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Targeting the ITGAV-VEGFR2 Complex with Polyphyllin B Normalizes Tumor Vessels to Combat $KRAS^{G12D}$-Mutant Pancreatic Cancer

Qiaoyan Wang
Lanzhou University
Zijiao Yang
Lanzhou University
Xinle Yang
Gansu University of Traditional Chinese Medicine
Zeping Zhang
Lanzhou University
Shengfu Che
Lanzhou University
Xueling Zhang
Lanzhou University
Jumei Yang
Lanzhou University Second Hospital
Wengui Shi
ery_danwg@lzu.edu.cn
Lanzhou University Second Hospital
Zuoyi Jiao
Lanzhou University Second Hospital

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Abstract

Antiangiogenic immunotherapies are reshaping the solid tumors treatment, but pancreatic ductal adenocarcinoma (PDAC) remains poorly responsive owing to its unique vascular microenvironment. Here we identify ERK hyperactivation driven by $KRAS^{G12D}$ mutation as a primary determinant of antiangiogenic resistance in PDAC. Analysis of clinical specimens and the KPC ($KRAS^{G12D}$; $Trp53^{R172H}$; Pdx1-Cre) spontaneous PDAC model revealed that tumors with ERK hyperactivation constitute a prominent subgroup that derives little benefit from antiangiogenic treatment. Using an ERK-KTR reporter–based high-throughput screen of 434 natural products, we identified polyphyllin B as an ERK inhibitor capable of sensitizing tumors to the antiangiogenic agent rivoceranib. In co-culture system, polyphyllin B and rivoceranib combination suppresses PDAC cell proliferation and migration, as well as the tube formation and sprouting of human umbilical vein endothelial cells (HUVECs). In KPC mice, the combination therapy inhibited tumor progression by preventing excessive angiogenesis, normalizing tumor vasculature, and reprogramming the immunosuppressive microenvironment (TME). Mechanistically, VEGFR2 forms a complex with integrin alpha V (ITGAV) to sustain ERK activation and drive aberrant angiogenesis, whereas polyphyllin B directly binds ITGAV and disrupts the ITGAV/VEGFR2 complex, thereby sensitizing tumors to antiangiogenic therapy. Translationally, Pluronic F127-based nanodelivery system co-encapsulating polyphyllin B and rivoceranib exhibited robust antitumor efficacy with favorable tolerance in patient-derived xenograft (PDX) model. Collectively, our findings reveal the ITGAV/VEGFR2-ERK axis as a clinically actionable target and establish polyphyllin B as a vascular-normalizing agent for $KRAS^{G12D}$-mutant PDAC treatment.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal solid tumors, with late diagnosis, rapid progression, early metastasis, and limited benefit from current chemotherapy, targeted therapy, and immunotherapy [1]. KRAS mutations are present in the vast majority of PDAC cases, and $KRAS^{G12D}$ is the predominant subtype, accounting for approximately 35–40% of tumors [2]. Despite recent therapeutic advances, overall survival remains poor, underscoring the need for strategies that address not only tumor-intrinsic oncogenic signaling but also the unique tumor microenvironment (TME) of PDAC, which is characterized by aberrant angiogenesis, poor vascular perfusion, persistent hypoxia, and profound immunosuppression, all of which restrict drug delivery and limit immune-cell infiltration [3, 4]. In this context, antiangiogenic therapy has gained renewed attention, not only for its capacity to restrain pathological neovascularization, but also for its potential to normalize tumor vessels, improve perfusion, relieve hypoxia, and enhance the efficacy of immunotherapy. Thus, antiangiogenic-based combination regimens may offer a promising strategy to remodel the immunosuppressive TME of PDAC and improve therapeutic responses.

In recent years, combinations of antiangiogenic agents and immune checkpoint inhibitors have shown promising efficacy as first-line treatment for several solid tumors [5–7]. However, the clinical benefit of this strategy has not been consistent. Among 21 completed phase III trials, 12 (57.1%) failed to

demonstrate a significant overall survival benefit, including the JAVELIN Renal 101, COSMIC-312, LEAP-002, and IMagyn050 trials [8–11]. These findings highlight the need to identify the biological factors that determine the response to antiangiogenic–immunotherapy combinations. VEGF/VEGFR-targeted therapies, particularly VEGFR2 inhibitors, have shown therapeutic activity in hepatocellular carcinoma, renal cell carcinoma, gastric cancer, and non-small cell lung cancer, but their efficacy in PDAC has been disappointing. This limited response may be attributed to the highly heterogeneous and poorly perfused vasculature of PDAC, the dense stromal barrier, and the narrow or transient window of vascular normalization [12, 13]. More importantly, oncogenic KRAS-driven proangiogenic signaling may bypass or attenuate VEGFR2 blockade, making VEGF/VEGFR inhibition alone insufficient to achieve durable vascular remodeling.

In $KRAS^{G12D}$-mutant PDAC, sustained activation of the RAF-MEK-ERK cascade not only supports tumor cell proliferation, survival, and invasion, but also actively reshapes the vascular microenvironment [14, 15]. ERK hyperactivation and hypoxic environment enhance the secretion of proangiogenic mediators, including VEGF, FGF, and PDGF, which act on endothelial cells to increase VEGFR signaling activity and promote endothelial proliferation, migration, tube formation, and sprouting, thereby establishing a tumor cell-endothelial cell crosstalk loop that drives aberrant angiogenesis. Dysfunctional vasculature aggravates hypoxia, restricts drug delivery, and reinforces an immunosuppressive TME [16–18]. Although direct ERK inhibition represents a rational strategy to suppress KRAS-driven signaling, clinically investigated ERK inhibitors, such as ulixertinib, LY3214996, and MK-8353, have shown limited efficacy in solid tumors, with development constrained by adaptive pathway reactivation, insufficient therapeutic durability, and dose-limiting toxicities. Moreover, ERK inhibitors have not been clinically established as vascular-normalizing or antiangiogenic agents in PDAC [19, 20]. These limitations highlight the need for alternative ERK-modulating strategies that can attenuate $KRAS^{G12D}$-driven proangiogenic crosstalk, enhance the efficacy of VEGFR2 blockade, and improve vascular normalization in $KRAS^{G12D}$-mutant PDAC.

Natural products offer a rich source of structurally diverse bioactive compounds with multi-target therapeutic potential. Monomers derived from traditional Chinese medicine (TCM), in particular, have been increasingly recognized for their anti-inflammatory, immunomodulatory, and antiangiogenic activities in cancer treatment [21, 22]. In this study, high-throughput screening using an ERK-KTR reporter identified polyphyllin B as an ERK-modulating natural compound. Polyphyllin B disrupted the VEGFR2-ITGAV complex, inhibited aberrant angiogenesis, promoted vascular normalization, and enhanced T cell infiltration, thereby suppressing PDAC progression. Moreover, an F127-based nanodelivery system co-encapsulating polyphyllin B and rivoceranib achieved robust antitumor efficacy in PDAC PDX models. Together, these findings establish a rational and translatable antiangiogenic combination strategy for $KRAS^{G12D}$-mutant PDAC.

