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Authors: Rajesh M, Mukhopadhyay P, Bátkai S, Godlewski G, Haskó G, Liaudet L, Pacher P

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Pharmacological inhibition of poly(ADP-ribose) polymerase inhibits angiogenesis

Mohanraj Rajesh[1], **Partha Mukhopadhyay**[1], **Sándor Bátkai**[1], **Grzegorz Godlewski**[1], **György Haskó**[2], **Lucas Liaudet**[3], and **Pál Pacher**[1,*]

[1]*Laboratory of Physiological Studies, NIAAA, National Institutes of Health, Bethesda, Maryland, USA* [2]*Department of Surgery, UMDNJ-New Jersey Medical School, Newark, NJ 07103, USA* [3]*Department of Intensive Care Medicine, University Hospital, 1011 Lausanne, Switzerland*

Abstract

Poly(ADP-ribose)polymerase (PARP) is a nuclear enzyme which plays an important role in regulating cell death and cellular responses to DNA repair. Pharmacological inhibitors of PARP are being considered as treatment for cancer both in monotherapy as well as in combination with chemotherapeutic agents and radiation, and were also reported to be protective against untoward effects exerted by certain anticancer drugs.

Here we show that pharmacological inhibition of PARP with 3-aminobenzamide or PJ-34 dose-dependently reduces VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells *in vitro*.

These results suggest that treatment with PARP inhibitors may exert additional benefits in various cancers and retinopathies by decreasing angiogenesis.

Keywords

Poly(ADP-ribose)polymerase (PARP); angiogenesis; proliferation; migration; tube formation; HUVEC; 3-AB; PJ-34

Introduction

Poly(ADP ribose) polymerase (PARP), also known as poly(ADP ribose) synthetase (PARS), is a family of abundant nuclear enzymes of eukaryotic cells that participates in the regulation of DNA repair, gene transcription, cell cycle progression, chromatin function, genomic stability and cell death [1;2]. Pharmacological inhibitors of PARP are recently being considered as treatment for cancer both in monotherapy as well as in combination with chemotherapeutic agents and radiation [2;3;4;5;6;7] and entering clinical development as cytoprotective agents in cardiovascular diseases [2;4;8]. Angiogenesis is very important in the growth and metastasis formation of a variety of tumors, and increased angiogenesis also contributes to the development of blindness associated with diabetic retinopathy. In the present study we studied

Address correspondence to: Pál Pacher M.D., Ph.D., F.A.P.S., Section on Oxidative Stress and Tissue Injury, Laboratory of Physiological Studies, National Institutes of Health/NIAAA, 5625 Fishers Lane, MSC-9413, Bethesda, Maryland 20892-9413, USA., Phone: (301) 443-4830; Fax: (301)480-0257; E-mail: pacher@mail.nih.gov or ppacher@lycos.com.

the effects of PARP inhibition with 3-AB and PJ-34 on VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells in vitro.

Materials and Methods

Reagents

Human recombinant $VEGF_{165}$ was purchased from R&D systems (Minneapolis, MN). PARP inhibitor 3-aminobenzamide was obtained from Sigma, and PJ-34 was from Inotek Pharmaceuticals (Boston, MA).

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 according to manufacture's instruction. Cells were grown in either 100 mm dishes or 6 well multi-dish chambers coated with 0.2 % gelatin. Cells were used within passages 3 to 7. Prior to treatment, cells were acclimatized in endothelial basal medium containing 1% FBS (Invitrogen, CA) 6 hrs and then treated.

Cell viability assays

Cells were grown in 96 well plates and treated with either aminobenzamide (3-AB) or PJ 34 with varying concentrations for 6 hrs in growth factor free medium containing 1 % FBS. Then effects of the PARP inhibitors on cell viability were determined using commercially available MTT assay (Promega, WI). Each experiment was repeated in triplicate and the entire set of experiments was repeated three times.

