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## Poly(ADP-ribose) polymerase inhibition decreases angiogenesis

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### Abstract

Inhibitors of poly(ADP-ribose)polymerase (PARP), a nuclear enzyme involved in regulating cell death and cellular responses to DNA repair, show considerable promise in the treatment of cancer both in monotherapy as well as in combination with chemotherapeutic agents and radiation. We have recently demonstrated that PARP inhibition with 3-aminobenzamide or PJ-34 reduced vascular endothelial growth factor (VEGF)-induced proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro*. Here we show dose-dependent reduction of VEGF- and basic fibroblast growth factor (bFGF)-induced proliferation, migration and tube formation of HUVECs *in vitro* by two potent PARP inhibitors 5-aminoisoquinolinone-hydrochloride (5-AIQ) and 1,5-isoquinolinediol (IQD). Moreover, PARP inhibitors prevented the sprouting of rat aortic ring explants in an *ex vivo* assay of angiogenesis. These results establish the novel concept that PARP inhibitors have antiangiogenetic effects, which may have tremendous clinical implications for the treatment of various cancers, tumor metastases and certain retinopathies.

### Keywords

Poly(ADP-ribose)polymerase (PARP); angiogenesis; proliferation; migration; tube formation; aortic ring assay; HUVEC; 5-aminoisoquinolinone-hydrochloride (5-AIQ); 1,5-isoquinolinediol (IQD); cancer

### Introduction

Poly(ADP-ribose) polymerase (PARP) is a family of nuclear enzymes of eukaryotic cells. PARP plays an important role in regulating DNA repair, gene transcription, cell cycle progression, chromatin function, genomic stability and cell death [1;2;3].

In preclinical testing, PARP inhibitors have demonstrated the ability to enhance the efficacy of various chemotherapeutic agents (e.g. methylating agents, DNA topoisomerase inhibitors, cisplatin), as well as radiation, against a broad spectrum of tumors (e.g. glioma, melanoma, lymphoma, colorectal cancer, head and neck tumors; [4;5;6;7;8;9;10;11;12;13;14;15;16;17; 18;19;20]), and were also reported to be protective against untoward effects exerted by certain

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anticancer drugs [21;22;23]. Recently, PARP inhibitors are being considered as promising treatment for cancer both in monotherapy as well as in combination with radiation and chemotherapeutic agents in humans.

Angiogenesis, the process of new blood vessel formation, is crucial for the development and progression of pathophysiological changes associated with a variety of disorders, including various cancers, tumor metastases and retinopathies. We have recently demonstrated that PARP inhibition with 3-aminobenzamide or PJ-34 reduced VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro* [24]. Here we show that two potent (structurally distinct from 3-AB and PJ-34) PARP inhibitors 5-aminoisoquinolinone-hydrochloride (5-AIQ) or 1,5-isoquinolinediol (5-ISQ) dose-dependently reduce both VEGF- and bFGF-induced proliferation, migration and tube formation of HUVEC *in vitro*. Additionally, we demonstrate that PARP inhibitors prevent the sprouting of rat aortic ring explants. These results establish the concept that pharmacological inhibition of PARP decreases angiogenesis, and predicts unexpected additional benefits of PARP inhibitors in various cancers and retinopathies.

## Materials and Methods

### Reagents

Human recombinant VEGF<sub>165</sub> was purchased from R&D systems (Minneapolis, MN). Human recombinant basic fibroblast growth factor (bFGF) was from Invitrogen (CA, USA). PARP inhibitors 5-aminoisoquinolinone-hydrochloride (5-AIQ) and 1,5-isoquinolinediol (IQD) were purchased from Calbiochem (San Diego, CA) and Sigma (St. Louis, MO), respectively.

### Cell Culture

Human umbilical vein endothelial cells (HUVEC) and endothelial cells growth medium EGM™ were purchased from Cambrex, (Walkersville, MD) and cells were cultured in EGM™ medium. Cells were used within passages 3 to 7. Prior to treatment, cells were conditioned in endothelial basal medium containing 1% FBS (Invitrogen, CA) 6 hrs and then used for the experiments.