Results

1. KRAS ^{G12D} -mutant-driven ERK hyperactivation promotes aberrant angiogenesis and limits the efficacy of antiangiogenic therapy in PDAC

To investigate the relationship between ERK activation and tumor angiogenesis in KRAS^{G12D}-mutant PDAC, we performed immunofluorescence analysis on clinical PDAC specimens. Compared with KRAS wild-type tumors, KRAS^{G12D}-mutant tumors exhibited a significantly higher density of CD31^{+} vessels, together with markedly increased p-ERK expression (Fig. 1A). The result suggested a potential association between KRAS^{G12D}-ERK signaling and the angiogenic phenotype of PDAC.

To define the role of KRAS^{G12D}-mutant in tumor-endothelial crosstalk within the vascular microenvironment, we established a co-culture system using KRAS^{G12D}-expressing BxPC-3 cells and HUVECs (Fig. 1B). Co-culture markedly increased p-ERK levels in both BxPC-3 cells and HUVECs (Fig. 1C and Figure S1A), indicating reciprocal activation of ERK signaling between tumor and endothelial cells. In parallel, the co-culture condition substantially enhanced the proangiogenic phenotype of HUVECs, as evidenced by increased tube formation, proliferative activity, and VEGF secretion (Fig. 1D-F). To determine whether this proangiogenic crosstalk depended on KRAS^{G12D} activity, we next established a co-culture system using KRAS^{G12D}-mutant PANC-1 cells and HUVECs and treated the cells with the selective KRAS^{G12D} inhibitor MRTX1133 (0.1 μmol/L). Under co-culture conditions, MRTX1133 markedly reduced p-ERK levels in both PANC-1 cells and HUVECs (Fig. 1G and Figure S1B), and suppressed HUVEC tube formation, proliferation, and VEGF secretion (Fig. 1H-J). Together, these findings show that KRAS^{G12D} promotes ERK hyperactivation to drive endothelial activation and aberrant angiogenesis in PDAC.

Rivoceranib is a small-molecule VEGFR2 tyrosine kinase inhibitor with antiangiogenic activity, however, it has not been established as a standard therapy for PDAC. To evaluate its therapeutic efficacy in KRAS^{G12D}-driven PDAC, we employed the KRAS^{G12D}; Trp53^{R172H}; Pdx1-Cre (KPC) genetically engineered mouse model, which spontaneously develops PDAC. Tumors derived from KPC mice were subcutaneously transplanted into C57BL/6 mice. When tumor volumes reached approximately 100 mm^3, mice were treated with rivoceranib (60 mg/kg, every 3 days) for seven doses (Fig. 1K). We found that rivoceranib monotherapy resulted in only modest tumor inhibition (Fig. 1L and M). To assess vascular remodeling, tumor sections were stained for CD31 and α-SMA, markers of endothelial cells and perivascular smooth muscle coverage, respectively. Immunofluorescence analysis revealed that rivoceranib only partially reduced aberrant CD31^{+} vasculature and produced minimal improvement in α-SMA^{+} vessel coverage, indicating limited vascular normalization. Notably, ERK activation remained largely sustained after rivoceranib treatment, with only weak suppression of p-ERK expression (Fig. 1N). Collectively, these findings suggested that persistent ERK activation and incomplete vascular normalization may contribute to the limited efficacy of KRAS^{G12D}-mutant PDAC to antiangiogenic therapy.

1. **Polyphyllin B is identified as a natural ERK inhibitor**

To identify natural compounds capable of suppressing $KRAS^{G12D}$-ERK signaling and enhancing antiangiogenic efficacy, we designed a multi-step screening strategy using a library of 434 traditional Chinese medicine (TCM) monomers (Table S1). The screening workflow integrated endothelial cell viability assays, an ERK kinase translocation reporter (ERK-KTR) system, and HUVEC tube formation assays (Fig. 2A).

In the primary screen, HUVECs were treated with each TCM monomer at 10 μmol/L in combination with rivoceranib at 10 μmol/L for 48 h, followed by cell viability analysis. This screen identified 22 compounds with prominent anti-proliferative activity against HUVECs (Fig. 2B). To further evaluate their effects on ERK signaling, we established an ERK-KTR reporter system. The reporter comprised an ELK1-derived ERK docking domain, a phosphorylation-inhibited nuclear localization signal (NLS), a phosphorylation-enhanced nuclear export signal (NES), and the fluorescent protein mClover. In ERK-inactive cells, the KTR signal is enriched in the nucleus, whereas VEGF-induced ERK activation promotes nuclear export of the reporter (Fig. 2C). Stable HUVECs expressing the ERK-KTR reporter were then generated and validated. VEGF stimulation induced robust cytoplasmic translocation of the KTR reporter, whereas treatment with ERK inhibitor 1 at 3 nmol/L markedly blocked this response, confirming the reliability of the system for monitoring ERK activity in endothelial cells (Fig. 2D). Using this platform, HUVECs were treated with VEGF at 50 ng/mL together with each of the 22 candidate compounds at 10 μmol/L for 30 min. Six compounds significantly suppressed VEGF-induced ERK activation: gossypol, hypericin, 6-methoxydihydroavicine, methylprotodioscin, atractyloside potassium salt, and Polyphyllin B, also known as formosanin C (Fig. 2E). We next performed tube formation assays to compare the antiangiogenic activity of these candidates combined with rivoceranib. Among them, Polyphyllin B exhibited the most potent inhibition of HUVEC tube formation (Fig. 2F). Given its strong suppression of VEGF-induced ERK activation and endothelial angiogenic activity, and because its role in tumor angiogenesis remains poorly defined, Polyphyllin B (abbreviated as PPB throughout the figure) was selected for further investigation (Fig. 2G).

