



Anti-tumor activity of cediranib, a pan-vascular endothelial growth factor receptor inhibitor, in pancreatic ductal adenocarcinoma cells

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Abstract

Purpose Pancreatic ductal adenocarcinoma (PDAC) is the most common and lethal subtype of pancreatic cancer, with a 5-year survival rate of < 3%. Early tumor dissemination, late diagnosis and insensitivity to conventional treatment are the major reasons for its high mortality rate. Members of the vascular endothelial growth factor (VEGF) family are overexpressed in PDAC and play important roles in its malignant progression, suggesting that VEGF-targeted therapies may interrupt the proliferation and motility of PDAC cells. Here, we evaluated the anti-tumor activity of cediranib, a pan-VEGF receptor inhibitor, on PDAC cells.

Methods Anti-proliferative effects of cediranib were determined using cell proliferation and crystal violet staining assays. Annexin V/PI staining, radiation therapy, and cell migration and invasion assays were carried out to examine the effects of cediranib on apoptosis, radio-sensitivity and cell motility, respectively. Quantitative reverse transcription-PCR (qRT-PCR) and Western blot analyses were applied to elucidate the molecular mechanisms underlying the anti-tumor activity of cediranib.

Results We found that cediranib decreased PDAC cell proliferation and clonogenic survival and induced apoptotic cell death through inhibition of the anti-apoptotic proteins cIAP1, XIAP, MCL-1 and survivin. Combination with cediranib synergistically increased the sensitivity of PDAC cells to chemotherapeutic agents such as gemcitabine and paclitaxel, and potentiated the effects of radiation therapy on PDAC cell growth inhibition and apoptosis induction. Furthermore, we found that treatment with cediranib impaired PDAC cell migration and invasion via expression reduction of the epithelial-to-mesenchymal transition (EMT) markers ZEB1, N-cadherin and Snail.

Conclusions Our data indicate that cediranib may exhibit anti-tumor activity in PDAC cells and provide a rationale for further investigation of the potential of VEGF receptor-targeted therapies for the treatment of PDAC.

Keywords Pancreatic ductal adenocarcinoma · VEGF family · Cediranib · Therapeutic sensitization

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1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common and lethal subtype of pancreatic cancer. PDAC is the fourth leading cause of cancer-related death in the United States and has been predicted to be the second cause of cancer-related death in the next decade [1, 2]. Despite a decreasing trend in the mortality rate for most common malignancies during recent years, the mortality rate of PDAC has been slightly increasing. Its current 5-year survival rate is < 3%, the lowest among all human neoplasms [3, 4]. Late diagnosis, early invasion, low immunogenicity and therapy resistance primarily account for the poor prognosis of this disease [5, 6].

Despite considerable improvements in therapeutic strategies that have been made, the mortality rate of PDAC still closely resembles its incidence rate [4]. As yet, surgical

resection is the only potential curative intervention followed by adjuvant chemotherapy, but only 15–20% of the patients may opt for surgery due to asymptomatic early stages of the disease. Even then, most patients experience disease relapse. On the other hand, resistance to the chemotherapeutic agents gemcitabine and paclitaxel, or the first-line FOLFIRINOX regimen (5-fluorouracil, leucovorin, irinotecan, oxaliplatin), is increasingly observed in patients with metastatic disease [7, 8]. Erlotinib, an EGFR small molecule inhibitor, is the only approved targeted therapy for PDAC, but its clinical benefit is marginal [9]. Therefore, there is a critical need for the development of novel and more efficacious therapeutic modalities to combat this devastating malignancy.

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is an essential step in tumor growth, metastatic dissemination and therapy resistance [10]. The vascular endothelial growth factor (VEGF) family plays a key role in angiogenesis [11]. This family comprises seven members, i.e., VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, VEGFF and placenta growth factor (PlGF). The corresponding tyrosine kinase receptors include VEGF receptor type 1 (VEGFR1), VEGFR2 and VEGFR3 [12]. Tumor-secreted VEGF ligands bind to VEGF receptors on endothelial, stromal and tumor cells, indicating that contribution of the VEGF family to human malignancies exceeds angiogenesis [13, 14]. In line with this notion, autocrine activation of VEGF receptors has been found to promote essential aspects of oncogenesis, including malignant progression, metastatic dissemination and therapy resistance [15, 16].

Evidence suggests that the VEGF family may contribute to malignant PDAC progression. VEGFR1 and 2 have been found to be overexpressed in PDAC and to promote tumor progression [17, 18]. VEGFR1 has also been found to induce epithelial-mesenchymal transition (EMT) [19], a process that enables a polarized epithelial cell to assume a mesenchymal and invasive phenotype [20]. VEGFR2 has been found to enhance local invasion of PDAC cells [18, 21], and it has been reported that the 5-year survival of patients with VEGFR2-positive tumors was 0% compared to 21% for patients with VEGFR2-negative tumors [22]. Furthermore, PDAC tissues have been found to show overexpression of VEGFR3 compared to normal human pancreatic tissues, and to drive lymphangiogenesis [23, 24]. In addition, VEGFA expression has been found to serve as an important predictor for liver metastasis in PDAC patients [25]. Taken together, these observations emphasize important roles of VEGF receptors in malignant PDAC progression and suggest that their therapeutic targeting may affect PDAC tumor cell growth, survival and motility.

Since PDAC is a hypo-vascular malignancy, it may be assumed that it is not likely to benefit from anti-angiogenic agents. This concept has, however, been disproven and both poorly and highly vascularized neoplasms have been found to

respond to anti-angiogenic therapies [8]. Here, we examined the activity of cediranib, a pan-VEGFR small molecule inhibitor, on PDAC cells. We found that cediranib exhibited a stronger anti-proliferative activity than other VEGFR-targeted inhibitors, diminished PDAC cell survival and inhibited PDAC cell migration and invasion. Moreover, we found that treatment with cediranib increased PDAC radio-sensitivity and enhanced the anti-proliferative efficacies of gemcitabine and paclitaxel.

2 Materials and methods

2.1 Antibodies and chemicals

Antibodies were purchased as follows: anti-HER3-neutralizing monoclonal antibody (clone H3.105.5) (Millipore); anti-Snail (clone C15D3), anti-XIAP (clone 3B6) and anti-cIAP1 (clone D5G9) (Cell Signaling Technology); anti-MCL1 (clone Y37) (Abcam); anti-Survivin (clone FL-142), anti-ZEB1 (clone H-3), anti-N-cadherin (clone 13A9) and anti- β -actin (Santa Cruz Biotechnology).