Proliferation assay

Endothelial cells (5×10^3) cells were suspended in 100 μl of growth factor free medium containing 1 % FBS and then treated with either VEGF 20 ng/ml alone or PARP inhibitors 3-AB (0.5–6 mM) and PJ 34 (0.5–6 μM) and seeded on to 0.2 % gelatin coated 96 well plates and allowed to proliferate for 24 hrs. Then BrdU labeling solution was added and incubated further for 12 hrs. After the incubation, the effect of PARP inhibitors on VEGF 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 medium was aspirated and 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 at 450 nm. The treatments were performed in triplicate and the experiment was repeated at least three times.

Migration assay

The migration assays were performed in a 24–well modified Boyden chamber as described earlier [9]. In brief, 8 μm cell culture inserts (BD Biosciences) coated with 0.2 % gelatin (Sigma) were placed over the bottom chamber containing 20 ng/ml VEGF as the chemo-attractant. Growth factor free medium containing 1% FBS served as negative control. 3×10^4 cells were suspended in 150 μl in growth factor free medium containing 1% FBS. Then the cells were pretreated with PARP inhibitors, for 30 min at 37°C in 5% CO_2 incubator. Then the HUVECs 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 inhibits on inhibiting the formation of tube-like structures on growth factor reduced Matrigel (BD Biosciences) were performed as described earlier [9;10]. In brief, 96 well plates were coated with 50 μl of growth factor reduced Matrigel and allowed to solidify at 37°C in 5% CO_2. During the incubation for the solidification of Matrigel, the cells were treated exactly as described as above and seeded on to the solidified matrix. After 12 hrs of incubation at 37°C in 5% CO_2, 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 (μm) for three photographic fields per experimental condition. Each treatment was performed in duplicate and the set of experiment was independently repeated three times.

Statistical analysis

Values are represented as mean ± SD. The statistical significance of the data was analyzed using students '*t*' test. $P < 0.05$ was considered as significant.

Results

PARP inhibitors did not affect cell viability in HUVECs

As shown in Fig.1, and consistently with results of numerous previous studies ([11;12;13]; reviewed in [1]) 3-AB (0.5–6 mM) and PJ-34 (0.5–6 μM) did not decrease cell viability in HUVECs.

PARP inhibitors decrease VEGF- induced proliferation of HUVECs

As shown in Fig.2., VEGF enhanced the proliferation of HUVECs (measured by BrdU incorporation assay) by 4 fold (P<0001 vs. control) and both 3-AB and PJ-34 significantly inhibited the HUVECs proliferation in a dose dependent manner (P < 0.001 vs. VEGF). 3-AB or PJ-34 alone had no effect on the proliferation of HUVECs (data not shown).

PARP inhibitors decrease VEGF- induced migration of HUVECs

As shown in Fig. 3, VEGF significantly enhanced the migration of HUVECs in the order of ~3 fold (P < 0.001 vs. control). 3-AB and PJ-34 elicited concentration dependent inhibition in VEGF induced migration of HUVECs (Fig. 3A and B, P<0.001 vs. VEGF). On the other hand, 3-AB or PJ-34 by itself had no effect on migration and the results were comparable to that of vehicle control (data not shown).

PARP inhibitors decrease VEGF-induced tube formation in vitro in HUVECs

When HUVECs were placed on growth factor reduced Matrigel, in the presence of VEGF 20 ng/ml, endothelial cells differentiate in to tube- like structures mimicking the neoangiogenesis process *in vivo* (Fig. 4, P < 0.001 vs. control). When HUVECs were pretreated with 3-AB (0.5 – 6 mM) or PJ-34 (0.5 – 6.0 μM) and plated on to polymerized Matrigel, in the presence of VEGF (20 ng/ml), both 3-AB and PJ-34 dose-dependently inhibited the tube formation of HUVECs when compared with cells treated with VEGF alone (Fig. 4; $P < 0.001$ vs. VEGF). Quantification of tube length revealed that VEGF enhanced the tube formation by ~3 fold when compared to control cells and this was decreased by PARP inhibitors in dose-dependent manner. PARP inhibitors alone had no effect on tube formation of HUVECs (data not shown).

