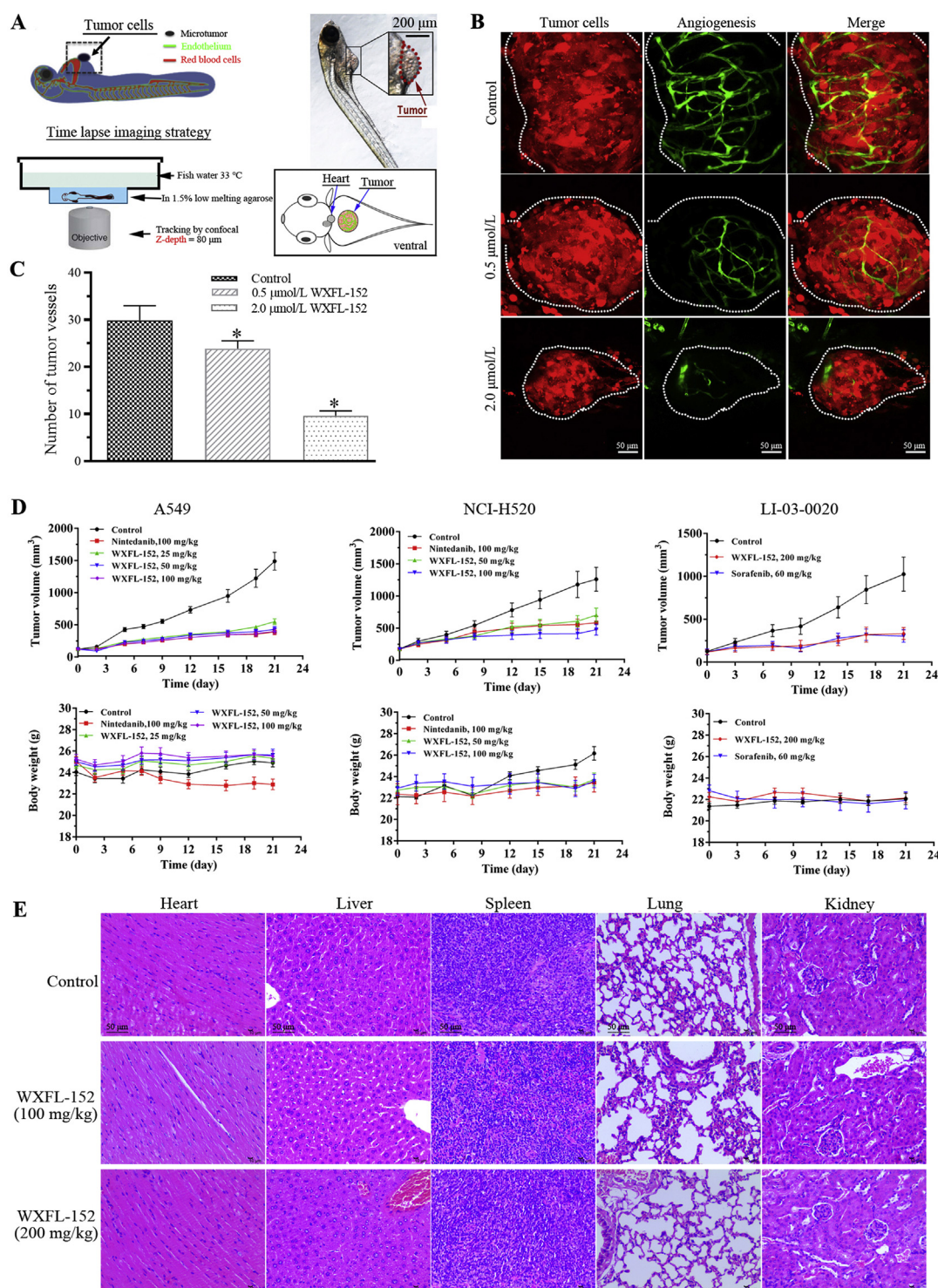


Figure 4 WXFL-152 inhibited tumor growth in subcutaneous xenograft models of transgenic zebrafish and mice. (A) The CT-26 cell line xenograft tumor model in the Tg (flk1: eGFP; Gata1: dsRed) double transgenic zebrafish model. (B) Typical images of tumor cells (red) and tumor angiogenesis (green) in transgenic zebrafishes captured by confocal. (C) The numbers of tumor vessels in each group, **P* < 0.05. Data are represented as mean ± SEM (*n* = 8). (D) WXFL-152 inhibited tumor growth in A549 and NCI-H520 cell lines subcutaneous xenograft models of mice and liver cancer PDX model. Except for the nintedanib (100 mg/kg) treatment group, the animals in the other treatment groups did not show obvious weight loss throughout the entire treatment period in the A549 lung cancer xenograft model. In the NCI-H520 lung cancer xenograft model, compared with the control group, the animals in the treatment groups showed a decrease in body weight. In the patient-derived tumor (LI-03-0020) xenograft model, there was no significant decrease in animal weight. (E) Typical H&E staining images of the vital organs (heart, liver, spleen, lung and kidney, × 400) of mice treated with WXFL-152.

Table 7 Tumor volumes, T/C and TGI after treatment of WXFL-152 in subcutaneous xenograft models of mice (mean $\pm$ SEM).

Group	Mouse number (n)	Tumor volume at Day 21 (mm ³)	T/C (%)	TGI (%)	P value
A549 Cell line model					
Control	9	1489 $\pm$ 138	/	/	/
WXFL-152 (50 mg/kg)	9	430 $\pm$ 42	29	78	<0.0001
WXFL-152 (100 mg/kg)	8	401 $\pm$ 38	27	80	<0.0001
Nintedanib (100 mg/kg)	9	391 $\pm$ 43	26	80	<0.0001
NCI-H520 Cell line model					
Control	6	1261 $\pm$ 186	/	/	/
WXFL-152 (50 mg/kg)	6	704 $\pm$ 107	58	51	0.0415
WXFL-152 (100 mg/kg)	6	478 $\pm$ 85	36	72	0.0014
Nintedanib (100 mg/kg)	6	585 $\pm$ 101	45	62	0.0057
Liver cancer PDX model					
Control	8	1026 $\pm$ 199	/	/	/
WXFL-152 (200 mg/kg)	6	334 $\pm$ 69	35	76	0.0097
Sorafenib (60 mg/kg)	6	304 $\pm$ 73	31	80	0.0182

The anti-tumor effects were evaluated by the relative tumor growth rate T/C (%) and tumor growth inhibition rate TGI (%) as described in the Experimental Section. In the A549 lung cancer xenograft model, 50 and 100 mg/kg WXFL-152 and 100 mg/kg nintedanib demonstrated similar significant suppression of cancer growth (T/C, 29%, 27% and 26%; TGI, 78%, 80% and 80%, $P < 0.0001$, respectively). In the H520 xenograft model, the T/C values were 58%, 36% and 45%, and the TGI values were 51%, 72% and 62% for the 50 and 100 mg/kg WXFL-152 and 100 mg/kg nintedanib treatment groups, respectively. WXFL-152 had better effects than nintedanib at the same dose of 100 mg/kg. In the liver cancer PDX model, 200 mg/kg WXFL-152 and 60 mg/kg sorafenib demonstrated similar therapeutic efficacies compared with the control group (T/C 35%, TGI 76%, $P = 0.0097$ for WXFL-152; T/C 31%, TGI 80%, $P = 0.0182$ for sorafenib). No obvious body weight loss observed throughout the entire treatment period except for the weight loss attributed to nintedanib (100 mg/kg) treatment in the A549 lung cancer xenograft model, suggesting that the administration of WXFL-152 was well tolerated. In addition, the vital organs (heart, liver, spleen, lung and kidney) of mice treated with WXFL-152 (100 and 200 mg/kg, *p.o.*, QD $\times$ 3 weeks) were collected and further analysed by H&E staining. No significant pathologic changes were observed in these organs of the nude mice (Fig. 4E), suggesting that there were no obvious toxicity with continuous treatment with WXFL-152.