Bay 11-7082 (a NF- κ B inhibitor), I-BET151 (a pan-inhibitor of bromodomain and extra-terminal domain (BET) proteins; epigenetic readers that regulate gene expression and are involved in cancer pathogenesis [26]), YM155 (a survivin inhibitor), navitoclax (an inhibitor of Bcl-xL, Bcl-2 and Bcl-w), AZD5582 (an inhibitor of XIAP, cIAP1 and cIAP2), abemaciclib (a CDK4/6 inhibitor), BI2536 (a polo-like kinase 1 inhibitor), buparlisib (a pan-PI3K inhibitor), LDC1267 (a TAM kinase inhibitor), dacomitinib (a pan-inhibitor of ERBB receptors), lapatinib (a dual EGFR/HER2 inhibitor) and the VEGFR small molecule inhibitors cediranib, nintedanib, pazopanib, regorafenib, sorafenib, tivozanib and vandetanib, were purchased from Adooq Bioscience (Irvine, CA, USA). All agents were dissolved in DMSO. The final concentration of DMSO did not exceed 0.1% [v/v] in all treatments.

Cetuximab (anti-EGFR mAb), bevacizumab (anti-VEGFA mAb), cisplatin (a DNA-damaging agent), 5-FU (a thymidylate synthase inhibitor), paclitaxel and docetaxel (taxane inhibitors of microtubule disassembly), irinotecan (a topoisomerase inhibitor) and gemcitabine (a nucleoside analogue that inhibits DNA synthesis) were purchased from the pharmacy of Shariati Hospital (Tehran, Iran). The drugs used are listed in supplementary Table 1.

2.2 Cell culture

The human PDAC cell lines AsPC-1, PANC-1, PaTu 8902 and MIA PaCa-2 were purchased from the National Cell Bank of Iran (Tehran, Iran) and were maintained at 37 °C and 5% CO₂ in a humidified incubator and cultured according

to NCBI recommendations. Cell line authentication was performed by STR profiling and by using the Cell ID™ system (Promega). The cells were regularly tested for mycoplasma contamination.

2.3 Cell viability and proliferation assays

Cell viability and proliferation were determined using MTT and bromodeoxyuridine (BrdU) incorporation assays, respectively. The PDAC cells were seeded in 96-well plates and treated with the indicated concentrations of the drugs for 48 h. The proportion of viable cells was determined by MTT assay using standard procedures. For the BrdU assay, the cells were incubated with 10 µl BrdU (Roche Molecular Biochemicals) at 37°C for 2 h. Next, the cells were fixed and DNA was denatured using 200 µl FixDenat solution provided with the kit. Following incubation with a peroxidase-conjugated anti-BrdU antibody at room temperature for 1 h, the cells were exposed to 100 µl of the substrate tetramethylbenzidine (TMB) for 30 min at room temperature. To stop the peroxidase reaction, 25 µl 1 M H₂SO₄ was added and the samples were read at 450 nm in an ELISA reader.

2.4 Crystal violet staining

The cells were seeded in 6-well plates and treated with drugs for 48 h. Next, they were fixed with ice-cold methanol and stained with crystal violet (0.5% w/v). Images were acquired using an inverted microscope.

2.5 Median-effect analysis of drug combinations

To determine the efficacy of combinational approaches, cells were treated with cediranib and different concentrations of the chemotherapeutics or targeted inhibitors for 48 h. Survival was determined by MTT assay (see above) and SynergyFinder was applied to analyze drug combinations. The Bliss independence model is widely used to analyze drug combinations. Through this model the observed combination response is compared with the predicted combination response, based on the assumption that there are no drug-drug interaction effects and that each contributes to a common result. Dose-response landscapes were generated and average synergy scores (delta scores) were calculated. Visualization of the synergy scores is depicted as a two-dimensional synergy map over the dose matrix. An average synergy score of 0 is considered additive, < 0 as antagonistic and > 0 as synergistic [27, 28].

2.6 Clonogenic assay

Cells were seeded in 6-well plates at a density of 1000 cells/well. After 24 h, the cultures were treated with cediranib for 48

h, after which the media were replaced with drug-free media in which the cells were maintained for another 10 d. The resulting colonies were stained with 0.5% crystal violet and counted using an inverted microscope. Plating efficiency (PE) and survival fraction (SF) were calculated using the following formula: PE = number of colonies/cells plated for untreated controls; SF = number of colonies/number of cells seeded × PE [29].

2.7 Radiation assay

Cells were seeded in 6-well plates and γ-irradiated at 2 Gy using a cobalt (Co 60) source. Next, the cultures were treated with cediranib for 48 h. Survival fraction and apoptosis were measured using MTT, crystal violet staining and annexin V assays.

2.8 Apoptosis assay

Induction of apoptosis was measured using an eBioscience™ Annexin V apoptosis detection Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Apoptotic cell death was evaluated using a Partec PAS III flow cytometer (Partec GmbH) and Windows™ FloMax® software (Partec).

2.9 Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed using a Light Cycler 96 instrument (Roche Molecular diagnostics). The primer sequences used are listed in supplementary Table 2. Target gene expression levels were normalized to β2-microglobulin (*B2M*) levels in the same reaction. For calculations the $2^{-\Delta\Delta C_T}$ formula was used, with $\Delta\Delta C_T = (C_{T \text{ Target}} - C_{T \text{ B2M}})_{\text{experimental sample}} - (C_{T \text{ Target}} - C_{T \text{ B2M}})_{\text{control samples}}$, where C_T is the cycle threshold.

2.10 Western blot analysis

Cells were lysed for 30 min in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS), after which equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma). β-actin was used as loading control and proteins were detected using a BM chemiluminescence detection kit (Roche Molecular Biochemicals).

2.11 Cell migration and invasion assays

Transwell cell migration and invasion assays were performed as described before [30].

2.12 Statistical analysis

All data were evaluated in triplicate against vehicle-treated control cells and collected from three independent experiments. Data were graphed and analyzed using GraphPad Prism Software 8.0.1 using one-way ANOVA and the unpaired two-tailed Student's *t* test. All data are presented as mean \pm standard deviation (SD).

3 Results

3.1 Drug sensitivity of PDAC cell lines

The sensitivity of PDAC cell lines to various chemotherapeutics and lapatinib was determined using a MTT assay. We found that the AsPC-1, PANC-1 and PaTu 8902 cell lines were more resistant to cisplatin, gemcitabine and 5-FU, compared to the MIA PaCa-2 cell line (Table 1; Supplementary Fig. 1).

