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Purpurin inhibits adipocyte-derived leucine aminopeptidase and angiogenesis in a zebrafish model

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ABSTRACT

Adipocyte-derived leucine aminopeptidase (A-LAP) is a novel member of the M1 family of zinc metallopeptidases, which has been reported to play a crucial role in angiogenesis. In the present study, we conducted a target-based screening of natural products and synthetic chemical libraries using the purified enzyme to search novel inhibitors of A-LAP. Amongst several hits isolated, a natural product purpurin was identified as one of the most potent inhibitors of A-LAP from the screening. In vitro enzymatic analyses demonstrated that purpurin inhibited A-LAP activity in a non-competitive manner with a K_i value of 20 M. In addition, purpurin showed a strong selectivity toward A-LAP versus another member of M1 family of zinc metallopeptidase, aminopeptidase N (APN). In angiogenesis assays, purpurin inhibited the vascular endothelial growth factor (VEGF)-induced invasion and tube formation of human umbilical vein endothelial cells (HUVEC). Moreover, purpurin inhibited in vivo angiogenesis in zebrafish embryo without toxicity. These data demonstrate that purpurin is a novel specific inhibitor of A-LAP and could be developed as a new anti-angiogenic agent.

1. Introduction

Angiogenesis is the process of new blood vessel formation by endothelial cells. This process plays a critical role in various pathophysiologic conditions including the growth of solid tumors, diabetic retinopathy, and rheumatoid arthritis [1]. The concept of inhibition of tumor growth by blocking angiogenesis was first proposed by Folkman in 1971 [2]. Since then, a number of angiogenesis inhibitors have been discovered to develop novel anticancer agents [3–5]. Drug target proteins crucial for angiogenesis in endothelial cells have been identified in parallel, such as vascular endothelial growth factor receptors (VEGFR) and matrix metalloproteases (MMPs) [6,7]. Aminopeptidases are amongst the drug target proteins for angiogenesis inhibition, which have emerged recently [8].

Aminopeptidases are metalloproteinases that remove amino acids from unblocked N-termini of peptides or proteins [9]. The N-terminal modification of peptides or proteins by aminopeptidase occurs either co-translationally or post-translationally and plays an important role in maturation and activation, as well as in the stability of proteins and peptides [10]. Accordingly, aminopeptidases participate in a wide range of biological processes including angiogenesis [11]. To date, three aminopeptidases including type-2 methionine aminopeptidase (MetAP2), aminopeptidase N (APN), and adipocyte-derived leucine aminopeptidase (A-LAP)/puromycin insensitive leucyl-specific aminopeptidase (PILSAP) have been reported to be involved in angiogenesis [8,12–16]. A-LAP and PILSAP is the identical enzyme which was isolated independently by two groups in different species. A-LAP was cloned from the human expressed sequence tag (EST) database [17], while PILSAP was isolated by the PCR amplification of rat pituitary mRNA and the

Abbreviations: A-LAP, adipocyte-derived leucine aminopeptidase; APN, aminopeptidase N; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA.

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subsequent cDNA-library screening [18]. Despite the important role of this enzyme in angiogenesis, only a few small molecule inhibitors of A-LAP have been identified so far [16].

Using a well-established fluorescent-based A-LAP activity measurement, we screened natural product and synthetic chemical libraries to identify novel A-LAP inhibitors. From the screening, purpurin was identified as one of the most potent hits among the natural product library tested. We subsequently conducted a series of experiments to validate inhibition of A-LAP activity and angiogenesis by purpurin both in vitro and in vivo. The present study demonstrates that purpurin is a novel inhibitor of A-LAP which can be applied to clinical studies for angiogenesis related diseases including cancer.

2. Materials and methods

2.1. Materials

Endothelial cell growth media (EBM-2) was purchased from Cambrex Bio Science (Walkersville Inc., MD, USA). Purpurin was purchased from TCI (Tokyo, Japan). 7-Leucine-4-amidomethylcoumarin (Leu-MCA) was from Peptide (Osaka, Japan), Matrigel was from BD Bioscience (Bedford, MA), Transwell plates were from Corning Costar (Cambridge, MA) and recombinant human VEGF was obtained from KOMA Biotech., Inc. (Seoul, Korea). A-LAP protein and its antibody were kindly provided by Masafumi Tsujimoto and Akira Hattori (The Institute of Physical and Chemical Research, Japan).