2.9. WXFL-152 decreased the tumor micro-vessel density in the subcutaneous xenograft models analysed by immunohistochemistry

CD31 is one of the standard markers for detecting vascular endothelial cells *in vivo*. To confirm the anti-angiogenesis mechanism of WXFL-152, anti-CD31 immunohistochemical analysis of tumors from mice in the subcutaneous xenograft and PDX models was performed. Compared with those in controls, the micro-vessel density of tumors from the WXFL-152 treated groups was significantly decreased. The areas of CD31 (mean $\pm$ SEM, $n = 3$) were 3.38 ± 0.49 , 1.05 ± 0.33 , 2.20 ± 0.29 and 1.27 ± 0.48 in the control, 100 mg/kg nintedanib, 50 and 100 mg/kg WXFL-152 groups in the A549 models, respectively (Fig. 5A and B), and were 4.96 ± 0.62 , 1.99 ± 0.04 , 1.96 ± 0.05 , 1.83 ± 0.26 in the control, 100 mg/kg nintedanib, 50 and 100 mg/kg WXFL-152 groups in the NCI-H520 model

(Fig. 5C and D), respectively. In the liver PDX model, the areas of CD31 in the control and 200 mg/kg WXFL-152 groups were 0.30 ± 0.10 and 0.19 ± 0.06 , respectively (Fig. 5E and F). These results indicated the significant anti-angiogenic activity of WXFL-152 even *in vivo* ($P < 0.05$), consistent with results that WXFL-152 inhibited HUVECs proliferation *in vitro* and tumor angiogenesis in a transgenic zebrafish model.

2.10. Pharmacokinetic study of WXFL-152 in rats and beagles given by oral or intravenous administration with a single dose or multi-doses

To profile pharmacokinetic characteristics of WXFL-152, including absorption, distribution, metabolism and excretion, systematic pharmacokinetic studies were performed. LC-MS/MS was used to analyse the concentration of WXFL-152 in the plasma of rats and beagles. The optimized quasi-molecular and fragment ions were selected for qualitative analysis of WXFL-152 (m/z , parent ion/product ion: 461.0/404.1, collision energy (CE): 33 V) and IS (m/z , parent ion/product ion: 393.0/373.10, CE: 18 V), respectively. Multiple reaction monitoring (MRM) mode was used to monitor both quasi-molecular/fragment ions of WXFL-152 and IS for the WXFL-152 concentration quantified in rat and beagle plasma. The protein precipitation method using acetonitrile was chosen as the extraction method of WXFL-152 and IS. As shown in Supporting Information Fig. S72–S79, no significant interference from rat and beagle plasma was observed at the retention times of WXFL-152 and IS. The retention times of WXFL-152 and IS were 1.09 and 1.49 min in rat plasma and 0.97 and 1.43 min in beagle plasma, respectively (Figs. S75 and S79). The calibration curve was linear over the concentration range of 2.0–2000 ng/mL for WXFL-152. The regression Eqs. (1) and (2) obtained by least-squares regression were

$$Y = 0.002113X + 0.0003743 \quad (1)$$

($R^2 = 0.9942$, Supporting Information Fig. S80) for WXFL-152 in rat plasma and

$$Y = 0.00152X - 0.00004393 \quad (2)$$

($R^2 = 0.9872$, Supporting Information Fig. S81) in beagle plasma (Y represents the peak-area ratio of an analyte to IS, and X represents the plasma concentration of the analyte). The lower limit