Discussion

Pharmacological inhibitors of PARP have been shown to decrease tumor growth and to restore sensitivity of resistant tumors to methylating agents or topoisomerase I inhibitors (drugs recently used for the treatment of various malignancies refractory to conventional chemotherapy), and to increase the sensitivity of cancer cells to radiotherapy [3;4;5;6;7;14;15;16;17;18;19;20;21;22]. The use of PARP inhibitors as anticancer agents exploits the fact that the repair of certain types of antitumor-agent related cellular damage relies almost exclusively on the functional integrity of PARP [3].

In the present study we demonstrate that pharmacological inhibition of PARP exerts antiangiogenic effects by reducing VEGF-induced proliferation, migration and tube formation of human umbilical vein endothelial cells *in vitro*. These results suggest that PARP inhibition may exert additional beneficial effects by decreasing angiogenesis-dependent growth in certain types of tumors and by inhibiting tumor metastasis formation. Interestingly, a semisynthetic tetracycline antimicrobial antibiotic minocycline, which has recently been reported to have potent PARP-1 inhibitory effects [23;24] also inhibits angiogenesis [25].

Antiangiogenetic potential of PARP inhibitors can further be exploited for the treatment of diabetic retinopathy, where the pathologically increased angiogenesis is the major cause of complications and blindness. PARP activation is also involved in the development of various diabetic complications (reviewed in [26]) including retinopathy [27;28] and PARP inhibitors exert protective effects against these pathologies [26].

Excitingly, PARP inhibitors were also reported to protect against nephrotoxicity of cisplatin [29] and cardiotoxicity of doxorubicin [30;31], which cause oxidative/nitrosative stress and consequent PARP overactivation and cell death. The cytoprotective effects of PARP inhibitors comprise of inhibition of the pathological overactivation of PARP, and blockade of the cell from entering into necrosis via over-utilization of cellular NAD^+ pools and promotion of cellular energetic failure [1;2].

Collectively, the present results suggest that treatment with PARP inhibitors may exert additional benefits in various cancers and retinopathies by decreasing angiogenesis.