3.2 Expression of VEGF family members in PDAC cells

To examine any potential association between resistance to certain chemotherapeutics and the expression of VEGF family members, a qRT-PCR analysis was carried out. By doing so, we found that apart from *VEGFB* and *VEGFC*, the expression of the other family members was higher in AsPC-1, PANC-1 and PaTu 8902 cells compared to that in MIA PaCa-2 cells (Fig. 1a). Moreover, we found that a higher expression of *VEGFD* was associated with gemcitabine resistance using Pearson's correlation test (Fig. 1b). The correlation coefficient (*r*) between *VEGFD* expression and gemcitabine concentration was 0.9987 (non-zero slope *p* = 0.0013).

Although gemcitabine remains a cornerstone of PDAC treatment in all stages of the disease, most patients develop resistance within weeks of treatment initiation, which results in a poor survival. Aberrations in a variety of cellular signaling pathways are known to promote gemcitabine resistance. In order to improve the therapeutic response to gemcitabine it is, therefore, essential to delineate the underlying resistance mechanisms

[31]. To confirm that the VEGF family drives gemcitabine resistance, we assessed the effects of cediranib on the proliferative response of gemcitabine-resistant PANC-1 cells to this chemotherapeutic agent. We found that combination with cediranib synergistically increased gemcitabine sensitivity (Fig. 1c-e).

Albumin-bound paclitaxel plus gemcitabine is a standard treatment option for metastatic PDAC [32]. Moreover, it has been reported that paclitaxel-induced VEGFA is an important mechanism of chemoresistance induction [33]. Here, we found a positive difference between *PLGF* expression and higher paclitaxel concentrations and, consistent with this, that cediranib synergistically enhanced the anti-proliferative effects of paclitaxel in PANC-1 cells (Fig. 1f-h). We also observed significant correlations between higher *VEGFR2* and *VEGFA* expression levels and higher concentrations of 5-FU and docetaxel, respectively (Supplementary Fig. 2).

3.3 Cediranib inhibits PDAC cell viability and proliferation

To compare the anti-proliferative effects of VEGFR small molecule inhibitors, including cediranib, nintedanib, pazopanib, regorafenib, sorafenib, tivozanib and vandetanib, on PDAC cells a MTT assay was performed. We found that cediranib exhibited a stronger anti-proliferative activity than the other anti-angiogenics (Fig. 2a and supplementary Table 3). The inhibitory effect of cediranib on cell viability and proliferation was also assessed using crystal violet staining and BrdU incorporation assays (Fig. 2b, c). Clonogenic survival represents the renewal potential and long-term response of tumor cells to anti-cancer agents [34]. We found that cediranib reduced the clonogenic growth of PDAC cells (Fig. 2d). Taken together, these data indicate that cediranib can significantly decrease the viability, proliferation and clonal growth of the PDAC cells.

Compared to the other cell lines in the present study, PANC-1 cells showed an intermediate resistance to cediranib (Fig. 2c). In an attempt to reveal the underlying mechanisms and enhance the sensitivity to cediranib, we assessed the growth capacity of these cells after treatment with cediranib and different inhibitors, including H3.105.5, Bay 11-7082, I-

Table 1 Sensitivity of PDAC cells to chemotherapeutics and lapatinib. Data represent averaged IC₅₀ values collected from three independent experiments, each performed in triplicate

Cell Lines	Sensitivity (IC ₅₀) ^a						
	Cisplatin (μ g/ml)	Gemcitabine (ng/ml)	5-FU (μ g/ml)	Docetaxel (μ g/ml)	Irinotecan (μ g/ml)	Lapatinib (μ M)	Paclitaxel (ng/ml)
AsPC-1	16.02	14854	33.17	7762	32.08	12.070	114
MIA	2.41	2.41	1.49	5527	21.77	5.831	35.4
PaCa-2							
PANC-1	14.87	38032	33.11	4739	30.99	8.981	49.93
PaTu 8902	42.21	3170	5.68	30653	32.22	11.890	74.58

^a Sensitivity is expressed as IC₅₀ for each cell line, which is the drug concentration that caused a 50% reduction in proliferation compared to vehicle-treated cells