3. Polyphyllin B suppresses ERK activation and promotes the antiangiogenetic effects of VEGFR2 inhibitor rivoceranib

To determine whether Polyphyllin B enhances rivoceranib-mediated inhibition of angiogenesis, HUVECs were treated with Polyphyllin B (1 μmol/L) and/or rivoceranib (10 μmol/L). In stable HUVECs expressing the ERK-KTR reporter, VEGF stimulation (50 ng/mL) induced cytoplasmic translocation of the reporter, reflecting ERK activation. Treatment with either Polyphyllin B or rivoceranib partially inhibited VEGF-induced ERK activation, whereas the combination produced the most pronounced suppression of ERK-KTR cytoplasmic translocation (Fig. 3A). Consistent with this effect on ERK signaling, western blot analysis showed that Polyphyllin B suppressed ERK phosphorylation in a time-dependent manner in both HUVECs and PANC-1 cells (Fig. 3B). Functionally, tube formation assays demonstrated that Polyphyllin B or rivoceranib alone reduced endothelial network formation, while the combination treatment resulted in the strongest inhibition of HUVECs tube formation (Fig. 3C). Wound-healing and migration assays further demonstrated that combined treatment markedly impaired HUVECs migration, with a greater inhibitory

effect than either monotherapy (Fig. 3D and Figure S2A). To further assess angiogenic sprouting, thoracic aortic rings isolated from C57BL/6 mice were treated with Polyphyllin B (1 μmol/L) and/or rivoceranib (10 μmol/L) for 7 days. Polyphyllin B enhanced the antiangiogenic effect of rivoceranib, as evidenced by reduced tip-cell sprouting (Fig. 3E). Similarly, EdU incorporation assays revealed a substantial reduction in proliferating HUVECs following combined Polyphyllin B and rivoceranib treatment (Fig. 3F). Collectively, these findings demonstrate that Polyphyllin B potentiates rivoceranib-mediated suppression of endothelial ERK activation, and significantly inhibits the antiangiogenetic capacity of vascular endothelial cells.

To further model the tumor vascular microenvironment, we established a PANC-1/HUVECs co-culture system and treated the cells with Polyphyllin B (1 μmol/L) and/or rivoceranib (10 μmol/L). Consistent with the results obtained in monocultured endothelial cells, combined treatment markedly suppressed tube formation and the percentage of EdU-positive nuclei in HUVECs under co-culture conditions (Fig. 3G and Figure S2B). Wound-healing assays further showed that the combination treatment impaired HUVEC migration within the co-cultured microenvironment (Fig. 3H). We next examined whether this combination also affected the malignant phenotype of pancreatic cancer cells. In PANC-1 cells, transwell migration and EdU incorporation assays revealed a pronounced decrease in cell migration and proliferation following combined Polyphyllin B and rivoceranib treatment (Fig. 3I and J). To assess ERK activity in tumor cells, stable PANC-1 cells expressing the ERK-KTR reporter were stimulated with VEGF (50 ng/mL). Polyphyllin B or rivoceranib alone partially reduced VEGF-induced ERK-KTR cytoplasmic translocation, whereas the combination produced the strongest inhibition of ERK activation (Figure S2C). Together, these results indicate that Polyphyllin B exhibits potent sensitizing effects on the VEGFR2 inhibitor rivoceranib to inhibit tumor angiogenesis.

4. Polyphyllin B combined with rivoceranib normalizes tumor vessels to inhibit the progression of PDAC

To evaluate the therapeutic potential of Polyphyllin B in antiangiogenic therapy *in vivo*, we established a syngeneic PDAC model by transplanting spontaneous tumors derived from KPC mice into C57BL/6 mice. When tumors reached a volume of 100 mm^3, mice were treated with rivoceranib (60 mg/kg/3 days), Polyphyllin B (1 mg/kg/3 days) or their combination for 21 days (Fig. 4A). We found that both the Polyphyllin B and rivoceranib monotherapy suppressed tumor growth, while the combination therapy showed the most significantly inhibition of tumor growth (Fig. 4B and Figure S3A). We next examined whether the enhanced therapeutic response was associated with suppression of ERK signaling. Immunohistochemical staining showed that combined Polyphyllin B and rivoceranib treatment substantially reduced p-ERK expression in tumor tissues compared to either agent alone (Fig. 4C). We then assessed vascular remodeling by immunofluorescence staining for CD31, an endothelial marker, and α-SMA, a marker of perivascular smooth muscle coverage. Combination treatment significantly reduced the density of aberrant $CD31^+$ tumor vessels and increased α-SMA^+ perivascular coverage, indicating improved vascular normalization (Fig. 4D and E). Consistently, HIF-1α staining revealed that the combination alleviated tumor hypoxia microenvironment, compared to the monotherapy groups

(Figure 4F and G). Achieving vascular normalization is an efficient strategy for reprogramming the immunosuppressive microenvironment. We further analyzed tumor-infiltrating lymphocytes by immunofluorescence staining, while Polyphyllin B or rivoceranib alone exerted only modest effects, their combination significantly increased the infiltration of both $CD8^+$ and $CD4^+$ T cells into tumors (Fig. 4H-K). Immunohistochemistry for Ki67 and H&E staining showed that combined treatment more effectively reduced tumor cell proliferation and disrupted tumor tissue architecture than either monotherapy (Figure S3B). In summary, these findings demonstrated that Polyphyllin B combined with rivoceranib prevents excessive angiogenesis, normalizes tumor vasculature, and reprograms immunosuppressive microenvironment in PDAC.

5. ITGAV is identified as a direct target of Polyphyllin B

To elucidate the molecular target of Polyphyllin B, we integrated transcriptomic profiling, structure-based virtual screening, and biochemical validation. RNA-seq analysis of Polyphyllin B-treated endothelial cells revealed that Polyphyllin B was closely associated with MAPK signaling and angiogenesis-related pathways (Fig. 5A). Transcriptomic heatmap and enrichment analysis further identified 615 Polyphyllin B-responsive genes that were significantly downregulated after treatment (Fig. 5B). Then 456 known ERK-interacting proteins were retrieved from the BioGRID database, and their protein structures were predicted using AlphaFold 3. These proteins were then subjected to virtual docking with Polyphyllin B using AutoDock Vina. This analysis yielded 433 candidate proteins with predicted binding energies below − 7.0 kcal/mol (Table S2). Intersecting these docking candidates with the Polyphyllin B-downregulated transcriptomic dataset identified 15 overlapping candidate targets. Based on the magnitude of transcriptional change and predicted binding affinity, ITGAV was prioritized as a potential target of Polyphyllin B (Fig. 5C). Detailed docking analysis suggested that Polyphyllin B fits within a putative binding pocket of ITGAV and forms hydrogen-bonding and hydrophobic interactions with key residues, including SER130, ARG129, and ASP314 (Fig. 5D).

To experimentally validate this predicted interaction, we performed drug affinity responsive target stability (DARTS) assays. Polyphyllin B protected ITGAV from pronase-mediated proteolytic degradation, indicating ligand-dependent stabilization of ITGAV (Fig. 5E). Consistently, cellular thermal shift assay (CETSA) analysis showed that Polyphyllin B markedly enhanced the thermal stability of ITGAV in cell lysates (Fig. 5F). To further assess binding-site specificity, we generated an ITGAV triple mutant (S130A/R129A/D314A) within the predicted Polyphyllin B-binding region. Mutation of the predicted contact residues abolished Polyphyllin B-mediated protection in DARTS assays and thermal stabilization in CETSA assays (Fig. 5E and F). These results support ITGAV as a candidate molecular target directly engaged by Polyphyllin B.