2.2. Cell culture and proliferation assay

Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium supplemented with 10% FBS and 1% antibiotics, and maintained in a CO_2 incubator adjusted at 37 °C. All experiments involving HUVECs were conducted with early passage of the cells (between passages 4 and 10). HeLa and C8161 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% antibiotics. The cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, Saint Louis, MO, USA) assay [19]. The cell viability was measured by staining of cells with trypan blue and counting under the microscope.

2.3. In vitro aminopeptidase assay

The in vitro enzyme activity assay of A-LAP was performed as described previously [17]. A-LAP (final conc., 500 ng/mL) and BSA (final conc., 10 μg/mL) were added in 25 mM Tris buffer (pH 7.4, 100 μL) with or without inhibitors in a black 96-well plate. The reaction was started by adding a substrate, 7-leucine-4 amidomethylcoumarin (Leu-MCA, final conc., 10 M) in a darkroom at 37 °C. After 1 h, the mixture was centrifuged, and the supernatant was collected for fluorescence reading at excitation and emission wavelengths of 360 and 460 nm, respectively. The activity of APN was determined as described previously [15].

2.4. In vitro angiogenesis assay

In vitro angiogenesis assays were performed using invasion and tube formation assays as described previously [15]. The invaded cells were counted under an IX70 fluorescence microscope (Olympus America, Melville, NY) for quantitative analysis.

2.5. In vivo zebrafish angiogenesis assay

Zebrafish embryos were incubated with 100 L of water containing drugs at one cell stage. In all experiments, final concentration of DMSO did not exceed 0.1%. After 28 h post fertilization (hpf), zebrafishes were washed and processed for vascular visualization assay, as described previously [20]. Briefly, tetramethylrhodamine isothiocyanate (TRITC)-dextran dye (Sigma) in 0.3× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $(CaNO_3)_2$, and 5.0 mM HEPES, pH7.6) was microinjected into the cardinal vein of zebrafish embryos, which results in labeling of the entire vascular system. Zebrafish embryos were visualized with an Olympus SZX12 stereomicroscope and photographed using an Olympus DP11 digital camera.

2.6. Knock-down of A-LAP using siRNA

siRNA against A-LAP (5′-CCCUCAAAUGGUCCCUUGCAAUCAU-3′ and 5′-AUGAUUGCAAGGGACCAUUUGAGGG-3′) were designed and obtained from Invitrogen (Grand Island, NY). For knock-down of A-LAP, HUVECs were transfected with the siRNA at a concentration of 600 nM using Lipofectamine 2000 reagent (Invitrogen) for 24 h according to the manufacturer's instruction. The knock-down of A-LAP by the siRNA was confirmed through reverse transcriptase polymerase chain reaction (RT-PCR for mRNA) and western blot (for protein levels) analyses.

2.7. Scratch-induced cell migration assay

The HUVECs were seeded in a 12-well plate and incubated until the cells reached 90% confluence. The cells were scratched using the tip of a micropipette, creating a linear line. The cells were then washed with PBS to remove floating cellular debris and incubated in the fresh medium with or without compound. The cell migration was regularly observed and photographed under IX70 microscopy at 100× magnification. The cell migration was quantified by counting the migrated cells from each wound edge.

2.8. Immunofluorescence

HUVEC were washed with PBS, fixed with 4% formaldehyde solution and permeabilized with 0.1% Triton X-100. The fixed cells were rinsed with PBS three times and incubated with blocking buffer containing 5% FBS in PBS at room temperature for 1 h. Anti-A-LAP antibody in blocking buffer was applied to the coverslips, and the incubation was continued for 1 h at room temperature. After washing with PBS three times, the cells were incubated with AlexaFluor 594-conjugated goat anti-rabbit IgG (Molecular Probe Inc., Eugene, OR) for 1 h at room temperature. The cells were washed with PBS and mounted with a mounting solution. The immunolabeled cells were observed under IX70 microscopy at 100× magnification.