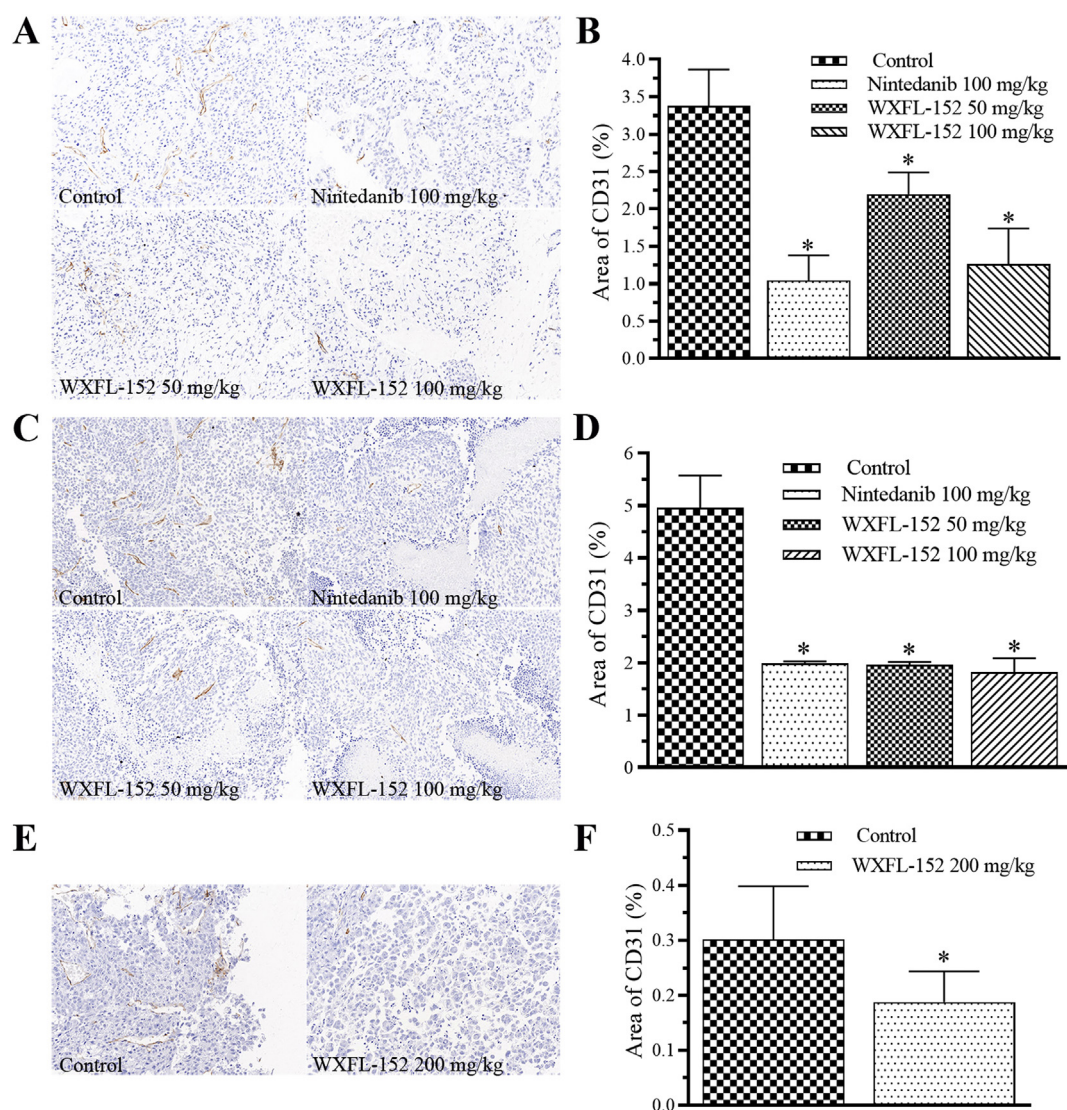


Figure 5 WXFL-152 decreased the tumor micro-vessel density in the subcutaneous xenograft models analysed by immunohistochemistry. (A) The typical CD31 staining images of tumor tissues from mice of A549 subcutaneous xenograft model ($\times 100$). (B) The staining areas statistics of CD31 in A549 subcutaneous xenograft model, $*P < 0.05$. (C) The CD31 immunohistochemical analysis of tumors from mice in the NCI-H520 subcutaneous xenograft ($\times 100$). (D) The staining areas statistics of CD31 in NCI-H520 subcutaneous xenograft model, $*P < 0.05$. (E) and (F) The micro-vessel density analysis of tumors from mice in the liver PDX model ($\times 100$, $*P < 0.05$). Data are represented as mean $\pm$ SEM ($n = 3$).

of quantification (LLOQ) was 2.0 ng/mL for WXFL-152 in both rat and beagle plasma. The precision and accuracy of LLOQ were acceptable, with relative standard deviation (RSD) values and relative error (RE) values within 20% for both analytes. The accuracies and precisions intra- and inter-day were within $\pm 15\%$. In rat plasma analysis, the precision and accuracy were 2.7%–10.5% and 97.0%–108.6% intra-day and 4.3%–13.7% and 92.5%–103.8% inter-day. In the beagle plasma analysis, the precision and accuracy were 2.9%–9.9% and 101.7%–108.1% intra-day and 5.4%–9.3% and 102.0%–110.6% inter-day, all within the acceptable limits. The results indicated that the present method was reliable and reproducible for the simultaneous quantitative analysis of WXFL-152 in rat and beagle plasma samples.

The recoveries (CV) of WXFL-152 were 81.9%–109.1% and 87.6%–94.1% at concentrations of 2.0, 800 and 1600 nmol/L ($n = 3$) from rat and beagle plasma, respectively. The IS-normalized matrix factors for WXFL-152 at concentrations of 4.0 and 2000 nmol/L were $90.9 \pm 12.1\%$ and $92.1 \pm 9.2\%$ in rat plasma, and $94.7 \pm 9.6\%$ and $92.4 \pm 3.8\%$ in beagle plasma, respectively (mean $\pm$ CV%, $n = 6$). These results suggested that there was no matrix effect under the tested conditions. The stability of the rat and beagle analytes was investigated at three concentrations under a variety of storage and process conditions. The data showed that the stability of quality control (QC) samples was acceptable when stored at room temperature for 24 h, placed in an auto-sampler (4 °C) for 58 h, and submitted to freeze-thaw cycles (9, 15 and 33 days, at -80 °C to room