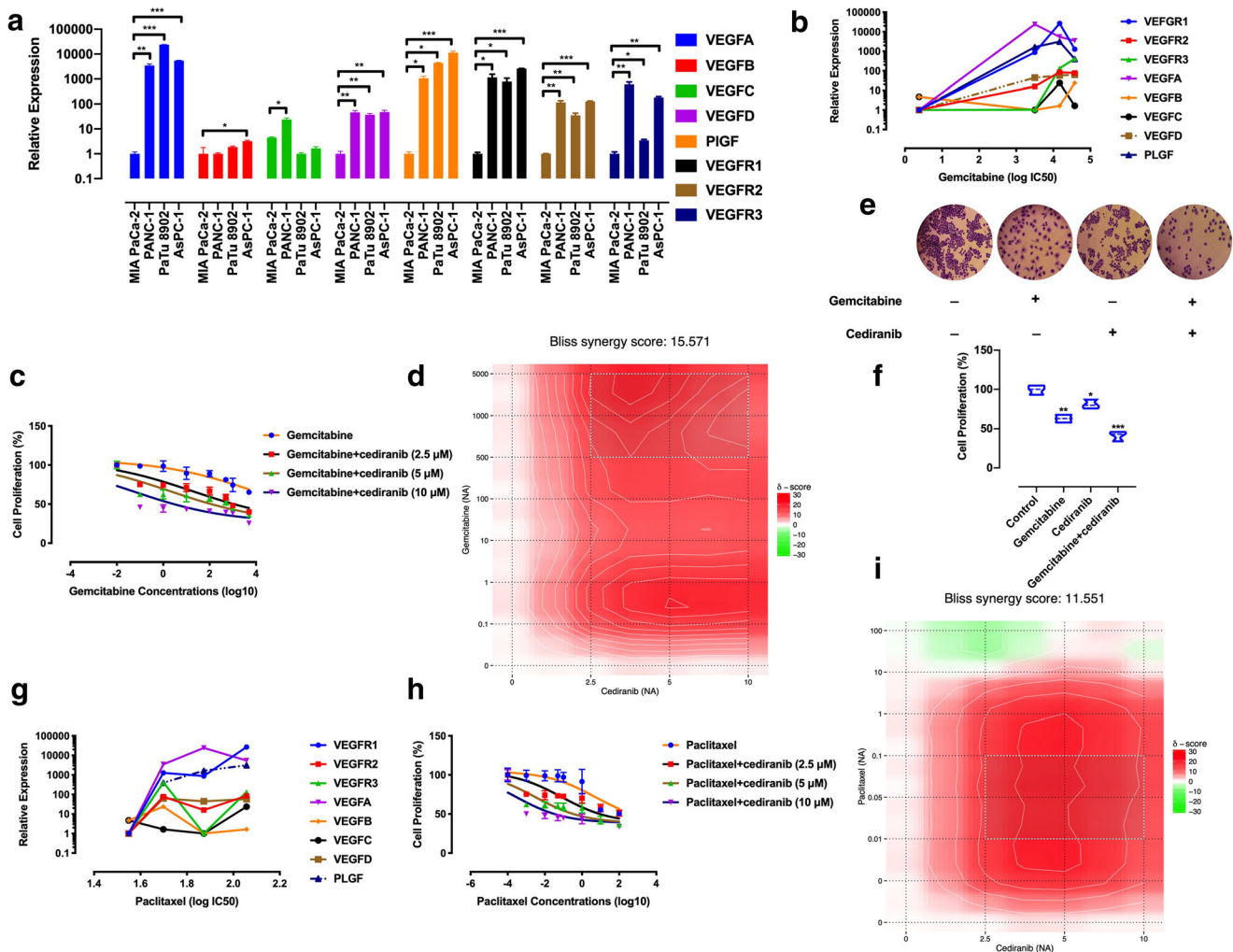


Fig. 1 Expression of VEGF family members in PDAC cells. **(a)** mRNA expression levels of the VEGF family members were determined by qRT-PCR. The data were generated in triplicate and collected from three independent experiments. The expression levels were normalized to β -actin in each cell line and then normalized to those in MIA PaCa-2 cells. Data are shown as mean \pm SD. Statistically significant values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were determined compared to the control. **(b)** Correlation of *VEGFD* expression and resistance to gemcitabine. PDAC cell lines with a higher *VEGFD* expression showed significantly higher gemcitabine IC_{50} values. **(c)** The effects of gemcitabine-cediranib on the proliferation of PANC-1 cells were investigated by MTT assay and depicted by IC_{50} shift analysis. The cell cultures were treated with gemcitabine (0, 0.1, 1, 10, 100, 500, 1000 and 5000 ng/ml) and cediranib (2.5, 5 and 10 μ M) for 48 h. **(d)** 2D synergy map of

gemcitabine-cediranib combination calculated by Bliss method. **(e, f)** The effects of the gemcitabine-cediranib combination on cell viability were assessed using crystal violet staining. The cells were treated with gemcitabine (5000 ng/ml) and cediranib (2.5 μ M) for 48 h, stained with crystal violet, imaged using an inverted microscope (images acquired at 10x magnification) and quantified using ImageJ software. **(g)** Association of *PLGF* expression and resistance to paclitaxel. A higher *PLGF* expression ($r = 0.9835$, $p = 0.0165$) positively correlated with paclitaxel resistance. **(h)** The effects of cediranib on the proliferative response of PANC-1 cells to paclitaxel was investigated by MTT assay and depicted by IC_{50} shift analysis. The cell cultures were treated with paclitaxel (0, 0.001, 0.01, 0.05, 0.1, 1, 10 and 100 ng/ml) and cediranib (2.5, 5 and 10 μ M) for 48 h. **(i)** Synergy map of paclitaxel-cediranib combination in PANC-1 cells

BET151, YM155, navitoclax, AZD5582, abemaciclib, BI2536, buparlisib, LDC1267, dacomitinib, lapatinib, cetuximab and bevacizumab. We found that BI2536 and buparlisib exhibited synergistic activities with cediranib on the inhibition of PANC-1 cell growth (Fig. 2e-h), suggesting that polo-like kinase 1 (PLK1) and PI3K/AKT blockade may enhance cediranib sensitivity in PDAC cells. Consistent with this, it has been reported that the PI3K/AKT pathway is deregulated in nearly 60% of PDAC patients and plays major roles in cell proliferation and survival [35]. PLK1, a serine/

threonine protein kinase, is essential for cell division and its elevated expression has been reported to correlate with tumor aggressiveness and a poor prognosis [36].

3.4 Cediranib induces PDAC cell apoptosis

We next measured the effects of cediranib on apoptosis induction. We found that cediranib induced apoptosis in all PDAC cells tested (Fig. 3a, b). To unravel the mechanisms underlying cediranib-induced apoptosis, we assessed the effect of the drug