### Proliferation assay

Endothelial cells ( $5 \times 10^3$ ) cells were suspended in 100  $\mu$ l of growth factor free medium containing 1 % FBS and then treated with VEGF 20 ng/ml or FGF 20 ng/ml alone or with PARP inhibitors IQD (4–16  $\mu$ M) or 5-AIQ (0.3–1.2  $\mu$ M) and seeded on to 96 well plates and allowed to proliferate for 24–36 hrs. Then BrdU labeling solution was added and incubated further for 12 hrs. After the incubation, the effect of PARP inhibitors on VEGF/FGF induced proliferation of the HUVECs was determined by the extent of BrdU incorporation using ELISA kit following the protocol supplied by the manufacturer (Roche Diagnostics, IN). In brief, after the incubation of cells with BrdU labeling solution, the cells were fixed and incubated with anti-BrdU antibody. After washing, the cells were incubated with secondary antibody conjugated with horse radish peroxidase. Finally, the extent of BrdU incorporation was determined colorimetrically by measuring the absorbance at 450 nm. The treatments were performed in triplicate and the experiment was repeated at least three times, as described [24].

### Migration assay

The migration assays were performed in a 24-well modified Boyden chamber as described earlier [24]. In brief, 8  $\mu$ m cell culture inserts (BD Biosciences) coated with 0.2 % gelatin (Sigma) were placed over the bottom chamber containing 20 ng/ml VEGF/FGF as the chemo-

attractant. Growth factor free medium containing 1% FBS served as negative control.  $3 \times 10^4$  cells were suspended in 150  $\mu$ l in growth factor free medium containing 1% FBS. Then the cells were pretreated with PARP inhibitors IQD or 5-AIQ, for 30 min at 37°C in 5% CO<sub>2</sub> incubator. Then the cell suspension was added to the upper chamber. After 8 hrs of incubation at 37°C, the non-migrated cells on the upper surface of the filter were removed by gentle swabbing with cotton tipped applicators. The cells that had migrated to the lower side of the chamber were fixed with 100 % methanol for 15 min at room temperature. After complete drying, the cells were stained with 0.5 % Giemsa stain (Sigma). 3–4 fields per insert were counted using 10X objective using Olympus IX81 microscope. The assays were performed in triplicate.

### **In vitro angiogenesis assay**

The effect of PARP inhibitors on inhibiting the formation of tube-like structures on growth factor reduced Matrigel (BD Biosciences) were performed as described earlier [24;25;26]. In brief, 96 well plates were coated with 50  $\mu$ l of growth factor reduced Matrigel and allowed to solidify at 37°C in 5% CO<sub>2</sub>. Cells treated with either VEGF, FGF alone or with IQD and 5-AIQ were carefully overlaid on to the solidified matrix. After 12 hrs of incubation at 37°C in 5% CO<sub>2</sub>, the medium was carefully aspirated and the cells were fixed with 4% formaldehyde and the images were captured using Olympus IX81 microscope. The tube length was quantified using the NIH Image J software. Results are represented as total tube length ( $\mu$ m) for three photographic fields per experimental condition. Each treatment was performed in duplicate and the set of experiment was independently repeated three times.

### **Ex vivo rat aortic ring assay**

The angioinhibitory properties of PARP inhibitors were further conformed using the cultured aortic ring explants in three-dimensional matrix gel according to the protocol described by Nicosia and Ottinetti (1990) with slight modifications [27]. In brief, thoracic aortas were dissected from 16-week old male Sprague-Dawley rats. Aortas were immediately transferred to the culture dish filled with cold and sterile serum-free Endothelial Cell Basal Medium 2 (EBM-2, Cambrex, USA) containing penicillin 100 IU/mL and streptomycin 100  $\mu$ g/mL (Gibco, USA). The peri-aortic fibro-adipose tissue was carefully removed with fine micro dissecting forceps and iridectomy scissors, taking special precaution not to damage the aortic wall. The aorta was cut in to 1.0 mm rings and rinsed several times with EBM-2. Washed aortic rings were then embedded between two 125  $\mu$ L layers of Growth Factor Reduced Matrigel (BD Biosciences, USA) in 48 well tissue culture plates. The matrigel was overlaid with EBM-2 containing penicillin 100 IU/mL, streptomycin 100  $\mu$ g/mL, 2% FBS and 2 % FBS (Invitrogen, CA). In certain experiments, the rings were incubated with either EBM-2 supplemented with VEGF/FGF (20 ng/ml) alone or with VEGF/FGF + IQD (16  $\mu$ M) or 5-AIQ (1.2  $\mu$ M), respectively. The cultures were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 8 days. The medium was replaced every 48 hrs. At the end of 8<sup>th</sup> day, the sprouting of endothelial cells differentiating into tube-like structures was documented using phase-contrast microscope (Olympus IX80). Each experiment was performed in triplicate. The sprout length was quantified using NIH image software.