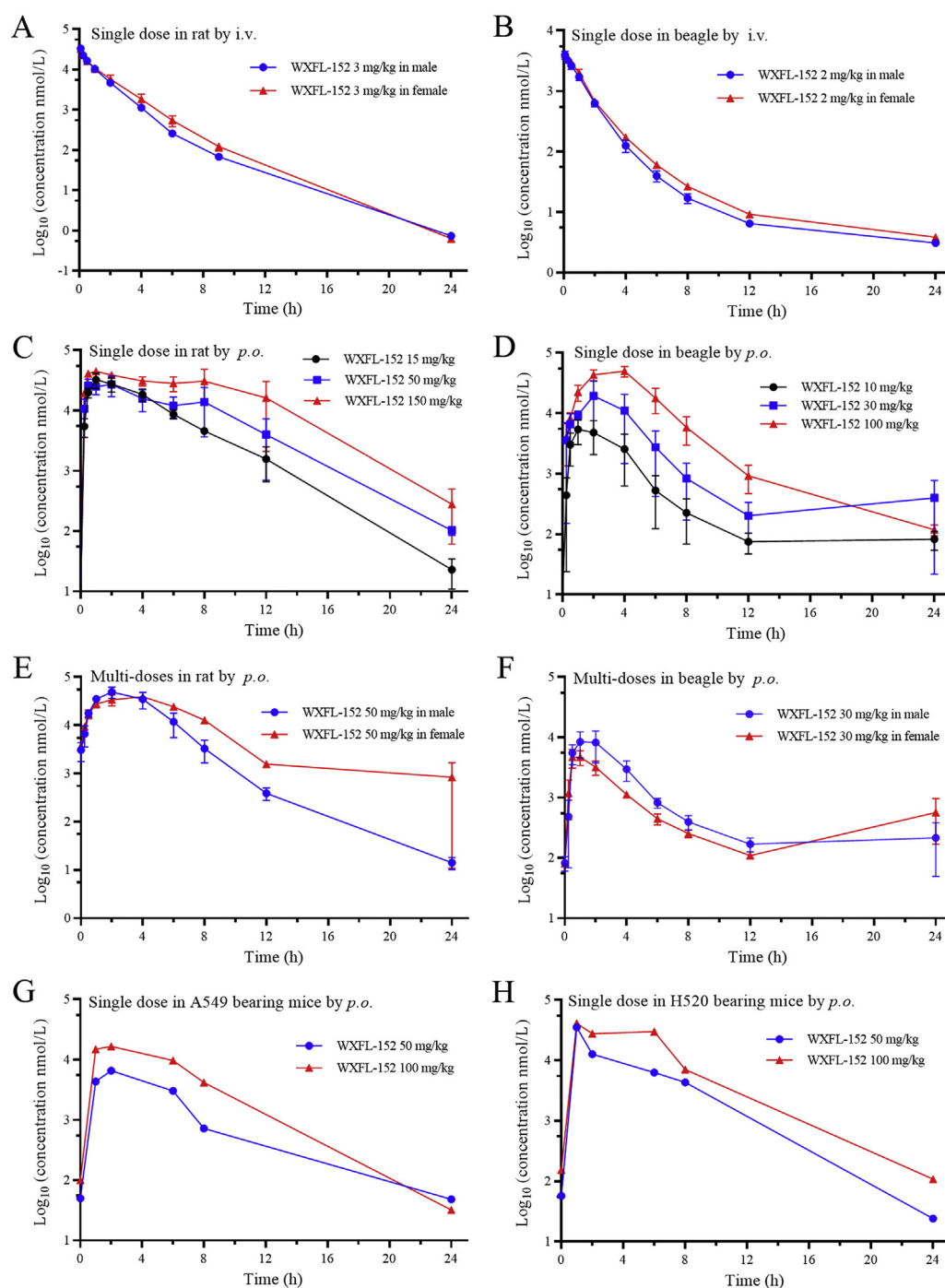


Figure 6 Pharmacokinetic study of WXFL-152 in mice, rats and beagles. (A) and (B) The plasma concentration in rat and beagle were measured at 24 h with intravenous injection of WXFL-152 (rat, 3 mg/kg; beagle, 2 mg/kg) by i.v. The plasma concentration–time curve of WXFL-152 in rats (male, $n = 3$; female, $n = 3$) was shown in (A), and that for beagles (male, $n = 3$; female, $n = 3$) was shown in (B). (C) and (D) The 24 h drug–time curves of single-dose WXFL-152 (rat, 15, 50 and 150 mg/kg; beagle, 10, 30 and 100 mg/kg) in rats and beagles (male and female, $n = 3$, respectively) administered *p.o.* (E) and (F) The 24 h drug–time curves of continuous multi-dose WXFL-152 (rat, 50 mg/kg/day; beagle, 30 mg/kg/day, for 7 days) in rats and beagles (male and female, $n = 3$, respectively) administered *p.o.* (G) and (H) The 24 h drug–time curves of continuous multi-dose WXFL-152 (50 and 100 mg/kg/day, for 21 days) in tumor-bearing mice (A549 and H520) administered *p.o.*

temperature, deviation within 15%). The dilution integrity was confirmed for rat and beagle QC samples that exceeded the up limit of quantitation (ULOQ). The results showed that the intra- and inter-day precision and accuracy of diluted samples were within the acceptable range.

The plasma concentration–time curves of WXFL-152 in rat and beagle plasma were examined by non-compartmental analysis. The drug–time curve of single-dose WXFL-152 (rat, 3 mg/kg; beagle, 2 mg/kg) in rats and beagles (male and female, $n = 3$, respectively) administered by intravenous injection (i.v.)

Table 8 The pharmacokinetic parameters of WXFL-152 in rat plasma after single or multi-doses ($n = 6$, 3 male and 3 female, mean $\pm$ SD).

Parameter	3 mg/kg (single dose, i.v.)	15 mg/kg (single dose, <i>p.o.</i>)	50 mg/kg (single dose, <i>p.o.</i>)	50 mg/kg/day (multi-doses, <i>p.o.</i> , 7th day)	150 mg/kg (single dose, <i>p.o.</i>)
$C_{\max}$ ($\mu\text{mol/L}$)	40.4 $\pm$ 3.0	26.0 $\pm$ 13.3	32.3 $\pm$ 11.3	46.5 $\pm$ 13.9	48.7 $\pm$ 9.5
$T_{\max}$ (h)	/	0.58 $\pm$ 0.20	0.54 $\pm$ 0.25	1.42 $\pm$ 0.67	1.58 $\pm$ 2.26
$t_{1/2}$ (h)	1.18 $\pm$ 0.18	2.21 $\pm$ 0.39	2.00 $\pm$ 0.55	2.17 $\pm$ 0.55	2.54 $\pm$ 0.80
V_{dss} (L/kg)	0.26 $\pm$ 0.05	/	/	/	/
CL (mL/min/kg)	3.24 $\pm$ 0.81	/	/	/	/
$\text{AUC}_{0-\text{last}}$ ($\mu\text{mol/L} \cdot \text{h}$)	35.0 $\pm$ 8.2	74.6 $\pm$ 36.3	110.0 $\pm$ 84.9	160.0 $\pm$ 60.3	232.0 $\pm$ 158.0
$\text{AUC}_{0-\text{inf}}$ ($\mu\text{mol/L} \cdot \text{h}$)	35.2 $\pm$ 8.3	77.7 $\pm$ 39.6	110.0 $\pm$ 84.9	151.0 $\pm$ 63.5	233.0 $\pm$ 158.0
Bioavailability (%)	/	42.0	18.7	/	13.0

$C_{\max}$, maximum plasma concentration; $T_{\max}$, time the maximum concentration occurred; V_{dss} , volume of distribution at steady state; CL, systemic clearance; AUC, area under the plasma concentration–time curve.

Table 9 The pharmacokinetic parameters of WXFL-152 in beagle plasma after single or multi-doses ($n = 6$, 3 male and 3 female, mean $\pm$ SD).

Parameter	2 mg/kg (single dose, i.v.)	10 mg/kg (single dose, <i>p.o.</i>)	30 mg/kg (single dose, <i>p.o.</i>)	30 mg/kg/day (multi-doses, <i>p.o.</i> , 7th day)	100 mg/kg (single dose, <i>p.o.</i>)
$C_{\max}$ ($\mu\text{mol/L}$)	4.36 $\pm$ 0.96	6.28 $\pm$ 6.57	16.20 $\pm$ 16.50	6.97 $\pm$ 5.26	47.80 $\pm$ 20.80
$T_{\max}$ (h)	/	1.25 $\pm$ 0.612	0.917 $\pm$ 0.585	1.08 $\pm$ 0.736	2.33 $\pm$ 0.816
$t_{1/2}$ (h)	4.91 $\pm$ 2.18	5.77 $\pm$ 2.17	5.25 $\pm$ 1.03	12.30 $\pm$ 12.60	2.99 $\pm$ 0.70
V_{dss} (L/kg)	1.45 $\pm$ 0.33	/	/	/	/
CL (mL/min/kg)	14.5 $\pm$ 2.74	/	/	/	/
$\text{AUC}_{0-\text{last}}$ ($\mu\text{mol/L} \cdot \text{h}$)	5.1 $\pm$ 0.9	19.6 $\pm$ 20.2	50.0 $\pm$ 58.8	23.7 $\pm$ 16.1	199.0 $\pm$ 90.5
$\text{AUC}_{0-\text{inf}}$ ($\mu\text{mol/L} \cdot \text{h}$)	5.13 $\pm$ 0.9	20.2 $\pm$ 20.2	51.8 $\pm$ 62.3	37.9 $\pm$ 31.9	230.0 $\pm$ 51.5
Bioavailability (%)	/	76.9	65.4	/	78.0

$C_{\max}$, maximum plasma concentration; $T_{\max}$, time the maximum concentration occurred; V_{dss} , volume of distribution at steady state; CL, systemic clearance; AUC, area under the plasma concentration–time curve.