6. Polyphyllin B disrupts the ITGAV–VEGFR2 complex to suppress ERK activation

To define the functional role of ITGAV in Polyphyllin B-mediated regulation of MAPK and angiogenesis-related signaling, we first established ITGAV-knockdown cells using shRNA (Figure S4A). Western blot analysis showed that ITGAV depletion reduced the phosphorylation of VEGFR2 and ERK, indicating that

ITGAV contributes to VEGFR2–ERK pathway activation (Figure 6A). Co-immunoprecipitation further confirmed an interaction between ITGAV and VEGFR2 (Fig. 6B). Consistently, proximity ligation assay (PLA) revealed close association between ITGAV and VEGFR2 in both HUVECs and PANC-1 cells. Notably, Polyphyllin B disrupted this interaction in a dose-dependent manner, supporting its ability to interfere with the ITGAV–VEGFR2 complex Figure 6C and Figure S4B.

We next examined whether genetic inhibition of ITGAV could influence the sensitizing effect of Polyphyllin B on rivoceranib. In HUVECs, shRNA-mediated ITGAV knockdown markedly enhanced the inhibitory effects of rivoceranib on tube formation, EdU incorporation, and wound-healing, indicating stronger suppression of endothelial network formation, proliferation, and migration (Fig. 6D-F). Similar effects were observed in PANC-1 cells, where ITGAV knockdown further increased rivoceranib-mediated inhibition of migration and proliferation, as assessed by EdU incorporation assays, transwell migration, and wound-healing assays (Figure S4C-E). To genetically verify ITGAV as the functional target of Polyphyllin B, we performed rescue experiments in ITGAV-knockdown HUVECs and PANC-1 cells. Wild-type ITGAV (Re-ITGAV) or a binding-site triple mutant (Re-ITGAV$^{S130A/R129A/D314A}$) was reconstituted in ITGAV- knockdown cells. In HUVECs expressing wild-type ITGAV, Polyphyllin B significantly enhanced the antiangiogenic activity of rivoceranib, as shown by reduced tube formation, EdU incorporation, and wound-healing. In contrast, this sensitizing effect was largely abolished in cells expressing mutant ITGAV (Fig. 6G-I). Consistently, in PANC-1 cells, combined Polyphyllin B and rivoceranib treatment markedly suppressed migration and proliferation in Re-ITGAV cells, but not in cells expressing mutant ITGAV (Figure S4F-H). Together, these results demonstrate that Polyphyllin B directly engages ITGAV and disrupts the ITGAV–VEGFR2 complex, thereby attenuating VEGFR2–ERK signaling and enhancing the antiangiogenic and antitumor efficacy of rivoceranib.

7. F127-based Polyphyllin B -Rivoceranib nanoparticle functions as potent antiangiogenic and antitumor agent

To assess the translational potential of Polyphyllin B and rivoceranib combination therapy, we first evaluated its systemic tolerability in tumor-bearing mice. Body weight, organ coefficients, and the histopathology of the heart, liver, spleen, lungs, and kidneys were examined after treatment. At the effective doses, the combination did not cause significant body-weight loss or measurable changes in organ coefficients (Figure S5A and S5B). Histological analysis showed no evident pathological alterations in the heart, spleen, lungs, or kidneys. However, inflammatory and fibrotic changes were observed in the liver of mice receiving the combination treatment, but not in the monotherapy or control groups (Figure S5C). These findings suggested that combination-associated hepatotoxicity could limit the translational potential of this regimen.

To improve the therapeutic feasibility of the combination, we developed an F127-based nanodelivery system co-encapsulating Polyphyllin B and rivoceranib (FRPn) through hydrogen-bonding and hydrophobic interactions (Fig. 7A). Transmission electron microscopy (TEM) revealed that FRPn formed relatively uniform spherical nanoparticles with an average diameter of 75.7 ± 3.0 nm Figure 7B.

Dynamic light scattering (DLS) showed a larger hydrodynamic diameter of 358.7 nm (Fig. 7C), reflecting the hydrated state of the particles in aqueous suspension. The zeta potential of rivoceranib was − 15.68 mV, whereas that of FRPn was − 16.55 mV after incorporation of Polyphyllin B and F127 Figure 7D. UV–visible spectroscopy showed that FRPn retained the characteristic absorption profiles of both Polyphyllin B and rivoceranib, confirming the successful incorporation of the two compounds. The drug-loading content and encapsulation efficiency were 22.9% and 55.5% for Polyphyllin B, and 12.3% and 29.7% for rivoceranib, respectively (Fig. 7E). We next characterized the release behavior of FRPn under physiological and tumor-relevant acidic conditions. FRPn exhibited pH-dependent drug release, with substantially faster release at pH 5.0 than at pH 7.4 (Fig. 7F). This acid-responsive release profile may facilitate drug liberation in the acidic tumor microenvironment.

We then evaluated the antiangiogenic activity of FRPn in the PANC-1/HUVECs co-culture system. FRPn inhibited HUVECs tube formation in a concentration-dependent manner (Fig. 7G). At a concentration of 1 μmol/L, FRPn also markedly impaired HUVECs proliferation in EdU incorporation assays and reduced HUVECs migration, as determined by wound-healing assays (Figure 7H and I). In PANC-1 cells, FRPn significantly suppressed migration and proliferation in transwell, wound-healing, and EdU incorporation assays (Fig. 7J-L). Collectively, these findings demonstrate that the F127-based FRPn nanodelivery system retains potent activity against both endothelial and tumor cells and may improve the therapeutic feasibility of Polyphyllin B–rivoceranib combination therapy for PDAC.

8. F127-based Polyphyllin B-Rivoceranib (FRPn) inhibits the progression of KRAS ^{G12D} PDAC in PDX models

To assess the translational potential of F127-based Polyphyllin B-Rivoceranib nanoparticles *in vivo*, we established a patient-derived xenograft (PDX) model using KRAS^{G12D}-mutant PDAC tissues obtained from a patient. When tumor volumes reached approximately 100 mm^3, mice were randomized and treated with FRPn for 21 days (Fig. 8A). Tumor volume measurements showed that FRPn markedly suppressed tumor progression compared with the other treatment groups (Fig. 8B and Figure S6A), indicating enhanced antitumor efficacy of the nanoparticle formulation in a clinically relevant PDAC model. Immunofluorescence analysis of PDX tumor tissues further supported the therapeutic activity of FRPn. Compared with the control and free-drug combination groups, FRPn treatment reduced CD31$^+$ vascular structures, while increasing α-SMA coverage around tumor vessels (Figure 8C). These findings suggest that FRPn not only inhibits abnormal angiogenesis but also promotes features of vascular normalization within the tumor microenvironment.