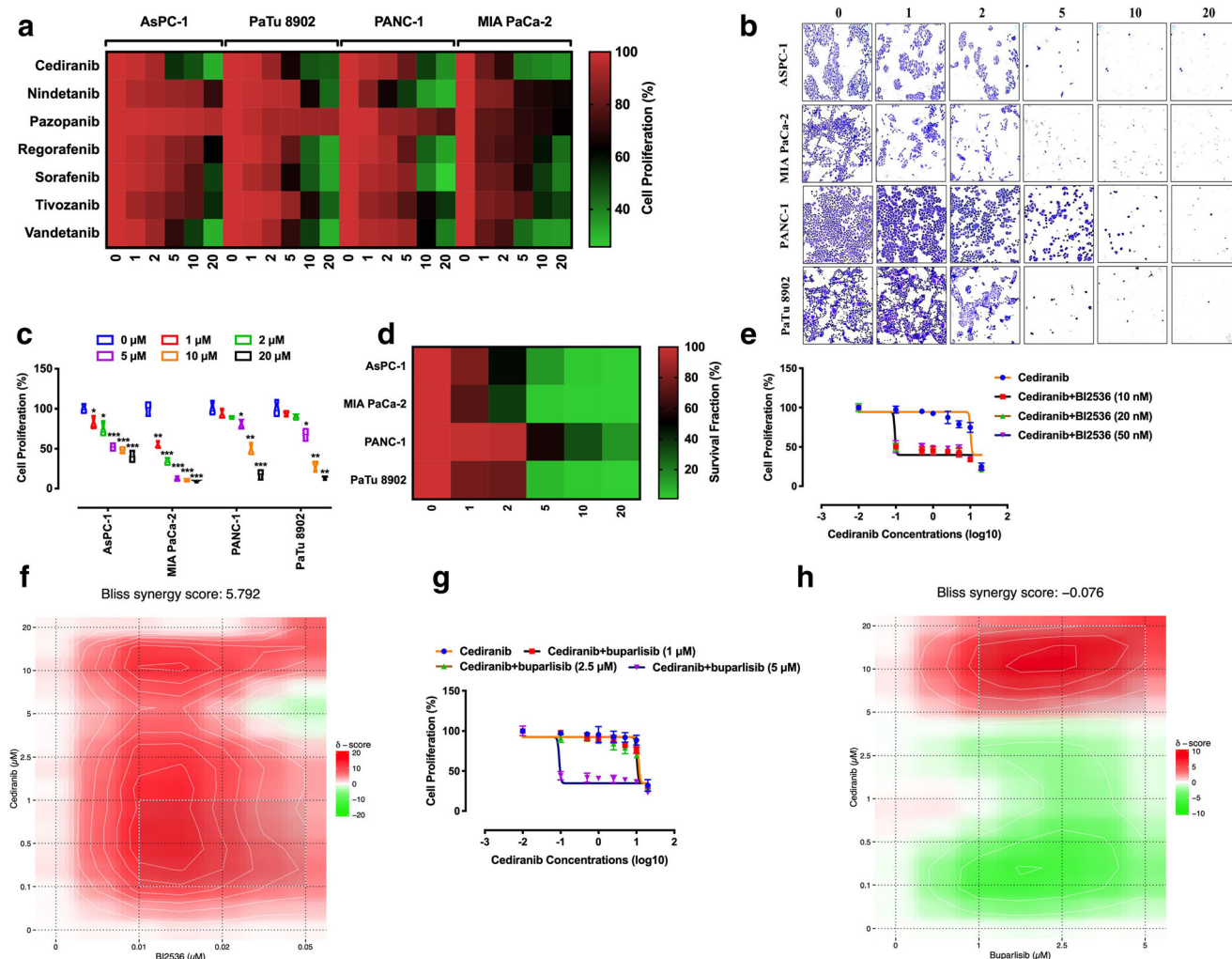


Fig. 2 Cediranib inhibits PDAC cell proliferation. **(a)** Comparisons of different concentrations (in μM) of the anti-proliferative effects of small molecule VEGFR inhibitors on PDAC cells were carried out by MTT assay. **(b, c)** The effect of cediranib on PDAC cell proliferation and viability illustrated by **(b)** crystal violet staining and **(c)** BrdU incorporation assay after 48 h treatment. **(d)** Cediranib inhibits the clonal proliferation of PDAC cells. **(e, f)** Inhibition of PLK1 synergistically enhances

sensitivity to cediranib in PANC-1 cells, as shown by IC_{50} shift and Bliss synergy analyses. The cell cultures were treated with cediranib (0, 0.1, 0.5, 1, 2.5, 5, 10 and 20 μM) and BI2536 (0.01, 0.02 and 0.05 μM) for 48 h. **(g, h)** Buparlisib, a pan-PI3K inhibitor, augments PDAC cell sensitivity to cediranib. The cell cultures were treated with cediranib (0, 0.1, 0.5, 1, 2.5, 5, 10 and 20 μM) and buparlisib (1, 2.5 and 5 μM) for 48 h

on known mediators of apoptotic cell death. Survivin, cellular inhibitor of apoptosis protein 1 (cIAP1), X-linked inhibitor of apoptosis protein (XIAP) and c-FLIP are well-known members of the IAP family that prevent apoptosis by inhibition of caspase activity, whereas the anti-apoptotic proteins MCL-1 and BCL-xL are known to prevent cytochrome c release from mitochondria [37, 38]. Our data indicate that cediranib decreased both the mRNA and protein levels of cIAP1, XIAP, MCL-1 and survivin (Fig. 3c, d), suggesting that cediranib may impede the proliferation of PDAC cells via apoptosis induction.

3.5 Cediranib impairs PDAC cell motility

Tumor invasion is triggered by break-down of the extracellular matrix (ECM) by a proteolytic cascade including cathepsin

B, urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs) [39]. Previous studies have revealed the relevance of this cascade in PDAC cell migration and invasion [40–42]. Overexpression of uPA and its receptor uPAR has been reported to be associated with a shorter post-operative survival, and increased MMP-2 and -9 levels with malignant PDAC progression [43, 44]. The lysosomal protease cathepsin B has been found to be upregulated in PDAC cells and to play a key role in invasion and malignant progression [45, 46]. Accordingly, it has been found that blockade of the VEGF family hinders this ECM-degrading cascade and diminishes the invasiveness of e.g. glioma and hepatocellular carcinoma cells [47, 48]. Here, we found that cediranib decreased the MMP-2, MMP-9, uPA, uPAR and CSTB (which encodes cathepsin B) expression levels (Fig. 4b).