showed that WXFL-152 had similar pharmacokinetic characteristics in male and female rats or beagles after 24 h (Fig. 6A and B). Fig. 6C and D showed the drug–time curve of single-dose WXFL-152 (rat, 15, 50 and 150 mg/kg; beagle, 10, 30 and 100 mg/kg) in rats and beagles (male and female, $n = 3$, respectively) administered *p.o.* (*per os*) in 24 h. Drug–time curves of multi-dose WXFL-152 (rat, 50 mg/kg/day; beagle, 30 mg/kg/day, for 7 days) in rats and beagles (male and female, $n = 3$, respectively) administered *p.o.* in 24 h were shown in Fig. 6E and F. To analyse the PK of continuous multi-dose WXFL-152 in the tumor-bearing mice, lung cancer-bearing mice (A549 and H520) treated with WXFL-152 (50 and 100 mg/kg/day, for 21 days) were given another dose of the same volume of WXFL-152 by *p.o.* The drug–time curves were shown in Fig. 6G and H. The PK parameters for rats and beagles were summarized in Tables 8 and 9. The $t_{1/2}$ values of WXFL-152 in rats and beagles varied with the dose and administration. The bioavailability (%) of WXFL-152 in rats decreased with increasing dose. The values were 42.0%, 18.7% and 13.0% when the doses were 15, 50 and 150 mg/kg in rats, respectively. This trend was not observed in beagles. The bioavailabilities (%) were 76.9%, 65.4% and 78.0% when the doses were 10, 30 and 100 mg/kg, respectively.

3. Conclusions

Angiokine signals such as VEGF/VEGFRs, FGF/FGFRs and PDGF/PDGFRs promote the proliferation and cellular migration

of ECs and pericytes, leading to angiogenesis in cancer. Multi-angiokine inhibitors targeting VEGFRs, FGFRs and PDGFRs, such as lenvatinib and nintedanib, have achieved highly favourable clinical benefits in recent years.

WXFL-152, which targets VEGFRs, FGFRs and PDGFRs, was identified from a novel series of 4-oxyquinoline derivatives based on a SAR study in this work. Molecular docking simulations revealed that the quinoline ring and the thiourea group were the main important groups in the binding site of its targets. The kinase inhibition assay showed the high affinity of WXFL-152 for VEGFRs, FGFRs and PDGFRs. Inhibition of endothelial cells and peripheral cells proliferation simultaneously can achieve a better anti-angiogenesis effect. The results of proliferation assays on HUVECs and HBVPs indicated that WXFL-152 could inhibit the proliferation of ECs induced by hVEGF-165 and pericytes induced by PDGF-BB in tumor angiogenesis.

To further confirm the molecular mechanisms of WXFL-152, Western blot assay was used to investigate its biological function in cellular level that blocked angiogenic signalling pathways (including VEGF–VEGFRs/ERKs, FGF–FGFRs/ERKs, and PDGF–PDGFRs/ERKs) in HUVECs and HBVPs. Furthermore, the molecular mechanism of WXFL-152 in transgenic zebrafish and mice bearing tumor models was revealed by the analysis of confocal microscopy images and immunohistochemistry (IHC). *In vivo*, the significant anti-cancer effects of WXFL-152 were confirmed in multiple preclinical tumor xenograft models in mice

with excellent tumor growth inhibition. In addition, pharmacokinetic studies of WXFL-152 demonstrated highly favourable bioavailability under single- and multi-dose continuous oral administration in rats and beagles.

In conclusion, WXFL-152 is a novel and effective triple-angiokinase inhibitor with clear PD and PK in tumor therapy. Recently, WXFL-152 as a novel clinical candidate is being tested in phase Ib clinical trials. Further clinical trials will focus on the treatment of solid tumors and pulmonary fibrosis.

4. Experimental

4.1. Chemistry

All starting chemicals and reagents were commercially available and used without further purification. Reaction progress was usually monitored by thin-layer chromatography (TLC) or LC–MS. The products were separated and purified by conventional preparative TLC and HPLC. ^1H nuclear magnetic resonance (^1H NMR) and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker Company, Karlsruhe, Germany) using TMS as an internal standard and $\text{DMSO}-d_6$ or CDCl_3 as a solvent. The chemical shifts were reported in parts per million (ppm) relative to TMS. The mass spectral (MS) data were obtained on LC triple quadrupole mass spectrometer system (LC–MS/MS 8050 system, Shimadzu Corporation, Kyoto, Japan). The purity of compound was tested by reverse-phase HPLC (XBridge column, C18, 150 mm $\times$ 4.6 mm, 5 μm) using a Shimadzu 20A LC system (Shimadzu). The detailed synthesis methods, including reagents and conditions of all compounds mentioned in this study (Schemes 1–5), were described in our previous patent²⁷. All of the compounds identification were provided in the Supporting Information. The synthesis route of WXFL-255 (lead compound) and WXFL-152 were shown in Schemes 1 and 6. The detailed methods were as follows.

4.1.1. 4-(3-Chloro-4-(3-cyclopropylthioureido)phenoxy)-7-methoxyquinoline-6-carboxamide (compound WXFL-255, lead compound)

The detailed synthesis methods, including reagents and conditions of WXFL-255 was described in our previous research²⁰. Briefly, as shown in Scheme 1, methyl 4-amino-2-methoxybenzoate (**1**) reacted with trimethyl orthoformate and 2,2-dimethyl-1,3-dioxane-4,6-dione to yield compound **1A**. Subsequent heating **1A** at 220 $^\circ\text{C}$ triggered intramolecular cyclization to give quinolone **1B**, which upon hydrolysis of methyl ester, chlorination of the ketone and aminolysis of the acyl chloride led to the desired chloroquinoline **1E**. Then 3-chloro-4-nitro-phenol was heated with **1E** to afford nitro compound **1F**, which was reduced by iron to provide the amine **1G**. The following isothiocyanate from the treatment of **1G** with 1,1'-thiocarbonyldi-2(1H)-pyridone coupled with cyclic propylamine to produce the lead compound WXFL-255, yellow solid, 90 mg, Yield 15.63%. ^1H NMR (400 MHz, methanol- d_4) δ 9.03 (s, 1H), 8.93 (d, J = 6.78 Hz, 1H), 7.91 (br. s., 1H), 7.57–7.68 (m, 2H), 7.39 (d, J = 8.78 Hz, 1H), 7.21 (br. s., 1H), 4.22 (s, 3H), 2.73 (br. s., 1H), 0.53–1.09 (m, 4H). LC–MS (ESI) m/z $[\text{M}+\text{H}]^+$: 443.0.