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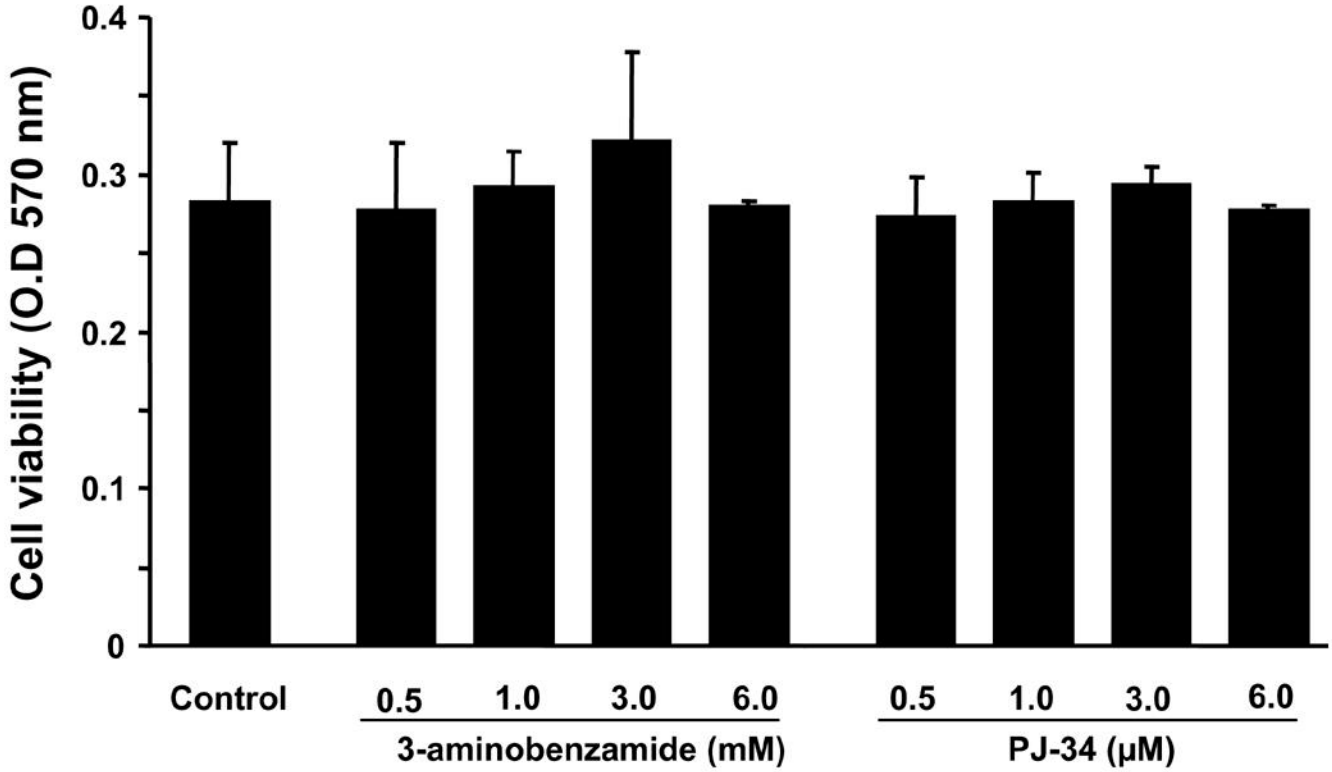

Fig.1. PARP inhibitors did not affect cell viability of HUVECs

Cells were grown in 96 well plates and treated with either aminobenzamide (3-AB) or PJ-34 with varying concentrations as indicated for 6 hrs. Then the cytotoxic effects of the PARP inhibitors were determined using commercially available MTT assay, n = 3.