2.9. Statistical analysis

Results are expressed as the mean ± standard error (SE). Student's *t* test was used to determine statistical significance between control and test groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Discovery of a natural product purpurin as an A-LAP inhibitor

The assay of A-LAP enzyme activity was performed based on the detection of the enzymatic degradation products of the fluorogenic

substrate of A-LAP, Leu-MCA. To identify new A-LAP inhibitors, a compound library consisting of 400 natural products and 600 synthetic chemical libraries [15] were screened against A-LAP activity. As a result, purpurin was isolated as one of the most potent inhibitors of A-LAP from the natural product library (Fig. 1A). Purpurin inhibited A-LAP activity in a dose-dependent manner with an IC_{50} value of 20 M (Fig. 1C). To examine the specificity of enzyme inhibition, we tested the effect of purpurin on the activity of aminopeptidase N (APN), another member of M1 family of zinc metallopeptidases. Purpurin did not inhibit the activity of APN at the concentration up to 50 μM, demonstrating relative specificity of purpurin to A-LAP (Fig. 1D). Next, we performed the kinetics analyses of the inhibition of A-LAP activity by purpurin. The Lineweaver–Burk plot of inhibition showed that purpurin noncompetitively inhibited the activity of A-LAP (Fig. 1E). The K_i value of the inhibition was determined as 20 M from the Dixon plot of the inhibition (Fig. 1F). These data demonstrate that purpurin is a non-competitive natural product inhibitor of A-LAP.

3.2. Purpurin inhibits of HUVEC proliferation and angiogenesis

Angiogenesis is a multistep physiological process of forming new capillaries from preexisting blood vessels. Three major steps of angiogenesis have been reported, which include the local proliferation of endothelial cells, degradation of subendothelial capillary basement membranes, and formation of vascular sprouts [21]. Each step of angiogenesis can be analyzed in vitro using well-established cell-based assays which are being widely used to develop angiogenesis inhibitors or activators. We first examined the effect of purpurin on HUVEC proliferation using the MTT assay. Purpurin inhibited the proliferation of HUVECs in a dose dependent manner with an IC_{50} value of 30 M (Fig. 2A). However, the proliferation of other types of cells such as HeLa (cervical carcinoma) and C8161 (melanoma) was not significantly inhibited by purpurin at the same concentration range used for HUVEC. These data suggested that purpurin selectively inhibited the proliferation of endothelial cells over the other types of mammalian cells. To see if the inhibition of HUVEC proliferation by purpurin was through cytotoxic effects on the cells, we conducted trypan blue exclusion assay for the HUVEC treated with purpurin. Purpurin did not significantly affect the viability of HUVEC up to 20 M treatment (Fig. 2B). A marginal decrease in the HUVEC viability was observed between 40 and 60 M, and the significant cytotoxicity was observed over 80 M treatment with purpurin. Thus, the concentrations equal to or lower than 20 M of purpurin were used for all in vitro angiogenesis assays.

We, next, conducted the chemotactic invasion assay of HUVEC. HUVEC were loaded on the porous membrane of an upper transwell chamber which was pre-coated with Matrigel. VEGF was added to the lower chamber as a chemo-attractant to facilitate