4.1.2. 4-(4-Amino-3-chloro-2-fluorophenoxy)-7-methoxyquinoline-6-carboxamide (compound **b**)

4-Chloro-7-methoxyquinoline-6-carboxamide compound **a** (710.4 g, 3.00 mol), sodium *tert*-butoxide (353 g, 3.67 mol) and 4-

amino-3-chloro-2-fluorophenol (600 g, 3.71 mol) were dissolved in DMSO (21.3 L) at 20–35 $^\circ\text{C}$. Under the protection of nitrogen, the reaction system was stirred at room temperature for 20 min and then stirred at 96–100 $^\circ\text{C}$ for 2 h. The reaction was detected by HPLC. The resulting mixture was cooled to room temperature. Water (28.4 L) was added, and the mixture was filtered and dried *in vacuo* to obtain compound **b** (purple-grey solid, 994 g). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.65 (s, 2H), 7.87 (br. s., 1H), 7.75 (br. s., 1H), 7.51 (br. s., 1H), 7.17 (br. s., 1H), 6.72–6.70 (d, J = 8.00 Hz, 1H), 6.50 (s, 1H), 5.83 (s, 2H), 4.01 (s, 3H). MS m/z $[\text{M}+\text{H}]^+$: 362.0.

4.1.3. 4-(3-Chloro-4-(3-cyclopropylthioureido)-2-fluorophenoxy)-7-methoxyquinoline-6-carboxamide (WXFL-152)

Compound **b** (990 g, 2.49 mol) and 1,1'-thiocarbonyldi-2(1H)-pyridone (1020.5 g, 4.39 mol) were dissolved in THF (19.8 L) at 20–35 $^\circ\text{C}$. Under the protection of nitrogen, the reaction system was stirred at 61–65 $^\circ\text{C}$ for 2 h. The reaction was detected by HPLC. The resulting mixture was cooled to room temperature. Cyclopropylamine (328.7 g, 5.76 mol) was added dropwise and stirred for 20 min, then filtered. The filtrate was collected. Water (140 L) was added, and the mixture was stirred for 30 min, filtered, and dried *in vacuo* to obtain WXFL-152 (light-brown solid, 664 g, Yield 57.9%). ^1H NMR (400 MHz, CD_3OD) δ 9.05 (s, 1H), 8.95 (d, J = 6.4 Hz, 1H), 7.86–7.72 (m, 1H), 7.66 (s, 1H), 7.55–7.51 (m, 1H), 7.22 (m, 2H), 4.22 (s, 3H), 2.76 (m, 1H), 0.94 (m, 2H), 0.79 (m, 2H). MS m/z $[\text{M}+\text{H}]^+$: 461.1.

4.1.4. 4-(3-Chloro-4-(3-cyclopropylthioureido)-2-fluorophenoxy)-7-methoxyquinoline-6-carboxamide methanesulfonate (WXFL-152 methanesulfonate)

To a solution of WXFL-152 (650 g, 1.41 mol) in EtOH (18.85 L) was added MsOH (128.76 g, 1.34 mol) dropwise at 55–65 $^\circ\text{C}$. The resulting mixture was cooled to room temperature and stirred for 12 h. The mixture was filtered and dried *in vacuo* to obtain WXFL-152 methanesulfonate (white solid, 703 g; Yield 89.6%, purity 98.93%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.39 (s, 1H), 9.07 (d, J = 6.4 Hz, 1H), 8.74 (s, 1H), 8.74–8.37 (m, 1H), 8.01 (s, 1H), 7.93 (s, 1H), 7.81–7.62 (m, 2H), 7.71 (s, 1H), 7.07–6.95 (m, 1H), 4.10 (s, 1H), 3.03–2.69 (m, 1H), 2.39 (s, 3H), 0.78–0.59 (m, 4H). ^{13}C NMR (400 MHz, $\text{DMSO}-d_6$) δ 182.6 (s, –C), 165.4 (s, –C), 165.0 (s, –C), 161.0 (s, –C), 151.0 (s, –C), 149.1 (d, –CH), 148.5 (s, –C), 143.7 (s, –C), 128.7 (s, –C), 124.7 (s, –CH), 121.0 (s, –CH), 113.8 (s, –C), 103.2 (s, –CH), 101.6 (s, –CH), 56.9 (s, –CH₃), 39.8 (s, –CH₃), 6.6 (s, –CH₃). MS m/z $[\text{M}+\text{H}]^+$: 483.1. The mesylate form of WXFL-152 was used in biological test in this study.

4.2. Cell lines and cell culture

Non-small cell lung cancer lines (A549 and NCI-H520) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$). HUVECs isolated from human umbilical veins and human brain vascular pericytes (HBVPs) isolated from human brains were obtained from ScienCell Research Laboratories, Inc. (Catalog: 8000 and 1200, CA, USA). HUVECs were grown in endothelial cell medium (ECM, Cat. #1001, ScienCell Research Laboratories, Inc.) supplemented with 5% FBS. HBVPs were grown in Pericyte Medium (PM, Cat. #1201, ScienCell Research Laboratories, Inc.) supplemented with

5% FBS. HUVECs and HBVPs at passages 3–8 were used in all studies. All cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C³³.

4.3. Molecular docking analyses

Molecular docking analyses were performed as described in our previous study³⁴. Briefly, the AutoDock Vina program (Scripps Research Institute, CA, USA) was used for molecular docking simulations³⁵. WXFL-152 was prepared as a pdbqt file using Auto Dock Tools. The crystal structure of FGFR1 complexed with ponatinib (PDB ID: 4V04) and VEGFR2 complexed with sorafenib (PDB ID: 4ASD) were selected as the protein models. Then, all the water molecules and solvent molecules were removed, Gasteiger–Marsili charges were added, and non-polar hydrogens were then merged into their respective heavy atoms, as previously described³⁶. A total of 20 docking poses were generated for WXFL-152, and the other parameters for Vina were set to their default settings. The docking results were viewed using the PyMOL program.

4.4. Kinase inhibition assay

A total of 31 human kinases (Invitrogen, CA, USA) were incubated with WXFL-152 (0.17–10,000 nmol/L, diluted with DMSO). The kinase activities were analysed by Invitrogen Z'-LYTE[®] Kinase detection technology according to its protocol and an Agilent Bravo Automated Liquid Handler (G5409A, CA, USA). Briefly, 2.5 µL/well WXFL-152 was added to 384-well microplate assays and centrifuged (5810R, Eppendorf, Hamburg, Germany) for 1 min (1000 rpm), and 5 µL/well kinase solutions were added and centrifuged for 1 min (1000 rpm). After incubation for 15 min at 23 °C, another 2.5 µL ATP solution was added per well and centrifuged for 1 min (1000 rpm). After incubation for 60 min at 23 °C, the reaction was stopped by the addition of 5 µL stop buffer. Envision (PerkinElmer, MA, USA) was used to analyse the readouts after incubation at 23 °C for 60 min. The IC₅₀ values were calculated using the XLfit5 205 formula (IDBS, Guildford, UK).