### **Statistical analysis**

Values are represented as mean  $\pm$  SD. The statistical significance of the data was analyzed using one way ANOVA or Student's *t* test. *P* < 0.05 was considered as significant.

## Results

### PARP inhibitors IQD and 5-AIQ inhibitors VEGF and FGF induced proliferation of HUVECs

As shown in Fig. 1 A&B, VEGF and FGF induced the proliferation of HUVECs by ~ 4.5 and 5.5 fold respectively ( $P < 0.00001$  vs. control). On the other hand when HUVECs were pretreated with IQD (Fig. 1A) or 5-AIQ (Fig. 1B), elicited dose dependent inhibition in both VEGF and FGF stimulated cells. Also at the indicated concentrations both IQD and 5-AIQ did not found to have cytotoxic effects on HUVECs (data not shown).

### PARP inhibitors decrease VEGF- and FGF-induced migration of HUVECs

Both VEGF and FGF when used at 20 ng/ml enhanced the migration of HUVECs by ~ 3.5 fold ( $P < 0.00001$  vs. control) Fig. 2A–C. When HUVECs were pretreated with increasing concentrations of IQD or 5-AIQ (Fig. 2), both these inhibitors were found to decrease the migration of HUVECs in concentration dependent fashion. IQD or 5-AIQ by itself had no effect on the migration of HUVECs and the results were comparable to that of vehicle control (data not shown).

### PARP inhibitors attenuate VEGF- and FGF-induced tube formation of HUVECs

As shown in Fig. 3 A, VEGF and FGF significantly enhanced the tube formation of HUVECs by 2.3 fold over control ( $P < 0.00001$ ) when plated in growth-factor reduced matrigel. Pre-treatment of HUVECs with IQD or 5-AIQ dose-dependently inhibited both VEGF- and FGF-induced tube formations (Figure 3A–C). IQD or 5-AIQ by itself had no effect on the tube formation and the results were comparable to that of vehicle control (data not shown).

### PARP inhibitors inhibit angiogenesis by preventing the sprouting of rat aortic ring explants

As shown in Fig. 4 VEGF and FGF at 20 ng/ml markedly stimulated the sprouting of rat aortic rings ( $P < 0.00001$  vs. control), which was largely reduced by both IQD (16  $\mu$ M) or 5-AIQ (1.2  $\mu$ M).

## Discussion

Inhibitors of PARP show considerable promise in the treatment of cancer both in monotherapy as well as in combination with chemotherapeutic agents and radiation [4;5;6;7;8;9;10;11;12;13;14;17;18;19]. Targeting PARP may prevent tumor cells from repairing DNA themselves and developing drug resistance, which may make them more sensitive to various chemotherapeutic agents (e.g. methylating agents, DNA topoisomerase inhibitors, cisplatin), as well as radiation therapy. In preclinical testings, PARP inhibitors markedly enhance the effect of the above mentioned chemotherapeutic agents and radiation against a broad spectrum of tumors (e.g. melanoma, glioma, lymphoma, colorectal cancer, head and neck tumors; [4;5;6;7;8;9;10;11;12;13;14;17;18;19]). Furthermore, PARP inhibitors were also reported to exert cytoprotective effects against cardiotoxicity of doxorubicin [22;23] and nephrotoxicity of cisplatin [21]. The cytoprotective effects of PARP inhibitors involve inhibition of the pathological overactivation of PARP, and blockade of the cells from entering into necrosis via over-utilization of cellular  $NAD^+$  pools eventually leading to cellular energetic failure [1;2].