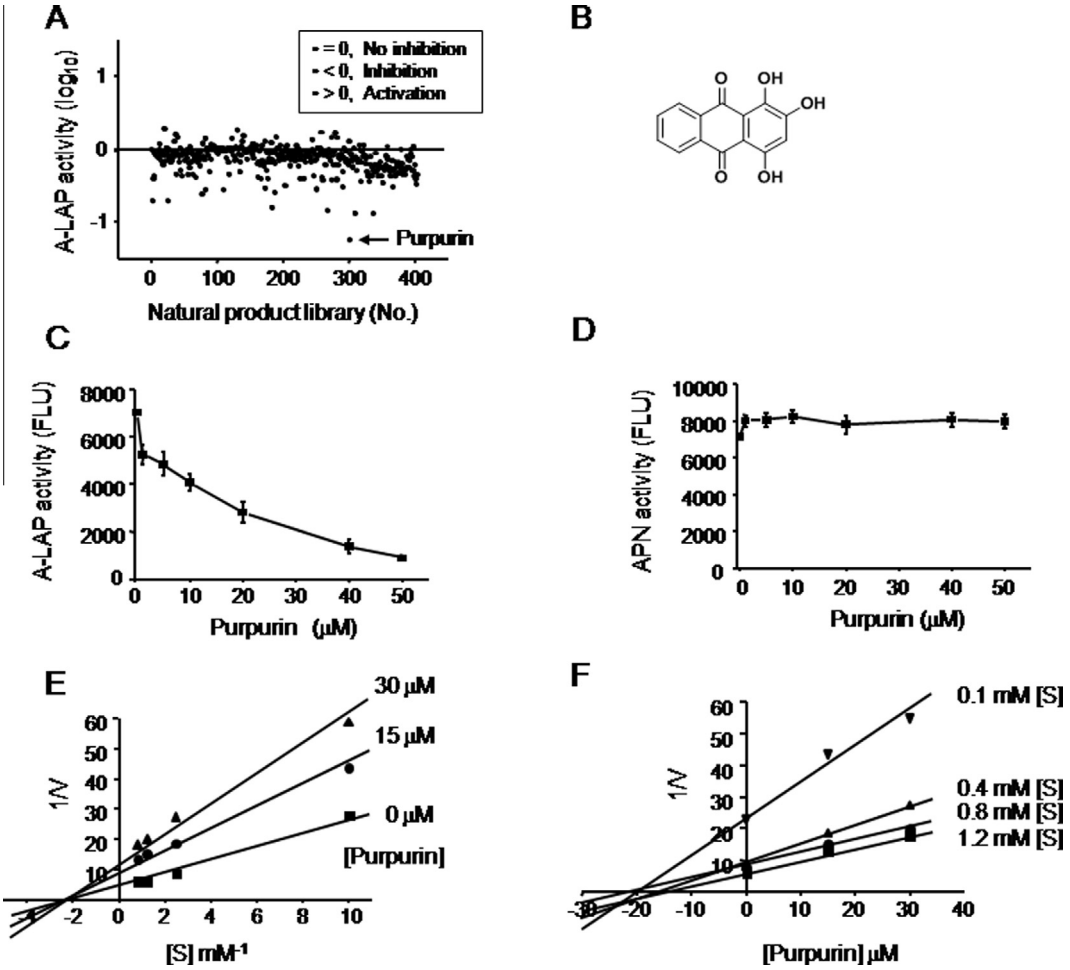

Fig. 1. Chemical screen identified a natural product purpurin as an inhibitor of A-LAP. (A) A-LAP (500 ng/mL) was incubated with Leu-MCA (10 M) in the presence or absence of drugs for 1 h at 37 °C. The substrate fluorescence was measured at the excitation and emission wavelengths of 360 nm and 460 nm, respectively. The ratio of A-LAP activity (drug treatment/control) was converted into log10 scale and plotted. (B) Chemical structure of purpurin is shown. (C and D) Effects of various concentrations of purpurin on A-LAP (C) and aminopeptidase N (APN, D) are shown. Data represent mean ± SE from three independent experiments. (E) Lineweaver–Burk plot of inhibition of A-LAP by various concentrations of purpurin is shown. (F) Dixon plot of A-LAP activity at various substrate concentrations is shown.