We also conducted toxicological evaluations in mice treated with FRPn. Serum biochemical analysis showed no overt hepatotoxicity or nephrotoxicity, as reflected by liver function markers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), alkaline phosphatase (ALP), and gamma-glutamyl transferase (gamma-GT), as well as renal function markers, including creatinine (CREA), blood urea nitrogen (BUN), and uric acid (UA) (Fig. 8D). Consistently, histological examination of major organs, including the heart, liver, spleen, lung, and kidney, revealed no apparent pathological

abnormalities in mice treated with either the free Polyphyllin B-rivoceranib combination or FRPn (Fig. 8E). In addition, body weight and organ indices remained largely unchanged across treatment groups (Figure S6B and S6C), supporting the favorable tolerability of FRPn in vivo. As summarized in Fig. 8F, Polyphyllin B targets the ITGAV-VEGFR2 axis to suppress $KRAS^{G12D}$-ERK-driven proangiogenic signaling, thereby enhancing rivoceranib-mediated antiangiogenic therapy, promoting vascular normalization, and improving the immunotherapeutic response in $KRAS^{G12D}$-mutant pancreatic cancer. Together, these findings show that FRPn improves the efficacy and tolerability of Polyphyllin B-rivoceranib therapy in $KRAS^{G12D}$-mutant PDAC PDX models and support its translational potential for PDAC treatment.

Discussion

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal solid malignancies, with limited responses to conventional chemotherapy, targeted therapy, and immunotherapy [1, 23]. The poor therapeutic outcome of PDAC is driven not only by the high prevalence of oncogenic KRAS mutations, particularly $KRAS^{G12D}$, but also by extensive stromal desmoplasia, vascular insufficiency, and an immunosuppressive TME. The vascular microenvironment is a central yet complex determinant of PDAC progression. Unlike many highly vascularized solid tumors, PDAC frequently displays a hypovascular phenotype, with compressed, poorly perfused, and structurally abnormal vessels embedded within a dense extracellular matrix. These tortuous and dysfunctional vessels impair drug delivery and immune cell infiltration while promoting hypoxia, stromal activation, and metastatic dissemination [24–26]. Therefore, angiogenic signaling remains functionally important in PDAC despite its relatively low vascular density.

Antiangiogenic agents, particularly VEGFR2 inhibitors, can induce a transient state of vascular normalization, thereby improving perfusion, drug delivery, and antitumor immune activity [27]. However, the therapeutic benefit of antiangiogenic therapy in PDAC has been limited, partly because the vascular normalization window is short-lived and difficult to sustain. Extending or reinforcing this window through rational combination therapy may therefore be essential for sensitizing PDAC to antiangiogenic treatment [28]. Although blockade of the VEGF-VEGFR pathway has provided an important therapeutic paradigm in oncology, its clinical translation in PDAC has remained challenging. A key limitation is that antiangiogenic monotherapy may be insufficient in tumors driven by strong oncogenic and stromal compensatory programs [29].

ERK is a central effector of oncogenic KRAS signaling and provides a mechanistic link between tumor-cell-intrinsic signaling and angiogenic remodeling. VEGFR2 is the major receptor mediating VEGF-driven endothelial proliferation, migration, and survival, and its downstream signaling converges on MAPK-ERK and PI3K-AKT pathways [30, 31]. In PDAC, oncogenic KRAS can reinforce proangiogenic programs through ERK-dependent transcriptional regulation, thereby coupling malignant cell signaling to endothelial activation and stromal remodeling [32]. Our data support the functional relevance of this ERK-VEGFR2 regulatory axis, as suppression of ERK-associated signaling attenuated endothelial

activation, angiogenic phenotypes, and tumor progression. In this study, we found that $KRAS^{G12D}$ PDAC exhibits severe vascular abnormalities and identified Polyphyllin B as an ERK signaling inhibitor capable of sensitizing PDAC to rivoceranib-based antiangiogenic therapy.

Despite the biological relevance of ERK inhibition, the clinical development of ERK inhibitors in PDAC has been constrained by modest efficacy and dose-limiting toxicity. Moreover, ERK inhibition has not been fully established as a practical antiangiogenic strategy in PDAC [33]. These limitations prompted us to search for alternative ERK-modulating agents that could suppress KRAS-driven proangiogenic signaling while improving the therapeutic window of VEGFR2 inhibition. Natural products, particularly monomers derived from traditional Chinese medicine (TCM), represent a structurally diverse source of bioactive compounds with potential antitumor, anti-inflammatory, immunomodulatory, and antiangiogenic activities [21, 34]. Using an ERK-KTR reporter-based screening platform, we identified Polyphyllin B from a library of 434 TCM monomers as a potent suppressor of ERK signaling.

Polyphyllin B is a steroidal saponin and a major bioactive constituent of Paris polyphylla. Although its antitumor activity has been reported in several cancers, including pancreatic, breast, and bladder cancers [35], our data extend its functional scope by identifying Polyphyllin B as an angiogenesis-modulating agent in PDAC. Mechanistically, DARTS and CETSA confirmed direct binding to ITGAV. Given the established roles of integrin αV-containing receptors in tumor adhesion, matrix remodeling, endothelial communication, and stromal signaling, this interaction is highly relevant to the PDAC microenvironment [36, 37]. Co-immunoprecipitation and proximity ligation assays further showed that ITGAV associates with VEGFR2, and Polyphyllin B disrupted this complex in a dose-dependent manner. These findings support a model in which Polyphyllin B interferes with ITGAV-VEGFR2 coupling and suppresses downstream ERK-associated angiogenic signaling. Functionally, Polyphyllin B inhibited endothelial proliferation, migration, and sprouting, indicating that its benefit lies not only in direct tumor cell effects but also in sensitizing the vascular compartment to antiangiogenic therapy. In KPC mice, the combination of Polyphyllin B and rivoceranib suppressed aberrant angiogenesis, promoted vascular normalization, and alleviated the immunosuppressive TME through inhibition of ERK activation. Together, these results suggest that Polyphyllin B enhances rivoceranib efficacy by targeting a complementary ITGAV-VEGFR2-ERK axis. Importantly, free-drug combination treatment was accompanied by liver inflammation and fibrotic injury, underscoring the need for a delivery system that maintains antitumor activity while reducing off-target toxicity.

Nanodrug delivery systems have attracted increasing interest for improving the translational potential of natural products and combination therapies, particularly by enhancing solubility, stability, tumor accumulation, and controlled drug release [38]. Pluronic F127, an amphiphilic copolymer composed of hydrophilic polyethylene oxide and hydrophobic polypropylene oxide blocks, is widely used as a pharmaceutical excipient [39]. To improve the therapeutic index of Polyphyllin B and rivoceranib, we developed an F127-based nanodelivery system, FRPn, for co-encapsulation and coordinated delivery of both agents. TEM, DLS, and UV-vis spectrophotometry confirmed successful nanoparticle formation, drug loading, and pH-responsive release under acidic conditions mimicking the tumor

microenvironment. This formulation provided a practical strategy to enhance local drug availability while limiting systemic exposure.