Angiogenesis is crucial for the growth and metastasis formation of a variety of cancers, and pathologically increased angiogenesis also contributes to the development of complications (e.g. blindness) associated with various retinopathy (e.g. diabetic retinopathy). We have recently demonstrated that PARP inhibition with 3-aminobenzamide or PJ-34 reduced VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells *in vitro* [24]. In the present study we confirm these observations using two potent structurally distinct PARP inhibitors 5-aminoisoquinolinone-hydrochloride and 1,5-isoquinolinediol.

Additionally, to strengthen the concept, we also demonstrate antiangiogenic potential of PARP inhibitors against FGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells, and by using an ex vivo rat aortic ring assay of angiogenesis. Consistently with these results, minocycline (a semisynthetic tetracycline antimicrobial antibiotic), which has recently been described as a potent PARP inhibitor, [28;29] also inhibits angiogenesis [30]. These results suggest that PARP inhibitors may be beneficial in decreasing angiogenesis-dependent growth of certain types of cancers and cancer metastases.

Additional benefits of antiangiogenic effects of PARP inhibitors can also be expected in various retinopathies associated with increased angiogenesis (e.g. diabetic retinopathy), where over-activation of PARP was previously demonstrated to contribute to the pathophysiology [31;32;33].

Collectively, these results have established the concept that pharmacological inhibition of PARP decreases angiogenesis, and predicts additional benefits of PARP inhibitors in various cancers and retinopathies.

#### Acknowledgements

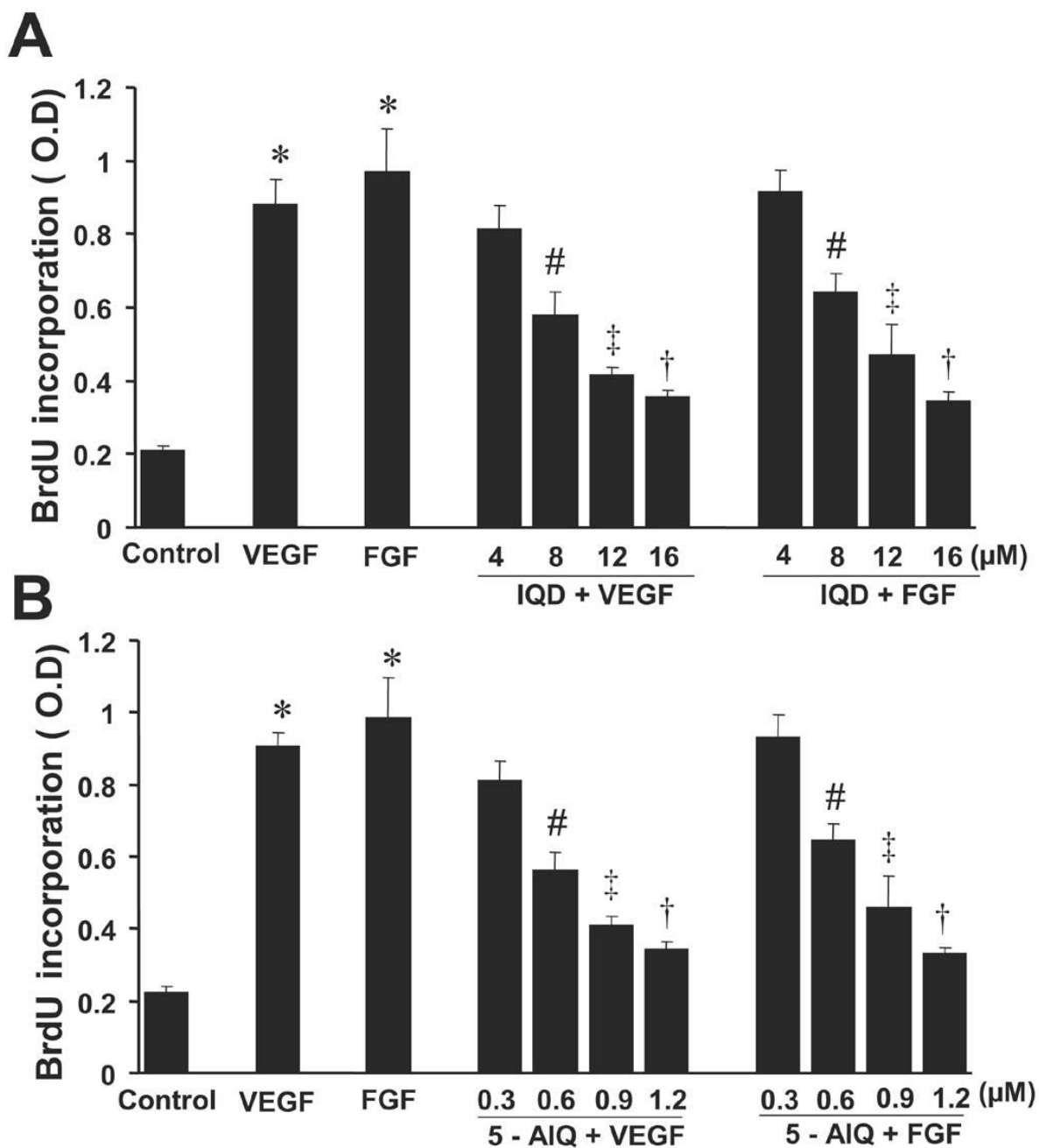
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**Fig.1. PARP inhibitors decrease VEGF- and FGF-induced proliferation of HUVECs**