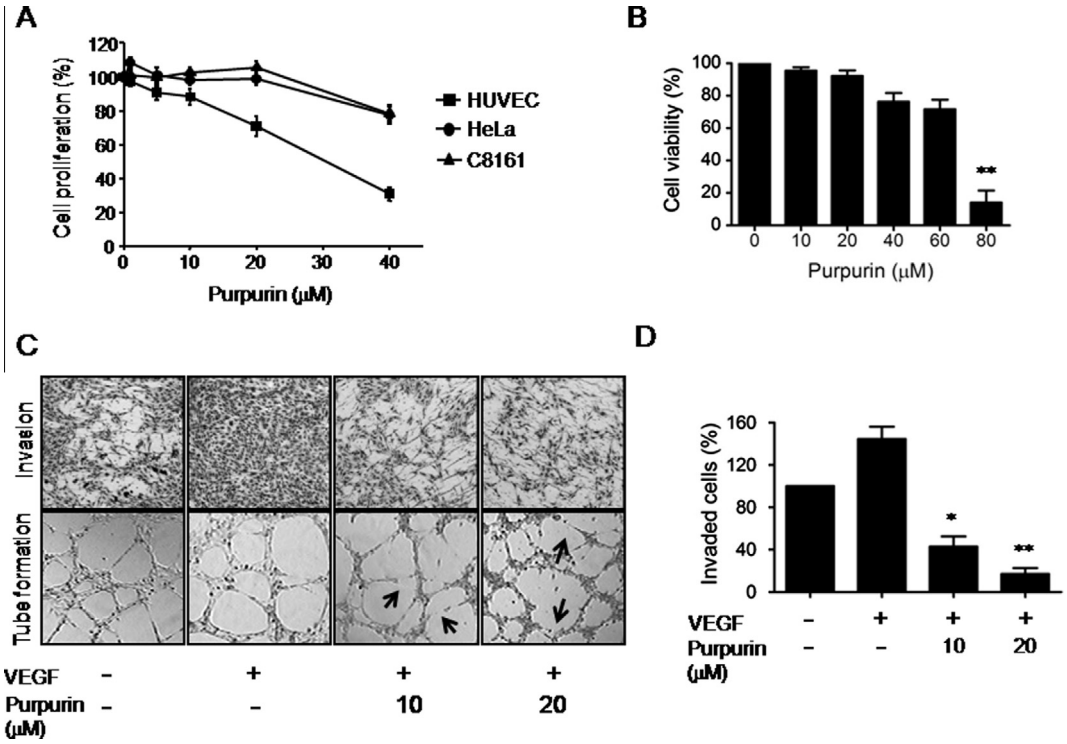

Fig. 2. Purpurin inhibited angiogenesis in vitro. (A) Cells (2×10^3 cells/well) were plated in 96-well plates and were treated with purpurin at various concentrations for 72 h. The cells proliferation was measured by an MTT assay. (B) HUVEC (5×10^3 cells/well) were plated in a 24-well plate and treated with purpurin for 72 h. The cell viability was measured by trypan blue staining. Data represent mean ± SE from three independent experiments. $^{**}P < 0.01$ versus no drug control. (C) For invasion assay, serum-starved HUVEC (7×10^4 cells/well) were seeded onto gelatin and Matrigel coated Transwell chambers. The migrated cells through Matrigel coated filter were stained with hematoxylin/eosin and counted under a microscope. For tube formation assay, serum-starved HUVEC (8×10^4 cells/well) were seeded on Matrigel in the presence or absence of VEGF. Photos were taken under the microscope (100× magnification) after 18 h of purpurin treatment. Arrows indicate broken tube networks, an indicative of angiogenesis inhibition. (D) Invaded cells from the data (C) were counted under a microscope and plotted using the Graphpad prism software. Data represent mean ± SE from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus VEGF control.