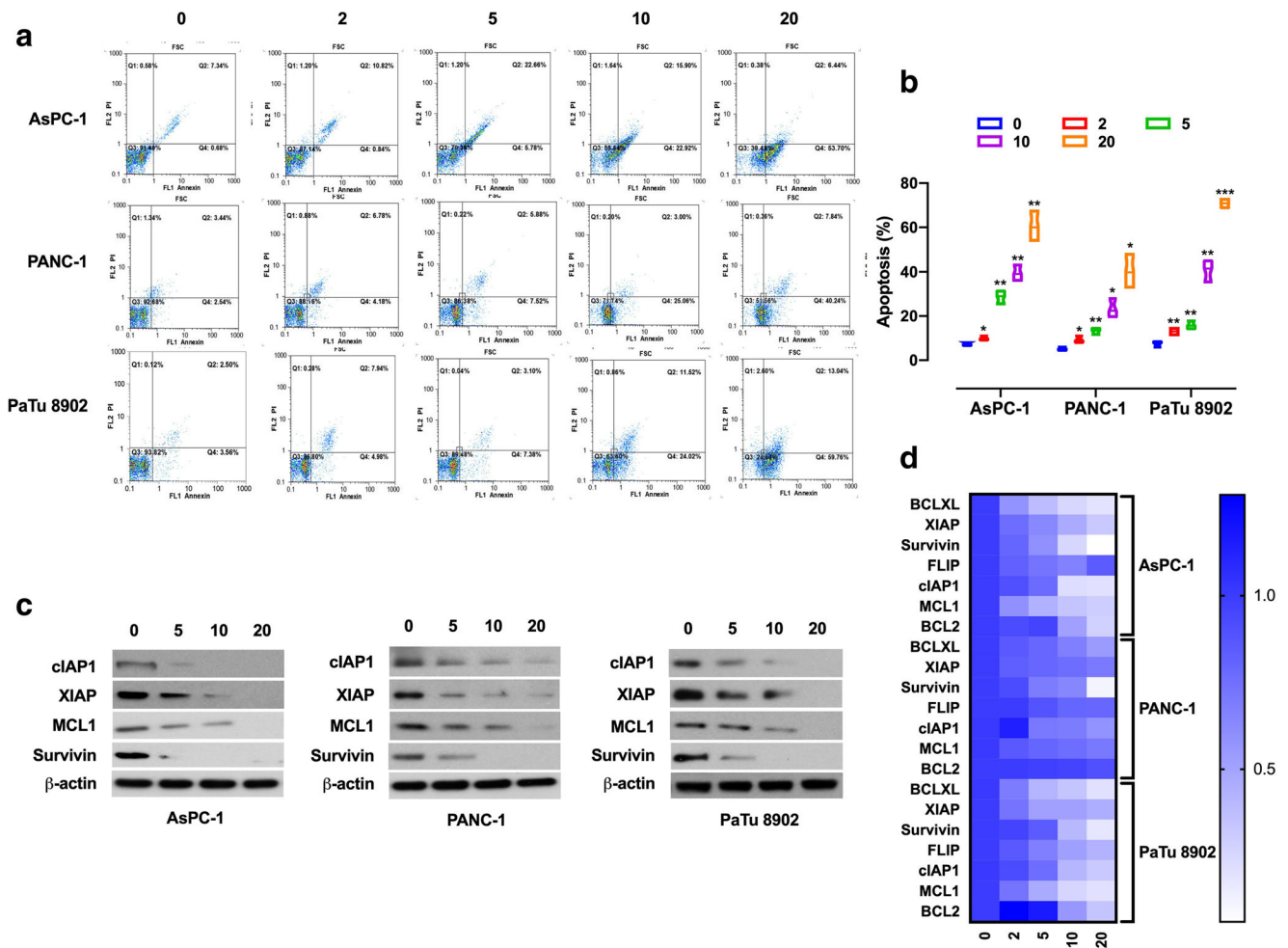


Fig. 3 Cediranib induces apoptosis in PDAC cells. **(a, b)** Apoptotic cell death was measured by annexin V staining after 48 h of cediranib treatment. Annexin V-positive cells are early apoptotic, whereas PI uptake indicates necrosis. Cells positive for both stains are late apoptotic. **(c)** Effects of cediranib on the expression of anti-apoptotic proteins

determined by Western blot analysis. The blots shown are representative of three independent experiments with similar outcomes. **(d)** Cediranib decreases the mRNA expression levels of the anti-apoptotic genes as measured by qRT-PCR after 48 h treatment

EMT contributes to early metastatic dissemination, a major hallmark of PDAC, and is known to be modulated by transcription factors such as Snail and ZEB1 [49, 50]. It has been found that VEGFR1 promotes the migration and invasion of PDAC cells via EMT induction [19]. We asked whether cediranib can reverse the EMTed phenotype. We found that cediranib decreased both the mRNA and protein levels of ZEB1, N-cadherin and Snail (Fig. 4a, b). Furthermore, we found that cediranib-mediated reduction in expression of the EMT markers and attenuation of the cathepsin B/uPA/MMP proteolytic cascade was associated with inhibition of PDAC cell migration and invasion (Fig. 4c, d).

3.6 Cediranib potentiates the radio-sensitivity of PDAC cells

Locally advanced unresectable disease is a common manifestation of PDAC and its management is, therefore,

challenging [51]. Although radiation therapy (RT) is used to improve local control and to palliate symptoms, the long-term survival is dismal because PDAC cells are relatively radioresistant [52–54]. RT has been found to induce VEGFA and to drive radio-resistance through activation of pro-survival pathways and attenuation of apoptosis [55, 56]. Preclinical studies suggest that combining antiangiogenics with RT may enhance the therapeutic efficacy of RT by affecting both tumor cells and the tumor vasculature [55, 57]. Due to the potent anti-tumor activity of cediranib, we questioned whether it may enhance the radio-sensitivity of PDAC cells. We found that the combination of cediranib and RT had a synergistic effect on PDAC cell growth inhibition. Moreover, we found that this combinational approach significantly increased the induction of apoptosis compared to each treatment alone (Fig. 5).

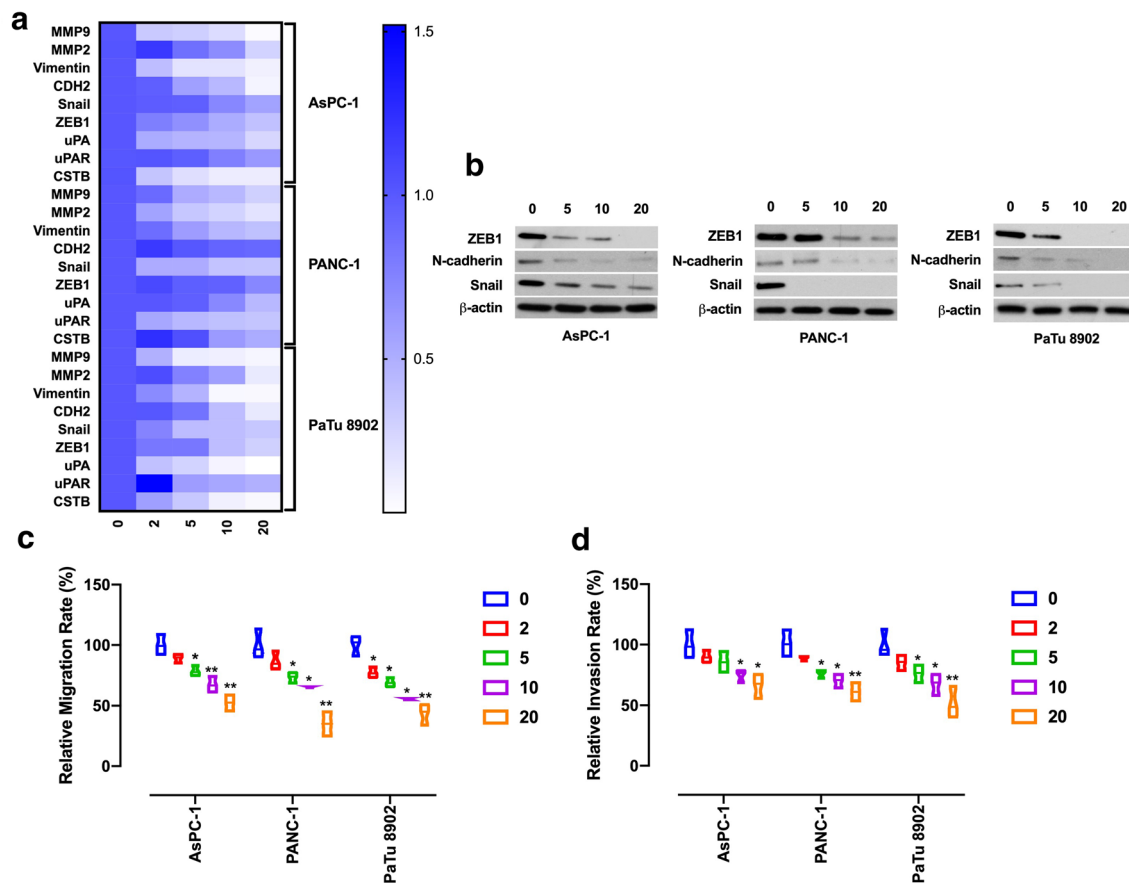


Fig. 4 Cediranib impairs PDAC cell motility. **(a, b)** The expression of EMT markers was determined by Western blot and qRT-PCR analyses after treatment with cediranib for 48 h. **(c, d)** Cediranib hampers cell migration and invasion. PDAC cells were treated with cediranib for 48 h after which equal numbers of cells were placed in 8- μ m porous culture inserts and allowed to migrate for 48 h. The migrated cells on