4.5. Proliferation, tube formation, scratch wound and migration assays of HUVECs

The CellTiter-Glo luminescent cell viability assay (Promega, Madison, USA) was used for the proliferation activity assay following the manufacturer's instructions³⁷. HUVECs in the logarithmic growth phase were collected and seeded in 384-well plates (1000 cells per well) with ECM basal medium containing 2% FBS. Twenty-four hours after seeding in 384-well plates, a series of WXFL-152 dilutions (final concentration from 10,000–0.038 nmol/L) with a final concentration of 20 ng/mL hVEGF-165 in ECM basal medium containing 2% FBS were added to the well by an Agilent Bravo Automated Liquid Handler (G5409A, Agilent Technologies Inc., CA, USA). Cell viability was detected after 72 h by an Envision microplate reader (PerkinElmer). The IC₅₀ values were calculated using the XLfit5 205 formula (IDBS). Recombinant human vascular endothelial cell growth factor (hVEGF-165) was obtained from PeproTech Inc. (Suzhou, China).

HUVEC tube formation was performed as previously described³⁸. First, 300 µL precooled Matrigel (growth factor reduced, BD Biosciences, NJ, USA) was coated on the bottom of

each 48-well plate. After incubation in a humidified atmosphere of 5% CO₂ at 37 °C for 1 h, HUVECs with 8×10^4 cells/well were seeded into the coated 48-well plate. Then, WXFL-152 at concentrations of 0.5, 5.0 and 50 µmol/L and/or stimulated with 50 ng/mL hVEGF-165 was added to each well after cells were seeded in triplicate. After 2, 4 and 6 h of co-culture, tube formation was visualized using an Olympus microscope (IX71, Olympus, Tokyo, Japan), and total tubes were analysed using Image-Pro Plus 7.0 software.

HUVECs were seeded in 6-well plates at a density of 4×10^5 cells/well and were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. After 24 h, a straight scratch was introduced into the monolayer of endothelial cells in each well. The cells were treated with WXFL-152 at concentrations of 0.5, 5.0 and 50.0 µmol/L in the ECM medium containing 20 ng/mL hVEGF-165 after being washed twice with DPBS (ScienCell Research Laboratories, Inc.). All of the straight scratches were captured by an Olympus microscope (IX71, Olympus) at 12 h. The width of the scratch was measured at two time points using Image-Pro Plus 7.0 software (Media Cybernetics, MD, USA).

For migration assays, HUVECs were seeded into the upper chamber of a transwell device (24-well plate, 2×10^4 cells/well) with the ECM medium containing 20 ng/mL VEGF-165 and 0.5, 5.0 and 50.0 µmol/L WXFL-152. Then, 600 µL ECM medium containing 20 ng/mL VEGF-165 with 10% FBS was added to the bottom of the transwell chamber. The plates were then incubated in a humidified atmosphere of 5% CO₂ at 37 °C for 24 h. The cells that migrated into the pores of the inserted filter were fixed with 4% PFA solution and stained with crystal violet. The stained migrated cells were counted manually under a fluorescence microscope (IX71, Olympus).

4.6. Proliferation assay of HBVPs

To test the effect of WXFL-152 on the proliferation of HBVPs, CCK-8 (Dojindo, Kumamoto, Japan) assay was performed as described in our previous study³⁹. HBVPs in the logarithmic growth phase were collected and seeded in 96-well plates using complete PM medium. After 24 h, the medium in 96-well plate was removed. HBVPs were subsequently treated with different concentrations of WXFL-152 (0–10 µmol/L) or nintedanib (0–10 µmol/L) with 20 ng/mL PDGF-BB in basal PM medium (1% FBS). After 48 h treatment, CCK-8 assay kit was used to detect the living cells. The IC₅₀ values were calculated by linear regression of plots.

4.7. Western blot analysis

HUVECs and HBVPs were pre-treated with WXFL-152 (0.5, 5.0 and 50.0 µmol/L) in ECM and PM basal medium with 1% FBS for 3 h at 37 °C with 5% CO₂. Following another 15 min of stimulation with 50 ng/mL VEGF-165 or 100 ng/mL bFGF for HUVECs and 50 ng/mL PDGF-BB (PeproTech Inc.), for HBVPs, cells were homogenized in cell lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na₃VO₄ and leupeptin, PMSF, and phosphatase inhibitor cocktails. The lysates were centrifuged at 13,000×g for 15 min. The protein concentration of the supernatant was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). Equivalent amounts of total proteins (30 µg) were loaded on 10% SDS-PAGE gel. After being transferred to PVDF membranes, the samples were

incubated with primary antibodies: VEGF receptor 2 rabbit mAb (CST, MA, USA), phosphor-VEGF receptor2 (Tyr1175) rabbit mAb (CST), FGF receptor1 (9740S, CST), FGF receptor2 rabbit mAb (BD), phosphor-FGF receptor1–4 rabbit mAb (BD), PDGF receptor β rabbit mAb (CST), phosphor-PDGF receptor β (Tyr1009) rabbit mAb (CST) and mouse anti- β -actin mAb. The secondary antibodies were goat anti-rabbit IgG (HRP)-conjugated (1:10,000) or goat anti-mouse IgG (HRP)-conjugated (1:10,000).

4.8. Tumor xenograft model in zebrafish

Pharmacodynamic studies in zebrafish were performed according to our previous research³⁷. Zebrafish experiments were approved by the Institutional Animal Care and Treatment Committee of State Key Laboratory of Biotherapy of Sichuan University (Chengdu, China). Tg (flk1: EGFP) zebrafish were bred and maintained normally (temperature, 28 °C; pH 7.2–7.4; 14 h on and 10 h off light cycle). Colon carcinoma cell line CT-26 (red fluorescence-labelled) was harvested and implanted into each zebrafish embryo through the perivitelline space using an electronically regulated air-pressure microinjector (PL1-90, Harvard Apparatus, NY, USA). WXFL-152 was added directly into the fish water at a final concentration of 2.0 and 0.5 $\mu\text{mol/L}$. DMSO was used as a vehicle control. Animals were maintained in 2 mL fish water that was changed daily. After 7 days of treatment, living zebrafish were anaesthetized with 0.003% tricaine. Digital micrographs were taken with a Zeiss Imager. Whole animal images were taken with a Zeiss Stemi 2000-C stereomicroscope with an AxioCam MRc5 digital CCD camera (Carl Zeiss Microimaging Inc., Jena, Germany). All images were taken in the same focal plane in bright-field mode and in transmitted light passing through RFP or GFP filters. To clearly image all vessels within xenograft models, 0.5–2 μm step z-stacks (512 $\times$ 512 focal planes, 50–200 μm in depth) were acquired using a 10 $\times$ or 20 $\times$ objective (Zeiss, Plan-Neofluar). Image capture and the measurement of tumor size, vessel length and vessel diameter were performed with AxioVision rel.4.8 software (Carl Zeiss Microimaging Inc.)⁴⁰.