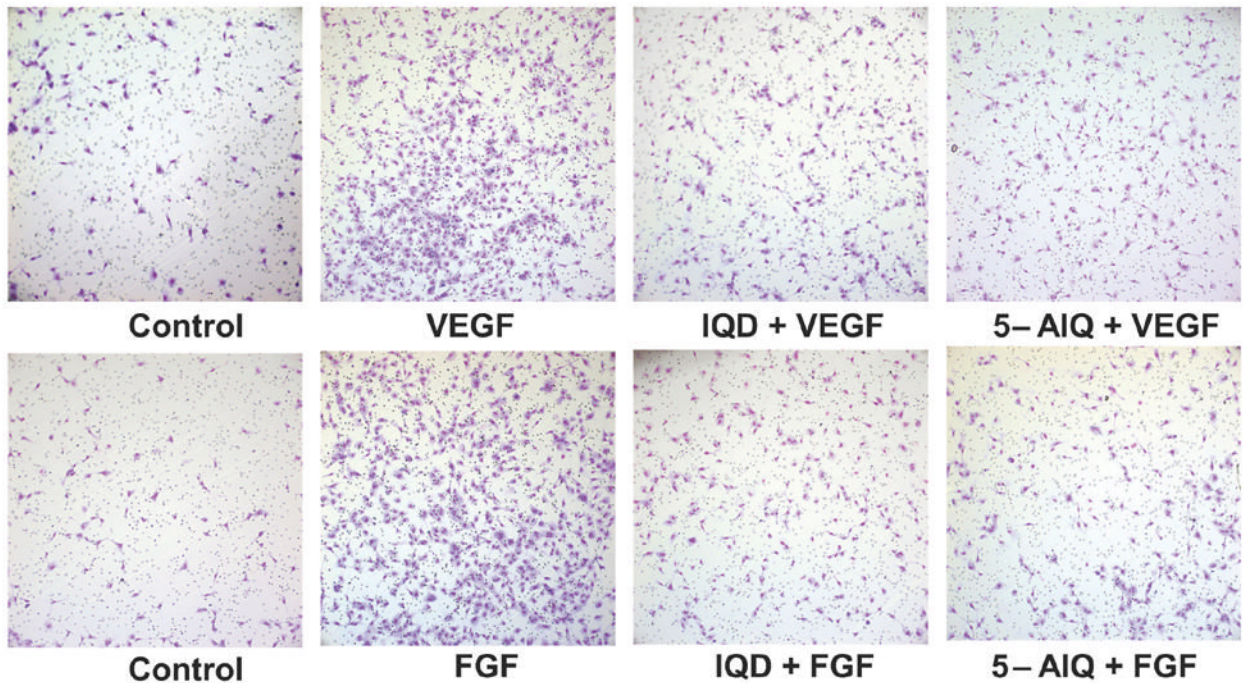
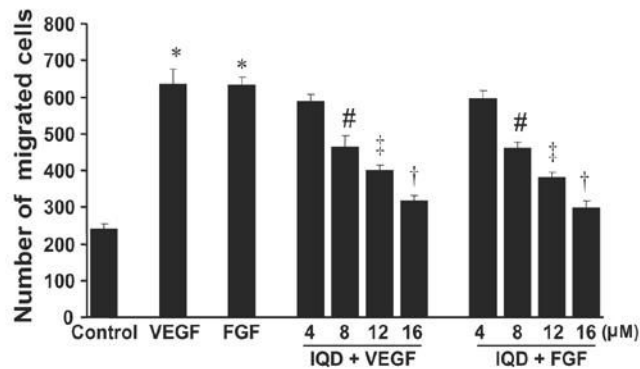
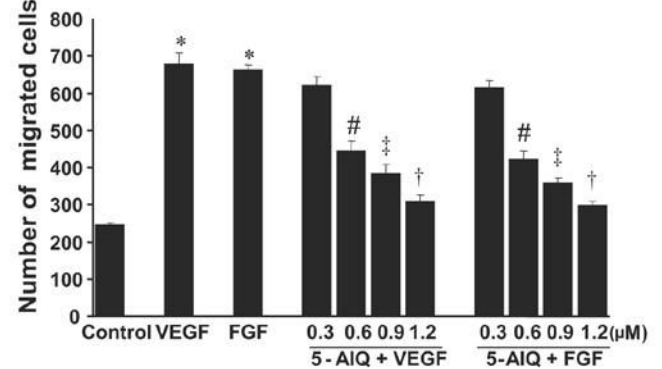
(A) IQD decreases VEGF- and FGF-induced proliferation of HUVECs.