The translational relevance of FRPn was further supported in $KRAS^{G12D}$ PDAC patient-derived xenograft models. FRPn significantly suppressed tumor progression compared with other treatment groups and reduced ITGAV expression and abnormal $CD31^{+}$ vascular structures while increasing α-SMA coverage. These findings indicate that FRPn retains the mechanistic activity of the Polyphyllin B-rivoceranib combination in a clinically relevant model and promotes vascular normalization in vivo. Importantly, toxicological evaluation showed that FRPn was well tolerated at efficacious doses, with no obvious hepatotoxicity or nephrotoxicity based on serum biochemical analysis and histological examination of major organs. Body weight and organ indices were also largely unchanged. These results suggest that F127-based co-delivery can improve the therapeutic window of Polyphyllin B-rivoceranib therapy.

Several limitations should be considered. First, although our data support the involvement of the ITGAV-VEGFR2-ERK axis, additional target-specific perturbation experiments would further clarify the causal contribution of this pathway to vascular normalization and tumor suppression. Second, while the $KRAS^{G12D}$ PDX model provides clinically relevant validation, broader evaluation across molecularly and stromally diverse PDAC models will be necessary to define predictive biomarkers of response. Third, the long-term pharmacokinetics, biodistribution, and safety profile of FRPn require further investigation before clinical translation.

In summary, this study identifies $KRAS^{G12D}$-mutant PDAC as a context in which antiangiogenic therapy may be potentiated through ERK pathway modulation. We demonstrate that Polyphyllin B directly targets ITGAV, disrupts the ITGAV-VEGFR2 interaction, and suppresses ERK-VEGFR2-associated angiogenic signaling. Utilizing both $KRAS^{G12D}$-mutant spontaneous PDAC mouse models and patient-derived xenograft (PDX) models, we showed that the combination of Polyphyllin B with rivoceranib, particularly when administered through an F127-based nanoparticle system, significantly inhibits tumor growth by preventing angiogenesis and promoting the normalization of tumor vasculature. Collectively, this study offers a translational strategy for identifying ERK inhibitors from traditional Chinese medicine (TCM) monomers, thereby contributing to the development of new approaches for antiangiogenic therapy in $KRAS^{G12D}$-mutant PDAC.

Materials and Methods

Animal Experiments

LSL-KrasG12D/+ mice, LSL-Trp53R172H/+ mice, and Pdx1-Cre mice were purchased from Model Organisms Center Inc. (Shanghai, China). LSL-KrasG12D/+ and LSL-Trp53R172H/+ mice were interbred with Pdx1-Cre mice to generate KrasG12D/+; LSL-Trp53R172H/+; Pdx1-Cre (KPC) mice. All mice were maintained on a C57BL/6 background and genotyped by PCR using the primers listed in Table S3. For the syngeneic transplanted tumor model, spontaneous pancreatic tumors derived from KPC mice were

excised, minced into small fragments, and subcutaneously implanted into 7-week-old female C57BL/6 mice. When tumor volumes reached approximately 100 mm^3, mice were randomly assigned to four groups receiving different route of administration: normal saline (I.P.), rivoceranib (P.O.,60 mg/kg/3 days, Selleckchem, #S5248), Polyphyllin B (I.P., 1 mg/kg/3 days, Selleckchem, #S3279), or a combination of the agents. Tumor volumes and body weights were monitored every 3 days throughout the treatment period. For the patient-derived xenograft (PDX) model, fresh tumor tissues from patients with $KRAS^{G12D}$-mutant PDAC were minced into small fragments and subcutaneously implanted into 7-week-old female NOD/SCID gamma mice (NSG; RRID: IMSR_NM-NSG-001; Model Organisms Center Inc.). When tumor volumes reached approximately 100 mm^3, mice were randomly divided into three groups receiving different route of administration: normal saline, Polyphyllin B (I.P.,1 mg/kg/3 days) plus rivoceranib (P.O., 60mg/kg/3 days), or FRPn (I.P., 1 mg/kg/3 days). Tumor growth and body weight were monitored during treatment. At the experimental endpoint, mice were euthanized, and tumors and major organs, including the heart, liver, spleen, lung, and kidneys, were collected for histological and immunohistochemical analyses. H&E staining was performed to evaluate tissue morphology and potential treatment-related toxicity. Whole-slide images were acquired using a TISSUEFAXS PLUS imaging system (TissueGnostics). Serum biochemical analyses of liver and kidney function were performed for toxicological evaluation. All animal procedures were approved by the Institutional Animal Care and Use Committee of The Second Hospital & Clinical Medical School, Lanzhou University and performed in accordance with institutional guidelines.

Cell culture

PUMC-HUVEC-T1 cells were purchased from Shanghai Fuheng Biotechnology Co., Ltd. HEK-293T cells and the human pancreatic cancer cell lines PANC-1 and BxPC-3 were obtained from the Chinese Academy of Sciences. PUMC-HUVEC-T1 cells were cultured in Endothelial Cell Medium (#1001, ScienCell) supplemented with 5% fetal bovine serum (FBS; #0025, ScienCell), 1% endothelial cell growth supplement (ECGS; #1052, ScienCell), and 1% penicillin/streptomycin/gentamicin (#P1410, Solarbio, China). PANC-1 and BxPC-3 cells were maintained in RPMI-1640 medium (#11875093, Gibco), whereas HEK-293T cells were cultured in DMEM (#11960044, Gibco), both supplemented with 10% FBS and 1% penicillin/streptomycin/gentamicin (#P1410, Solarbio, China). All cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Cell lines were routinely tested for mycoplasma contamination and authenticated by short tandem repeat DNA profiling.

ERK–KTRs analysis

The ERK kinase translocation reporter (ERK-KTR) was designed as previously described [40]. The ERK-nKTR plasmid pCC324 (#110165, Addgene; RRID: Addgene_110165) contains an ERK docking sequence derived from the ERK substrate Elk1, a phosphorylation-inhibited nuclear localization signal, a phosphorylation-enhanced nuclear export signal, and the fluorescent protein mClover. A lentiviral vector was constructed to coexpress the KTR-mClover biosensor and the nuclear marker mCherry-H2B from a

single transcript via a 2A peptide sequence. Lentiviruses were produced in HEK-293T cells and used to infect PUMC-HUVEC-T1 cells in medium containing 8 μg/mL polybrene. Infected cells were selected with 2 μg/mL puromycin to establish stable ERK-KTR reporter cells. For live-cell imaging, stable reporter cells were seeded in glass-bottom FluoroDishes and serum-starved for 24 h before treatment. Cells were treated with VEGF and/or rivoceranib as indicated and imaged using a Zeiss LSM 880 time-lapse confocal microscope under humidified conditions at 37°C and 5% CO_2. ERK activity was quantified as the nuclear mCherry/mClover fluorescence intensity ratio using ZEN Blue software.