the lower surface of the inserts were quantified by crystal violet staining. For invasion assay, the cells were placed into matrigel-coated inserts and allowed to invade through the matrigel layer for 48 h. Data shown represent mean \pm SD from three independent experiments, each performed in triplicate. Statistically significant values of * $p < 0.05$ and ** $p < 0.01$ were determined compared to the untreated control group

4 Discussion

We found that the pan-VEGF inhibitor cediranib exhibits a strong anti-proliferative activity in PDAC cells and diminishes their survival, migration and invasion. In addition, we found that cediranib increases the radio-sensitivity of PDAC cells and enhances the anti-proliferative efficacies of gemcitabine and paclitaxel.

Evidence indicates an important role of the VEGF family in PDAC tumorigenesis [58, 59]. Approximately 93% and 69% of PDAC patients exhibit VEGFA and VEGFR2 expression, which has been found to be associated with a poor prognosis [22, 25]. A recent study has shown that PDAC patient survival was correlated with VEGFR1 and VEGFR2 expression and that patients expressing these receptors exhibited a lower survival compared to those not expressing them [60]. Overexpression of VEGF family members, such as those representing the VEGFC/VEGFR3 axis, is believed to promote PDAC tumor growth and metastatic dissemination [16]. Moreover, high VEGFR2 expression has been found to

correlate with local invasion and a poor prognosis [22]. These findings suggest that the VEGF family may serve as an attractive therapeutic target to halt the motility and proliferation of the PDAC cells.

The VEGF family has been shown to foster therapy resistance in different human malignancies [61, 62]. For instance, VEGFC has been found to drive cisplatin resistance in bladder cancer cells and that its knockdown reverses the resistant phenotype [63]. In epithelial ovarian cancer cells, higher *VEGFR2* expression has been found to correlate with cisplatin resistance and apatinib, a VEGFR2 specific inhibitor, has been found to increase cellular sensitivity to cisplatin [64]. Here, we found that high *VEFGD* and *PLGF* expression levels correlate with PDAC cell resistance to gemcitabine and paclitaxel and that cediranib synergistically enhances the proliferative response of PDAC cells to these drugs. Together, these findings suggest that VEGF family members may promote chemoresistance and that its blockade may restore drug sensitivity in PDAC cells.

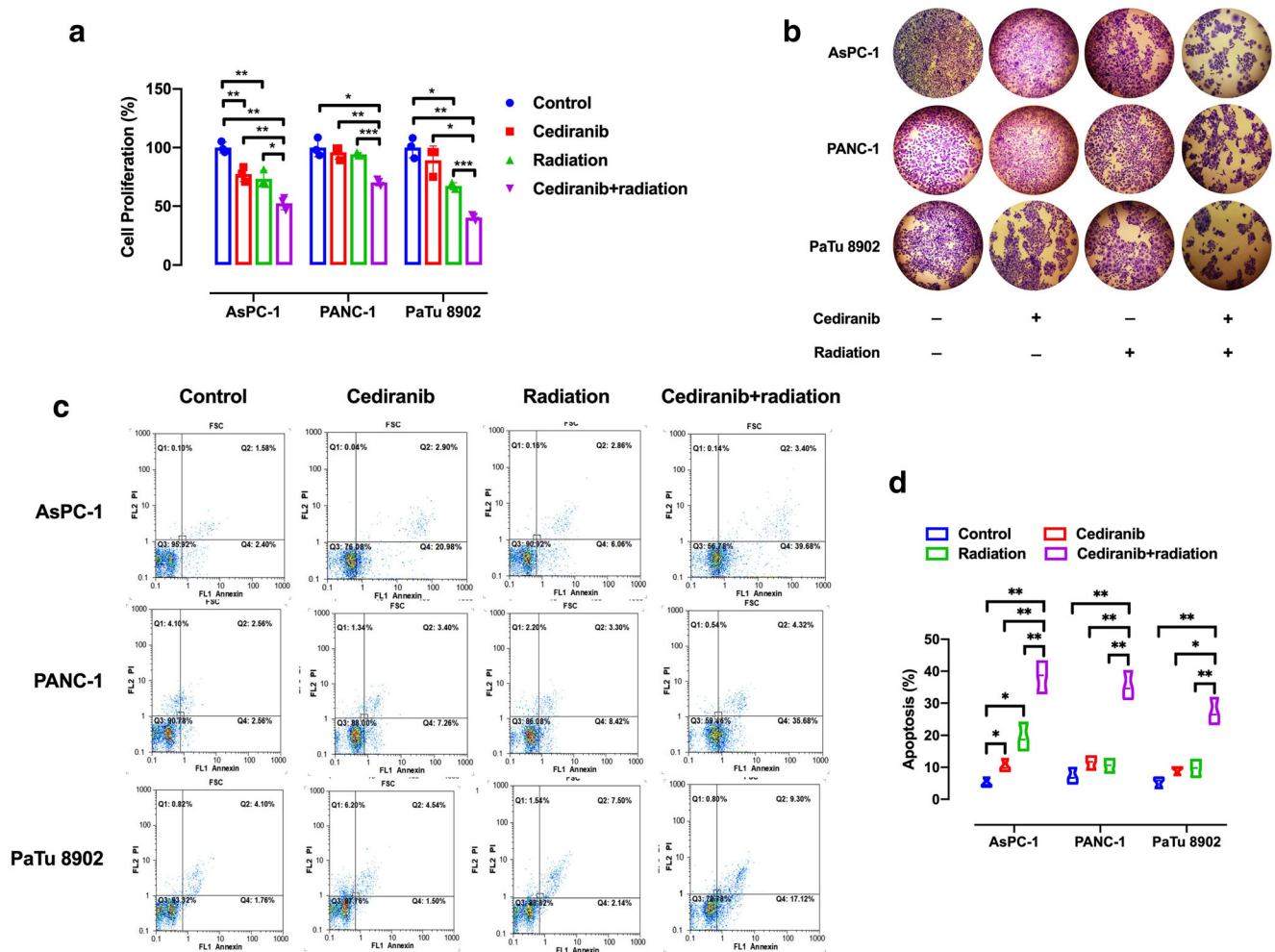


Fig. 5 Cediranib potentiates radio-sensitivity of PDAC cells. **(a, b)** The effects of cediranib (2.5 μ M) and radiation (2 Gy) on PDAC cell growth and viability were determined using MTT and crystal violet staining assays. **(c, d)** Cediranib augments the effects of RT on apoptosis induction.