Cells were treated with VEGF or FGF 20 ng/ml alone or with VEGF/FGF + IQD as indicated and the proliferation of HUVECs was determined by measuring the amount of BrdU incorporated using ELISA kit colorimetrically at 450 nm.

(B) 5-AIQ decreases VEGF- and FGF-induced proliferation of HUVECs.

\* P < 0.00001 vs. control; # P < 0.05 vs. VEGF or FGF; ‡ P < 0.01 vs. VEGF or FGF; † P < 0.001 vs. VEGF or FGF, n = 3.



**A****B****C**

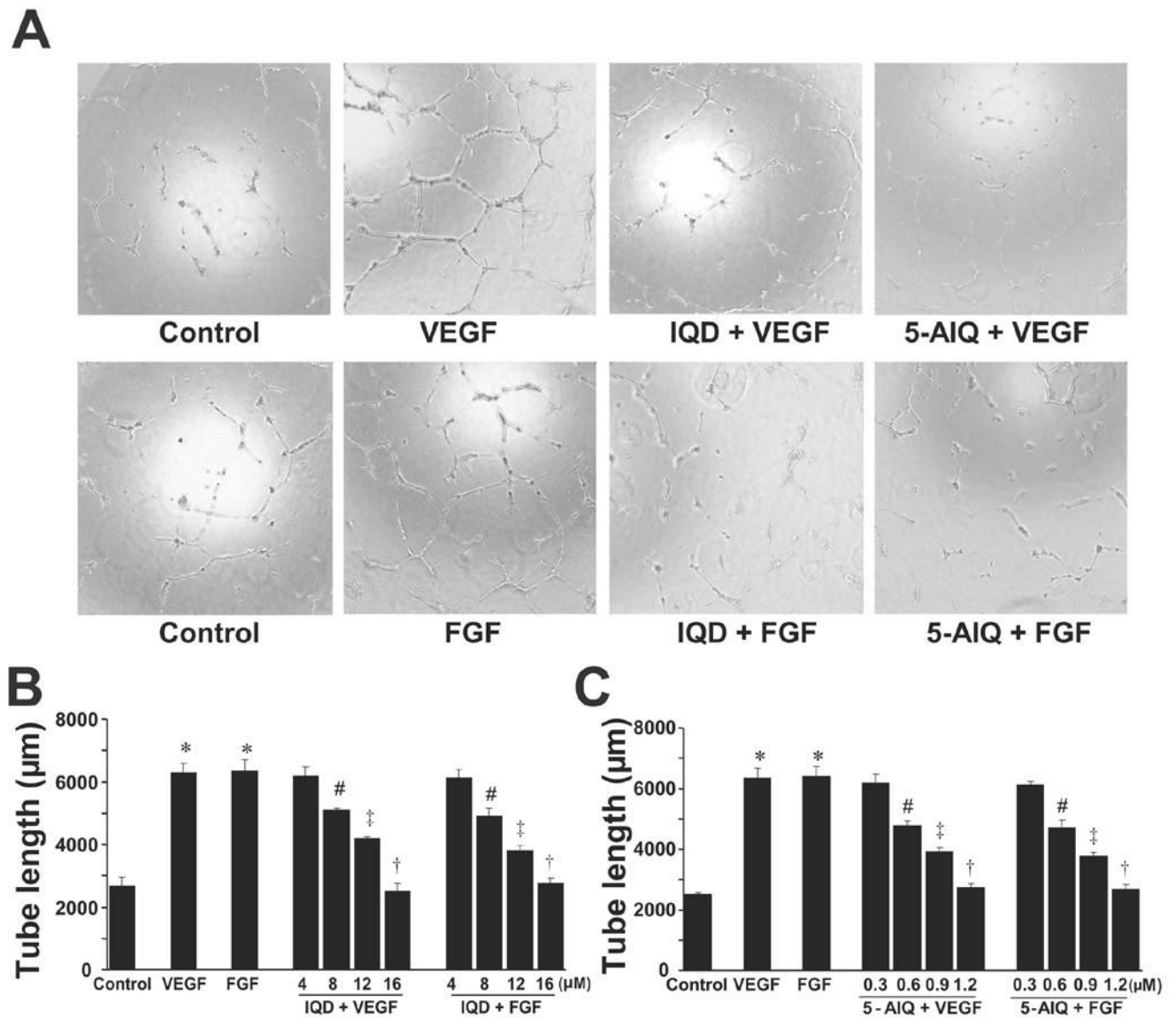
**Fig.2. PARP inhibitors attenuate VEGF- and FGF-stimulated migration of HUVECs**

(A) Representative fields of cell culture inserts containing HUVECs migrated in response to the treatments as indicated.

(B) Quantitative data on the effect of IQD as indicated.

(C) Quantitative data on the effect of 5-AIQ as indicated.

\*  $P < 0.00001$  vs. control; #  $P < 0.05$  vs. VEGF or FGF; ‡  $P < 0.01$  vs. VEGF or FGF; †  $P < 0.001$  vs. VEGF or FGF,  $n = 3$ .