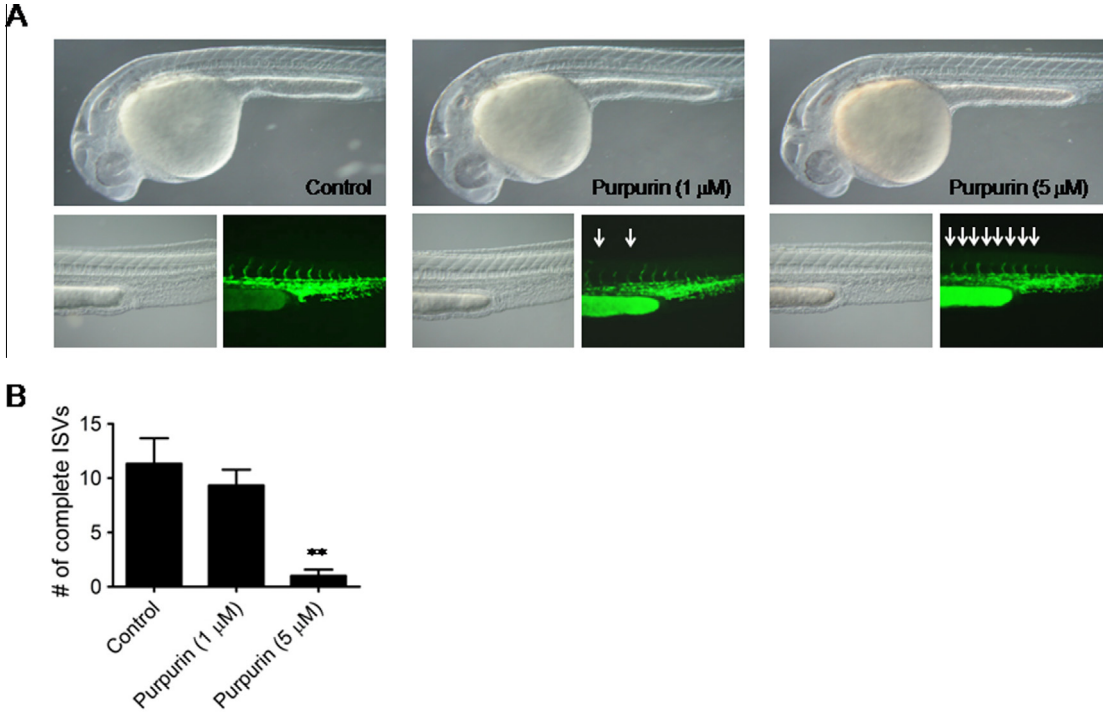

Fig. 3. Purpurin inhibited in vivo angiogenesis in a zebrafish angiogenesis model. (A) Twenty-eight hpf embryos were processed for vascular visualization using TRITC-dextran dye. Embryos in the vehicle treatment show complete ISV formation from dorsal aorta. Arrows indicate inhibition of ISV extension by purpurin. The morphology of embryos is apparently normal in all conditions. (B) The number of complete ISV was counted and plotted using the Graphpad prism software. $^{**}P < 0.01$ versus control.

the invasion and migration of HUVEC through 8 m pores in the upper chamber. The migrating cells through the pores were then fixed, stained with hematoxylin/eosin and counted under a microscope. As shown in the Fig. 2C (*upper panels*), VEGF significantly enhanced the number of invaded HUVEC compared to the basal level of invasion. Purpurin dose-dependently inhibited the VEGF-induced invasion of HUVEC in a non-cytotoxic concentration range (Fig. 2C, *upper panels* and Fig. 2D). Next, we analyzed the in vitro tube formation of HUVEC, an assay reminiscent of in vivo vascular sprout formation. HUVEC loaded on the Matrigel formed extensive tube-like networks which were significantly enhanced by addition of VEGF (Fig. 2C, *lower panels*). Purpurin inhibited the VEGF-induced tube formation of HUVEC in a dose-dependent manner. These results demonstrate that purpurin effectively inhibits VEGF-induced in vitro angiogenesis.

3.3. Purpurin inhibits in vivo angiogenesis

The zebrafish angiogenesis model has been rapidly established to develop novel small molecule regulator of angiogenesis as well as to study the regulation of vascular development in vertebrates, since a striking degree of anatomical and functional conservation of vascular pattern was found between zebrafish and other vertebrates including mammals [22]. Zebrafish embryos are cultured in an aqueous solution and are readily permeable to small molecules in the culture media allowing the study of small molecule regulation or perturbation of specific physiological processes of the organism [22,23]. We thus employed a well-established zebrafish angiogenesis model to evaluate the antiangiogenic activity of purpurin in vivo. Zebrafish embryos at one cell stage were incubated with culture media containing DMSO as a vehicle or purpurin. After 28 h post-fertilization (hpf), zebrafishes were washed and the entire vascular system was visualized by a microinjection of tetramethylrhodamine isothiocyanate (TRITC)-dextran dye into the cardinal vein of zebrafish embryos. Compared to the vehicle control, 1 M purpurin caused a partial inhibition of extension of the intersegmental vessels (ISVs) from the dorsal aorta (Fig. 3). Purpurin at 5 M inhibited the ISV extension from the dorsal aorta by over 50%. No apparent toxicity to the fish was observed by purpurin treatment. These data demonstrate that purpurin is a potent inhibitor of angiogenesis in vivo.