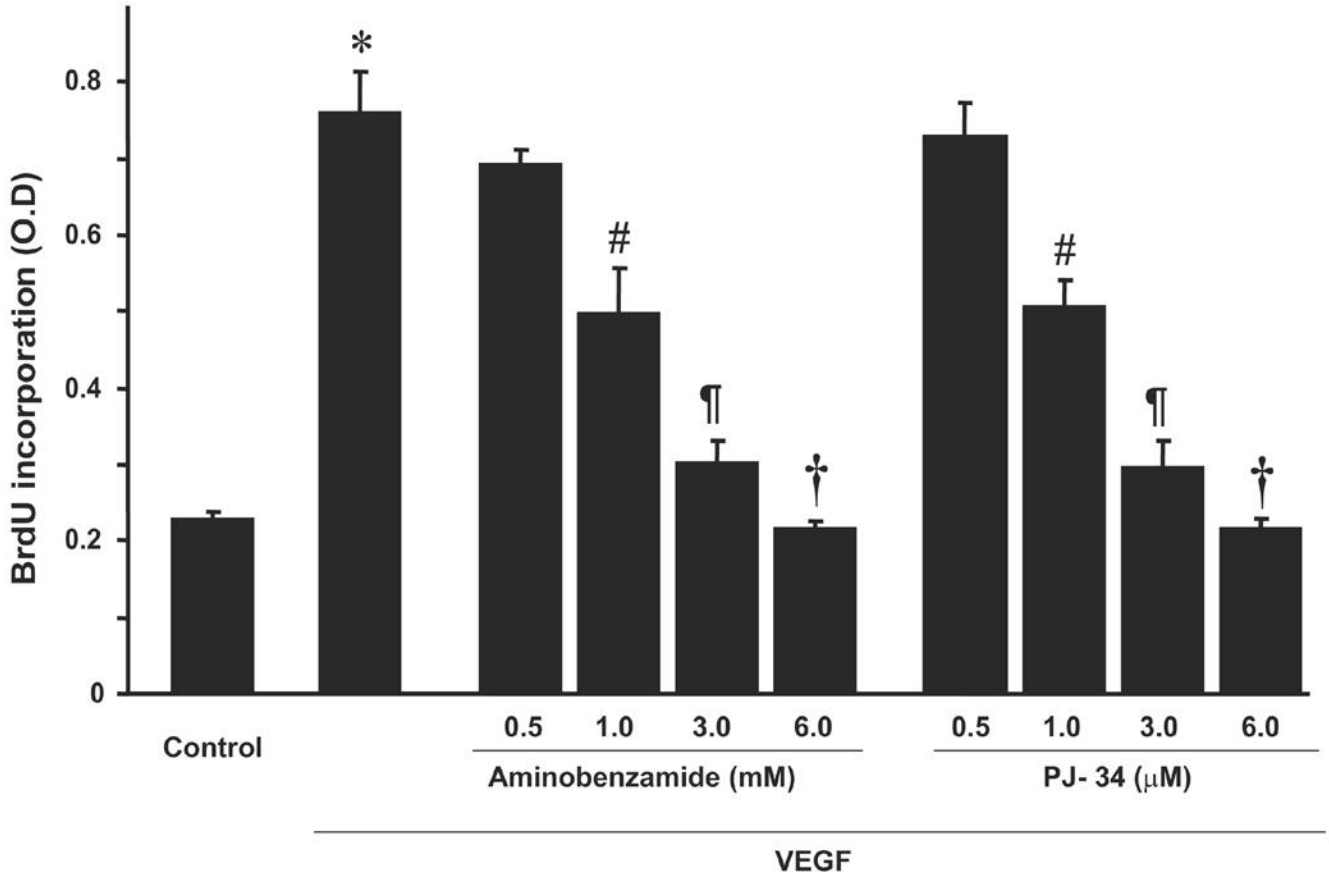

Fig.2. PARP inhibitors decrease VEGF-induced proliferation of HUVECs

HUVECs were treated with either VEGF alone or with PARP inhibitors as indicated and the proliferation of HUVECs were assed by measuring the rate of BrdU incorporation by ELISA kit colorimetricaly at 450 nm. * $P < 0.001$ vs. control ; # $P < 0.001$ vs. VEGF ; ¶ $P < 0.001$ vs. VEGF ; † $P < 0.001$ vs. VEGF ; n =3.