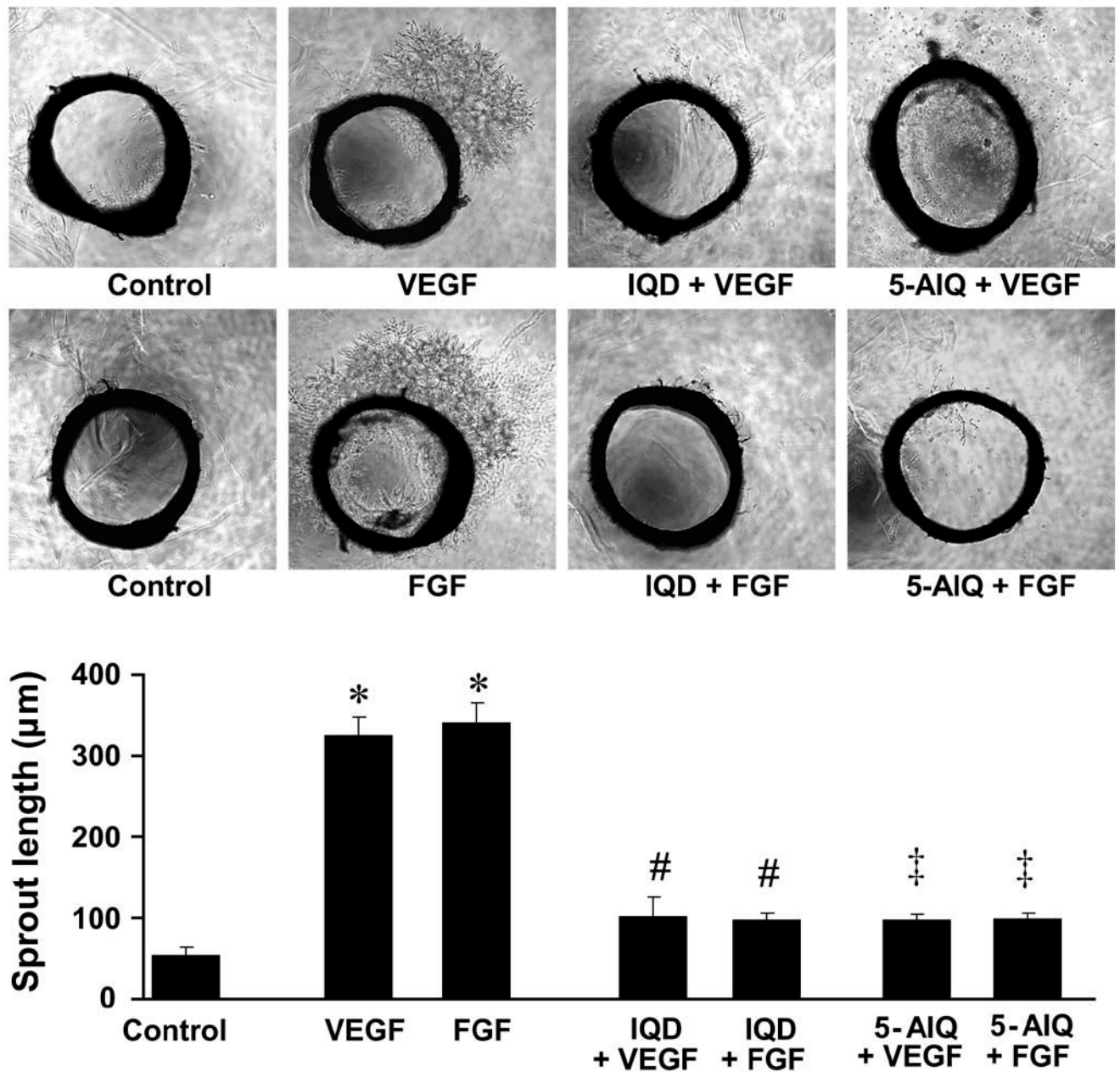
**Fig.3. PARP inhibitors attenuate VEGF- and FGF-induced tube formation of HUVECs**  
 HUVECs were treated with either VEGF/FGF or PARP inhibitors +VEGF/FGF and in vitro angiogenesis assays were performed using growth factor reduced Matrigel as described in the Methods section.

(A) Representative images of the tube formation of HUVECs when treated with treatments as indicated.

(B) Quantitative data on the effect of IQD on the tube formation of HUVECs.

(C) Quantitative data on the effect of 5-AIQ on the tube formation of HUVECs.

\*  $P < 0.00001$  vs. control; #  $P < 0.05$  vs. VEGF or FGF; ‡  $P < 0.01$  vs. VEGF or FGF; †  $P < 0.001$  vs. VEGF or FGF,  $n = 3$ .



**Fig.4. PARP inhibitors abrogate sprouting of rat aortic rings**

Representative images of rat aortic ring sprouting in response to treatments as indicated in the figure (above) and quantitative data on the sprout lengths (below).

\*  $P < 0.00001$  vs. control; #  $P < 0.05$  vs. VEGF or FGF; ‡  $P < 0.01$  vs. VEGF or FGF; †  $P < 0.001$  vs. VEGF or FGF,  $n = 3$ .