High-Throughput Screening of TCM Monomers

A multi-step screening strategy integrating cell viability assays, ERK-KTR reporter analysis, and tube formation assays was used to identify traditional Chinese medicine (TCM) monomers with antiangiogenic activity through suppression of ERK activation. For the primary viability screen, PUMC-HUVEC-T1 cells were seeded in 96-well plates at 2 x 10^3 cells per well. After 24 h, cells were treated with 434 TCM monomeric compounds from a commercial compound library (Selleck, #L8300) or rivoceranib at 10 μmol/L for 48 h. Cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8; AbMole, #M4839) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader. The top 22 candidate compounds from the primary screen were further evaluated using the ERK-KTR reporter system. Stable ERK-KTR reporter PUMC-HUVEC-T1 cells were seeded in 24-well plates at 2 x 10^4cells per well. Cells were treated with each candidate compound at 10 μmol/L for 4 h, followed by stimulation with VEGF at 50 ng/ml for 15 min. Fluorescence images were acquired using a Zeiss LSM 880 confocal microscope, and ERK activity was quantified based on reporter translocation. Finally, six leading candidates were assessed using tube formation assays. PUMC-HUVEC-T1 cells were seeded into Matrigel-precoated 24-well plates (#96992, Corning) at 4 x 10^5 cells per well and treated with candidate compounds at 10 μmol/L for 4 h. Cells were then stained with 0.05% crystal violet and imaged under a microscope. Angiogenic segments were quantified using the Angiogenesis Analyzer plugin in ImageJ.

Coimmunoprecipitation assay

Cells were transfected with the indicated plasmids and lysed on ice in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.2% Triton X-100, supplemented with phosphatase inhibitor (#P1081, Beyotime) and protease inhibitor cocktail (#ST506-2, Beyotime). The lysates were centrifuged at 13 800 x g for 10 min at 4°C, and the resulting supernatants were incubated with prewashed anti-Flag magnetic beads (#HY-K0207, MedChemExpress) for 2 h at 4°C under gentle rotation. The beads were washed three times with lysis buffer and resuspended in SDS loading buffer. Bound proteins were eluted by heat denaturation in a metal bath and analyzed by immunoblotting with the indicated antibodies.

Proximity ligation (PLA) assay

HUVECs and PANC-1 cells were fixed in 4% paraformaldehyde, and proximity ligation assays were performed using the Duolink In Situ Detection Reagents Red kit (#DUO92008, Merck) following the manufacturer's protocol. After blocking with Duolink Blocking Solution for 1 h at 37°C, cells were incubated overnight at 4°C with primary antibodies against VEGFR2 (#9698, Cell Signaling Technology) and ITGAV (#ZMS1030, Sigma-Aldrich). Cells were then incubated with Duolink In Situ PLA Probe Anti-Rabbit PLUS and Anti-Mouse MINUS for 1 h at 37°C, followed by ligation and amplification for 100 min at 37°C. Fluorescence images were captured using a Zeiss LSM 880 confocal microscope, and PLA-positive puncta were quantified manually.

Tube formation

For the tube formation assay, HUVECs were seeded in 24-well plates precoated with Matrigel (#96992, Corning) at a density of 4 x 10^5 cells per well. Cells were treated with rivoceranib, Polyphyllin B, or their combination and incubated at 37°C for 4 h. After treatment, cells were stained with 0.05% crystal violet and imaged under a microscope. Tube formation was quantified using the Angiogenesis Analyzer plugin in ImageJ software.

Aortic ring assays

Thoracic aortas were isolated from mice, cleaned of surrounding connective tissue, and briefly treated with collagenase I (#SCR103, Sigma-Aldrich). Under a stereomicroscope, the adventitia was carefully removed, and the aortas were cut into 0.5-2 mm rings. The aortic rings were embedded in Matrigel and cultured in Endothelial Cell Medium (#1001, ScienCell). Rings were treated with rivoceranib, Polyphyllin B, or their combination for 9 days. At the endpoint, the rings were permeabilized and stained with phalloidin-iFluor 594 (#ab176757, Abcam). Images were acquired using a microscope system, and microvessel sprouting was quantified using ImageJ software.

Transwell Invasion and Wound-Healing Assays

For the transwell invasion assay, HUVECs or PANC-1 cells were seeded into Matrigel-coated upper chambers at a density of 1 x 10^5 cells per chamber in serum-free medium. The lower chambers were filled with medium containing 10% FBS as a chemoattractant. After 24 h of incubation, invaded cells on the lower surface of the membrane were fixed, stained, imaged, and quantified. For the wound-healing assay, cells were seeded in 6-well plates at a density of 1 x 10^6 cells per well and grown to confluence. Linear scratches were generated using a sterile pipette tip, and detached cells were removed by washing. Cells were then cultured in serum-free medium containing the indicated treatments. Wound closure was imaged at 0 and 24 h and quantified using ImageJ software (RRID: SCR_003070).

EdU Staining Assay

HUVECs or PANC-1 cells were seeded in glass-bottom FluoroDishes and treated with rivoceranib, Polyphyllin B, or their combination as indicated. Cells were then incubated with EdU for 2 h, fixed, permeabilized, and stained using a BeyoClick EdU assay kit (#C0078S, Beyotime) according to the

manufacturer's instructions. Fluorescence images were acquired using a Zeiss LSM 880 confocal microscope.

Western blotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. After centrifugation at 13 800 x g for 10 min at 4°C, the clarified supernatants were collected for protein analysis. Equal amounts of protein were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk and incubated with the indicated primary antibodies overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were detected using an enhanced chemiluminescence system. Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. The following primary antibodies were used for western blotting: anti-GAPDH (1:1000, #60004-1-Ig, Proteintech), anti-phospho-ERK1/2 (1:500, #4370, Cell Signaling Technology), anti-ERK1/2 (1:1000, #ab184699, Abcam), anti-Flag (1:1000, #F1804, Sigma-Aldrich), anti-HA (1:1000, #26183, Invitrogen), anti-VEGFR2 (1:1000, #9698, Cell Signaling Technology), anti-phospho-VEGFR2 (1:500, #2478, Cell Signaling Technology), and anti-ITGAV (1:1000, #ZMS1030, Sigma-Aldrich).

Immunofluorescence staining

Cells or tissue sections were fixed, permeabilized, and blocked before incubation with the indicated primary antibodies overnight at 4°C. After washing, samples were incubated with fluorophore-conjugated secondary antibodies for 1 h at 37°C. Nuclei were counterstained with DAPI where indicated. Confocal fluorescence images were acquired using a Zeiss LSM 880 laser scanning microscope. The following primary antibodies were used: anti-CD31 (1:200, #ab28364, Abcam), Cy3-conjugated anti-alpha-SMA (1:150, #C6198, Sigma-Aldrich), anti-HIF-1alpha (1:300, #ab51608, Abcam), Alexa Fluor 647 anti-mouse CD4 (#100530, BioLegend), Alexa Fluor 647 anti-mouse CD8 (#100724, BioLegend), anti-phospho-ERK1/2 (1:500, #4370, Cell Signaling Technology), and anti-ITGAV (#ZMS1030, Sigma-Aldrich). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG (#ab150077, Abcam), Alexa Fluor 488 goat anti-mouse IgG (#ab150113, Abcam), Alexa Fluor 594 goat anti-rabbit IgG (#ab150080, Abcam), and Alexa Fluor 594 goat anti-mouse IgG (#ab150116, Abcam). For quantitative analysis, vascular smooth muscle coverage was calculated as the proportion of CD31-positive pixels colocalized with or adjacent to alpha-SMA-positive signals relative to total CD31-positive pixels. Tumor hypoxia was quantified as the ratio of HIF-1alpha-positive pixels to DAPI-positive pixels. Tumor-infiltrating T cells were quantified as the proportions of CD4-positive or CD8-positive cells. The expression levels of phospho-ERK1/2 and ITGAV in tumor tissues were quantified as the ratios of phospho-ERK1/2-positive or ITGAV-positive pixels to DAPI-positive pixels.