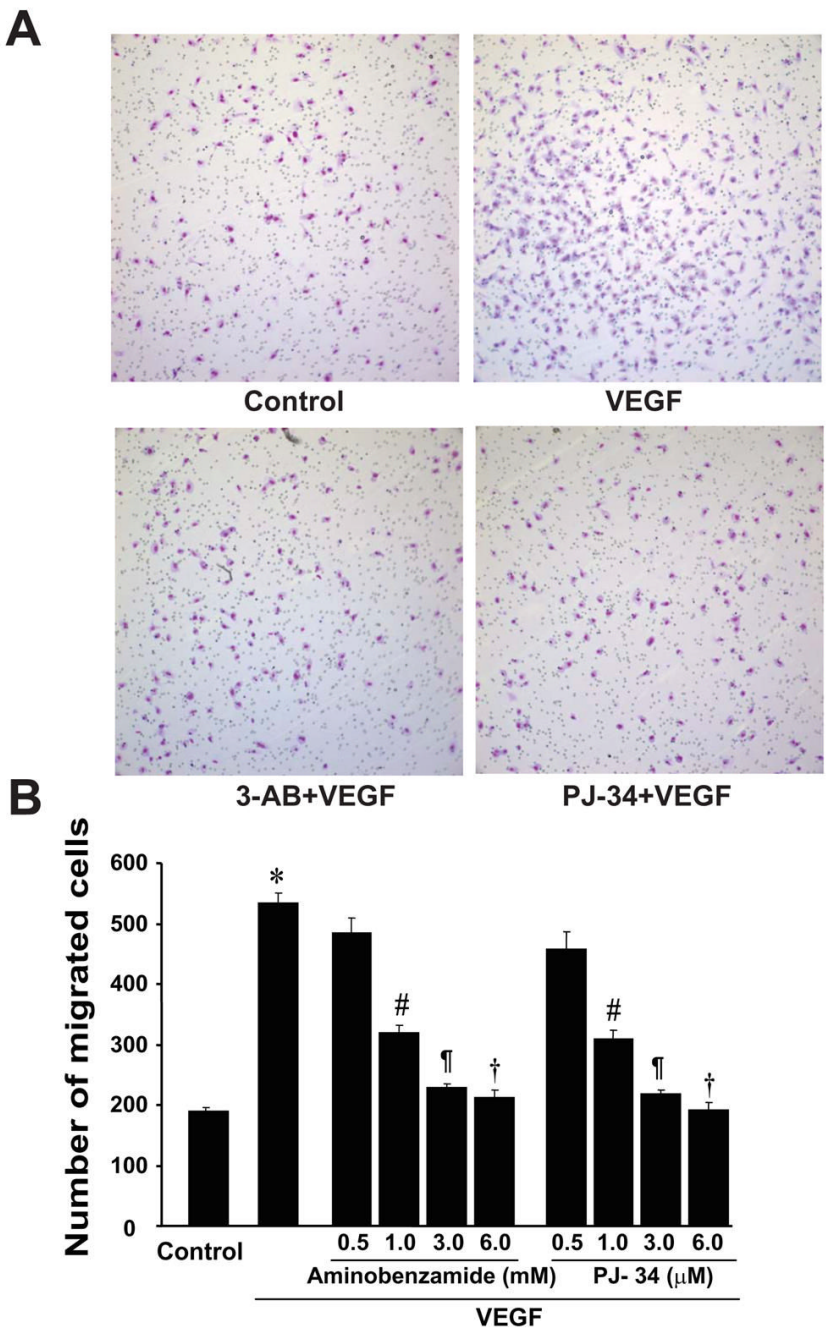

Fig.3. PARP inhibitors attenuate VEGF-induced migration of HUVECs

Cells were treated with either VEGF or PARP inhibitors as indicated and migration assays were performed using Boyden chamber.

(A) Depicts the representative images of HUVECs migrated in response to treatment with either vehicle control, 20 ng/ml VEGF alone, 6 mM 3-AB + VEGF or 6 μM PJ–34 respectively.

(B) Shows the quantification of data for cell migration in response to treatments. * $P < 0.001$ vs. control; # $P < 0.001$ vs. VEGF ; ¶ $P < 0.001$ vs. VEGF ; † $P < 0.001$ vs. VEGF ; n =3.