Data were analyzed by one-way ANOVA followed by Tukey's post hoc test and are represented as mean \pm SD. Statistically significant values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were determined compared to untreated controls

Nearly half of the PDAC patients who undergo surgery and adjuvant chemotherapy develop liver metastases within two years [49, 65]. EMT has been found to play critical roles in tumor invasion and metastasis in PDAC patients [66] and EMT mediators have been found to be overexpressed in these patients and to promote early-stage dissemination of tumor cells [50]. Snail and Slug have, for instance, been found to be expressed in 78% and 50% of PDAC patients and to drive malignant progression [67]. Additionally, ZEB1 expression has been found to correlate with a more aggressive phenotype and a poor outcome in PDAC patients [68].

Evidence indicates that VEGF family members may drive EMT [69]. VEGFR1 activation has been found to increase the expression of the mesenchymal markers vimentin, N-cadherin, Snail, Twist and Slug [19]. EMT may be induced by VEGFR2 through up-regulation of vimentin in tubular epithelial cells, and the VEGFC/VEGFR3 loop may enhance tumor cell motility via increased Slug expression [70, 71]. In

this context, we asked whether a pan-VEGFR inhibition approach would reverse the EMTed phenotype and attenuate PDAC cell motility. We found that cediranib impaired the migration and invasion of PDAC cells, which was associated with a decreased expression of the EMT markers ZEB1, N-cadherin and Snail.

Evasion of apoptosis is one of the hallmarks of PDAC cells and has been found to contribute to treatment failure [72, 73]. Defects in the apoptotic pathways and deregulation of the anti-apoptotic proteins survivin, BCL-2, BCL-xL and MCL-1 may play decisive roles in PDAC resistance to apoptosis [74]. Enhanced expression of XIAP, BCL-xL and survivin has been found to correlate with a shorter survival and a poor outcome in PDAC patients, and MCL-1 overexpression has been found to be linked to disease progression [75–77]. These apoptotic inhibitory proteins have been reported to be highly overexpressed in PDAC cells and to drive therapy resistance [72, 78–81]. It is believed that simultaneous inhibition of anti-apoptotic proteins

has more profound effects on PDAC cell death induction than single targeted approaches [82]. We found that cediranib induced apoptosis in PDAC cells through simultaneous suppression of the anti-apoptotic proteins cIAP1, XIAP, MCL-1 and survivin and revealed the molecular mechanism by which cediranib induces cell death in PDAC cells.

Despite recent advances and improvements that have been made in RT, the majority of PDAC patients do not achieve objective responses due to intrinsic or acquired radioresistance [54]. In this context, it has been reported that VEGFR1 induces radioresistance in head and neck squamous cell carcinoma (HNSCC) cells and that the VEGFA/VEGFR2 axis promotes radioresistance in glioma cells [83, 84]. RT has been found to induce expression of VEGF ligands, including VEGFA, in different neoplasms [85, 86]. Additionally, RT-induced activation of the PI3K/AKT pathway has been found to increase VEGFC expression in non-small cell lung cancer (NSCLC) cells, thereby promoting cell survival and proliferation [87].

It has been hypothesized that combination of anti-VEGF inhibitors with RT may yield a better anti-proliferative outcome than RT alone [88], but the addition of bevacizumab to a regimen of capecitabine-based chemoradiotherapy followed by gemcitabine did not result in an improvement in overall survival in PDAC patients [89]. Combining RT with multi-kinase inhibitors sorafenib and sunitinib, small molecule inhibitors of VEGF and platelet-derived growth factor receptors, has been suggested to exert strong effects on inhibition of clonogenic survival and induction of apoptosis in HNSCC cells [90]. Consistent with this, we found that cediranib augmented the effects of RT on inhibition of PDAC cell growth and induction of apoptosis. MCL-1 and survivin are among the critical mediators of radioresistance in PDAC cells [91, 92] and their inhibition by cediranib may be one molecular explanation for cediranib-mediated potentiation of the anti-survival effects of RT in PDAC cells in this study.

Cediranib is a pan-VEGFR inhibitor with potent antitumor activity in lung, colorectal, prostate, ovarian and mammary gland adenocarcinoma models *in vitro* and *in vivo* [93, 94]. This anti-angiogenic agent is currently in clinical trials for a range of human malignancies, including ovarian cancer (in combination with the PARP inhibitor olaparib) [95], metastatic or recurrent cervical cancer (in combination with carboplatin-paclitaxel) [96], prostate cancer (in patients with progressive disease after docetaxel-based therapy) and renal cell carcinoma [97, 98]. Among a panel of tyrosine kinase inhibitors, cediranib was found to be the only small molecule inhibitor that yielded a partial response in patients with advanced refractory osteosarcoma [99]. Collectively, these findings suggest that application of cediranib may represent a novel and promising VEGFR-targeted therapy with anti-tumor activity in a broad range of human malignancies.

We conclude that the present study provides mechanistic insights into the anti-tumor activity of cediranib in PDAC

cells. We found that cediranib showed a stronger anti-growth activity compared to other small molecule VEGFR inhibitors and potentiated the anti-proliferative efficacies of gemcitabine and paclitaxel. Moreover, we found that cediranib enhanced the effects of RT on inhibition of PDAC cell growth and apoptosis induction. Cediranib-mediated inhibition of PDAC cell motility was found to be associated with a reduction in expression of EMT markers. Further *in vivo* investigations are warranted to explore the anti-tumor activity of cediranib, including the potential of VEGF receptor-targeted therapies, in this dreadful malignancy.

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Author's contributions M.M. designed the research; Z.A., H.Y. A.Z. and P.G. conducted the research; M.M., J.T., K.A. and A.G. analyzed the data; M.M. and Z.A. wrote the paper; M.M. and S.H.G. were primarily responsible for the final content. All authors have reviewed and approved the final manuscript.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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