Lentiviral Production and Infection

The plasmids pLV3-U6-ITGAV-shRNA-CopGFP-Puro, pLV3-U6-ITGAV-FLAG-CopGFP, pCMV-EGFP-ITGAV-mutant-3HA-CopGFP, and pLV3-CMV-KDR-FLAG-EF1a-CopGFP were designed and obtained from MiaoLingPlasmid (Wuhan, China). The ERK-KTR plasmid was purchased from GeneChem (Shanghai,

China). For lentivirus production, the indicated transfer plasmids were co-transfected with the packaging plasmids psPAX2 and pMD2.G into HEK293T cells using PEI 40K transfection reagent (#G1802, Servicebio). Viral supernatants were collected, concentrated with PEG8000, and used to infect HUVECs or PANC-1 cells in the presence of polybrene. Stable cells were selected with puromycin at 2 μmol/L, and infection efficiency was confirmed by fluorescence microscopy.

Transcriptomic profiling and Target Screening

HUVECs were treated with Polyphyllin B (2 μmol/L) for 48 h and then harvested for total RNA extraction. RNA quality was assessed before library construction and sequencing. Raw FASTQ reads were processed to remove adaptor sequences and low-quality reads, and the resulting clean reads were used for downstream transcriptomic analysis. Differentially expressed genes were identified using the DESeq2 R package (RRID: SCR_015687). Genes with |log2 fold change| > 0.58 and an adjusted P value < 0.05 were considered differentially expressed. ERK-interacting proteins were retrieved from the BioGRID database, and their protein structures were predicted using AlphaFold 3. The predicted structures were subsequently subjected to molecular docking with Polyphyllin B using AutoDock Vina. Proteins with predicted binding energies below − 7.0 kcal/mol were retained, yielding 433 putative Polyphyllin B-binding ERK-associated proteins. Candidate targets were further identified by intersecting these docked ERK-associated proteins with genes downregulated after Polyphyllin B treatment.

Drug Affinity Responsive Target Stability (DARTS) Assay and Cellular Thermal Shift Assay (CETSA)

For the drug affinity responsive target stability (DARTS) assay, cells were lysed in RIPA buffer, and lysates were centrifuged at 13 800 x g for 10 min at 4°C. The supernatants were collected and incubated with Polyphyllin B 50 μmol/L or DMSO for 1 h at 37°C. Samples were then digested with pronase (#HY-114158, MedChemExpress) at the indicated concentrations for 15 min at 37°C. The digestion reaction was terminated by adding SDS loading buffer followed by boiling. Protein stability was analyzed by western blotting.

For the cellular thermal shift assay (CETSA), cell lysates were prepared and incubated with Polyphyllin B or DMSO as described for the DARTS assay. The lysates were then aliquoted and heated at the indicated temperatures ranging from 40 to 76°C for 5 min, followed by cooling to room temperature. SDS loading buffer was added, and samples were analyzed by western blotting to evaluate the thermal stability of the target proteins.

Preparation and Characterization of F127-Based Polyphyllin B–Rivoceranib Nanoparticles

F127-based Polyphyllin B-rivoceranib nanoparticles were prepared by a self-assembly method. Polyphyllin B and rivoceranib were separately dissolved in DMSO (#S3787, Selleck) at 10 mg/mL and then mixed with Pluronic F127 solution (#P2243, Sigma-Aldrich; 10 mg/mL) at the indicated ratio. The mixture was stirred at room temperature for 24 h, followed by dialysis against deionized water using

dialysis tubing (MW = 1000) for 48 h to remove free drugs and organic solvent. The resulting nanoparticle suspension was lyophilized for further use. The morphology of F127-Polyphyllin B-rivoceranib nanoparticles was examined by transmission electron microscopy (TEM; Hitachi, Japan). Hydrodynamic size distribution and zeta potential were measured using a 90Plus PALS dynamic light scattering system (Brookhaven Instruments, USA). Drug-loading content and encapsulation efficiency were determined by UV-vis spectrophotometry (Shimadzu, Japan). For in vitro drug release analysis, equal amounts of F127-Polyphyllin B-rivoceranib nanoparticles were placed in dialysis bags (MW = 1000) and immersed in PBS at pH 5.0 or pH 7.4. The release study was performed at 37°C with gentle shaking, and an equal volume of fresh PBS was replenished after each sampling. At predetermined time points, aliquots of the release medium were collected, and the concentrations of released Polyphyllin B and rivoceranib were quantified by UV-vis spectrophotometry.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 8.0. Data are presented as the mean ± SEM. Comparisons between two groups were assessed using a two-tailed, unpaired Student's t-test. For comparisons among more than two groups, one-way analysis of variance (ANOVA) was performed when the data met the assumptions of normality and homogeneity of variance. Tukey's multiple-comparisons test was then used for all pairwise comparisons. When each treatment group was compared with a single control group, Dunnett's test was used instead. Homogeneity of variance was assessed using Levene's test. When this assumption was not met, Welch's ANOVA followed by Dunnett's T3 test was performed. Statistical significance was defined as ns for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$. Unless otherwise stated, animal experiments included at least six mice per group, and cell-based experiments were performed with at least three independent biological replicates.

Declarations

Ethics approval and consent to participate

All animal procedures were conducted in accordance with institutional guidelines for animal welfare and were approved by the Animal Ethics Committee of Lanzhou University Second Hospital (D2026-57) and the Medical Ethics Committee of The Second Hospital of Lanzhou University (2024A-628).

Consent for publication

Not applicable. All patient data were anonymized and no individually identifiable information is included in this manuscript.

Competing interests

The authors declare no competing interests.

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Author Contribution

QYW, ZJY, and XLY designed, performed, and analyzed the experiments and prepared the figures and QYW wrote the manuscript. ZPZ, SFC and XLZ provided technical and clinical assistance and carried out all immunoassays. JMY, WGS and ZYJ participated in study design and coordination and helped to revise the manuscript. All authors read and approved the final manuscript.

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Data availability

Not applicable. This manuscript does not report data generation or analysis.

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