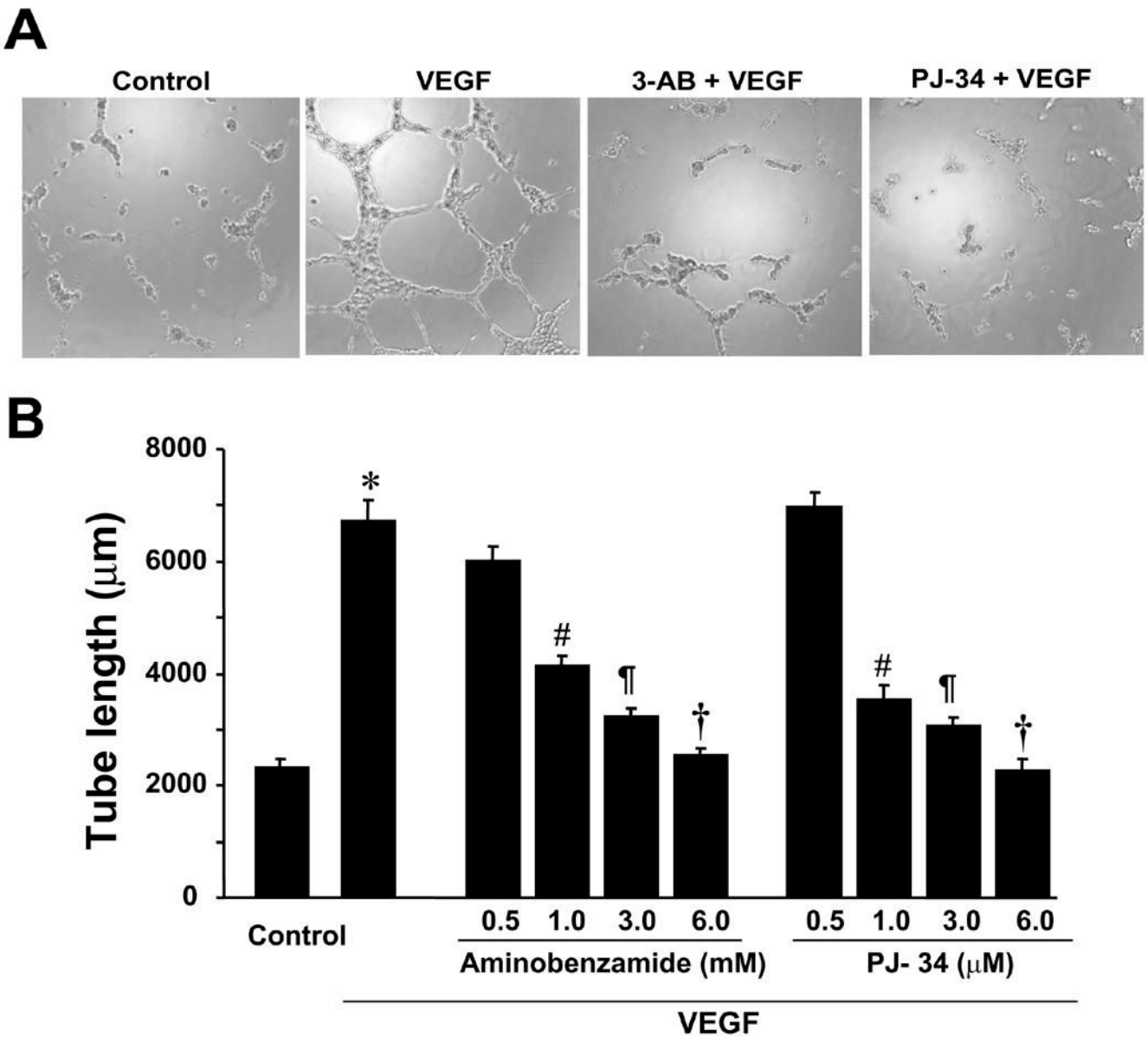

Fig.4. PARP inhibitors inhibit VEGF-induced tube formation in vitro

HUVECs were treated with either VEGF or PARP inhibitors+VEGF and an in vitro angiogenesis assays were performed using growth factor reduced Matrigel as described in the Methods section.

(A) Depicts the representative images of tube formation of cells treated with either VEGF alone or with 3-AB (6 mM) or PJ-34 (6 μM) showing the maximum inhibitory effect.

(B) Shows the quantitative data for the tube lengths in cells treated as indicated in the figure. * $P < 0.001$ vs. control ; # $P < 0.001$ vs. VEGF ; ¶ $P < 0.001$ vs. VEGF ; † $P < 0.001$ vs. VEGF ; n =3.