4.9. Pharmacokinetic study

To systematically reveal the pharmacokinetic characteristics of WXFL-152, a series of normalized PK methods, including HPLC–MS/MS conditions, standard solutions, calibration standards and QC sample, sample preparation, method validation, linearity of calibration curve and lower limit of quantification, were established as described in the Supporting Information. All animal experiments were approved by the Institutional Animal Care and Treatment Committee of WuXi AppTec. (Wuxi, China). Twenty-four Sprague–Dawley rats (12 male and 12 female, 180–220 g) were obtained from the Laboratory Animal Center of Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China), and 24 beagles (12 male and 12 female, 8–11 kg) were obtained from Beijing Marshall Biological Resources Co., Ltd. (Beijing, China). Twelve male or female animals (rats and beagles) were randomly divided into four treatment groups ($n = 3$). Animals in group 1 were administered a single dose of WXFL-152 in 20% PEG400 (3 mg/kg for rat and 2 mg/kg for beagle) by i.v. animals in group 2 (15 mg/kg for rat and 10 mg/kg for beagle), and 4 (150 mg/kg for rat and 100 mg/kg for beagle) were administered a single dose of WXFL-152 in 0.5%

methocel/0.2% Tween-80 *p.o.* The rats in group 3 (50 mg/kg/day for rat and 30 mg/kg/day for beagle) were given WXFL-152 in 0.5% methocel/0.2% Tween-80 for seven days by *p.o.* The animals were fasted, except for provision of water, for 12 h before the experiment. The diet was restored after 4 h of administration. Blood samples (0.2 mL for rat and 0.6 mL for beagle) were collected from the vein (tail vein for rats and saphenous vein of the forelimb for beagles) into heparinized 1.5 mL polythene tubes at continuous points in time after oral or intravenous administration of WXFL-152. The samples were immediately centrifuged at 3000 $\times g$ for 15 min. The plasma obtained was stored at -80 °C until analysis. The concentration of WXFL-152 in human plasma was detected by LC–MS/MS as described in the Supporting Information. Plasma WXFL-152 concentration–time data for each group were analysed using WinNonlin™ Version 6.3 software (Pharsight, Mountain View, CA, USA).

4.10. Anti-tumor activity and pharmacokinetic study in subcutaneous xenograft models

Female Balb/c nude mice (6–8 weeks old) were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China) and were acclimated for 1 week before the experiment. Animal experiments were approved by the Institutional Animal Care and Treatment Committee of WuXi AppTec. (Wuxi, China). All pharmacodynamics experiments of WXFL-152 were independently repeated twice. To establish a subcutaneous xenograft model, mice were injected subcutaneously with A549 and NCI-H520 cells (5×10^6 cells/100 μL). One or two weeks later, when the tumor sizes reached approximately 150 (100–200) mm^3 , the mice were divided into five groups in the A549 cell model and four groups in the NCI-H520 cell model, with 9 mice in each group. WXFL-152 (25, 50 and 100 mg/kg, QD $\times$ 3 weeks), nintedanib (100 mg/kg) and the control (vehicle) were administered *p.o.* once a day in the A549 cell model experiment. WXFL-152 (50 and 100 mg/kg), nintedanib (100 mg/kg) and the control (vehicle) were administered *p.o.* once a day in the NCI-H520 cell model experiment. The tumor volumes were measured using electronic digital callipers every 3–4 days and were calculated as $1/2 \times \text{length} \times \text{width} \times \text{width}$. The anti-tumor effects were evaluated based on the T/C (%) and TGI (%), as Eqs. (3)–(5):

$$T/C (\%) = T_{\text{RTV}}/C_{\text{RTV}} \times 100 \quad (3)$$

where T_{RTV} : mean value of mice RTV in treatment group; C_{RTV} : mean value of mice RTV in control group;

$$\text{RTV} = V_t/V_0 \quad (4)$$

where V_0 is the tumor volume of the mice starting treatment, V_t is the tumor volume of the mice at the end of treatment;

$$\text{TGI} (\%) = [1 - (V_{\text{ti}} - V_{\text{t0}}) / (V_{\text{i}} - V_{\text{0}})] \times 100 \quad (5)$$

where V_{ti} , average tumor volume of mice in treatment group, ended treatment; V_{t0} , average tumor volume of mice in treatment group, starting treatment; V_{i} , average tumor volume of mice in control group at the end of treatment; V_{0} , average tumor volume of mice in control group starting treatment.

To further analyse the PK characteristics in the tumor-bearing mice, animals in the WXFL-152 treatment groups were given another dose after 21 days of treatment. Orbital blood samples were collected from mice (A549-or NCI-H520-bearing mouse) into heparinized 1.5 mL polythene tubes at continuous points in time. The plasma samples were obtained, stored and analysed according to the method described in the pharmacokinetics study section.

4.11. Patient-derived tumor xenograft model

A liver cancer PDX model was established in accordance with China regulations concerning patient information and consent. A fresh specimen was collected from a patient diagnosed with liver cancer (LI-03-0020) and then xenografted subcutaneously in the interscapular space of 5-week-old male Balb/c nude mice (Shanghai Laboratory Animal Research Center) named P0. After five serial passages (P5), tumor pieces measuring approximately 30 mm³ were xenografted to every Balb/c nude mouse. When the average tumor volume reached 122 mm³, the mice were divided into three groups ($n = 6$). WXFL-152 (200 mg/kg), sorafenib (60 mg/kg) and a control (vehicle) were administered *via* oral administration once a day. Tumor volume and body weight were monitored twice a week throughout the treatment.

4.12. Histopathology and immunohistochemistry

Tumor tissues obtained from the mice in the A549, NCI-H520 and PDX models were collected for immunohistological analysis. At the end of the treatment, mice were sacrificed, and tumor tissues were obtained. IHC staining was performed to detect CD31 expression. Briefly, tissue peroxidases were inactivated by treatment with 3% H₂O₂ for 10 min. Then, the sections were pre-treated with antibody diluent solution containing 1% BSA, followed by 20 min incubation at room temperature with primary antibodies against CD31 (1:200 dilution, BD-550274, BD). Labelling was accomplished with HRP secondary antibodies (M2U522H, Biocare, CA, USA) and a DAB kit (ZSGB-BIO, Beijing, China) and then counterstained with haematoxylin for 5 min. The CD31 areas were captured and analysed to evaluate the micro-vessel density in tumor tissue. To observe drug toxicity, vital organs, including the heart, liver, spleen, lung and kidney, were collected and analysed by H&E staining after treatment with WXFL-152. The PerkinElmer Vectra system (PerkinElmer) with Inform 2.2.0 software was used to capture and analyse the image results. IHC staining intensity and quantity were examined by three independent pathologists who did not have authorship in this study.

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Author contributions

Jinliang Yang, Xiaoxin Chen, Yang Zhang, and Yuqin Yao designed this research. Yuqin Yao, Zhuowei Liu, Zhengxia Chen, Peng Li, Yang Zhang, and Chengjian Zhao performed the experiments. Yuqin Yao, Zhuowei Liu, Yang Zhang, Yuxi Wang, and Peng Li collected and interpreted data. Yuqin Yao and Manyu Zhao wrote this paper. Xiaoxin Chen and Jinliang Yang revised the paper. Jinliang Yang, Xiaoxin Chen, and Chaofeng Long were responsible for fund collection.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at <https://doi.org/10.1016/j.apsb.2020.04.002>.

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