3.4. Knock down of A-LAP expression by siRNA inhibits HUVEC angiogenesis

We identified purpurin as an inhibitor of A-LAP and angiogenesis. However, the causal relationship between A-LAP and angiogenesis inhibition by purpurin is obscure. To examine whether the

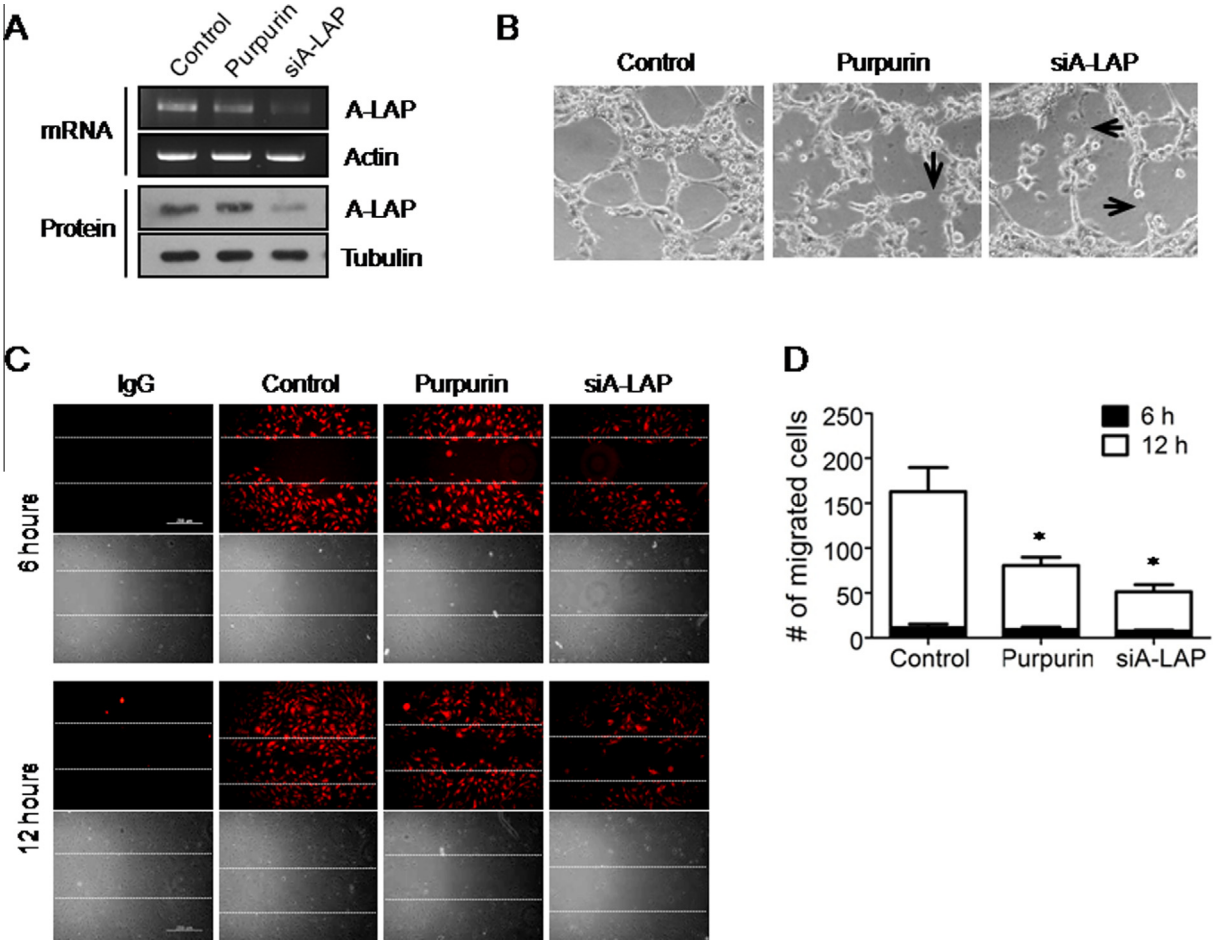

Fig. 4. Purpurin treatment recapitulated A-LAP knock-down phenotype in HUVEC. (A) mRNA and protein levels of A-LAP were analyzed from the HUVEC treated with either purpurin or siRNA for A-LAP (siA-LAP). Actin and tubulin were used as loading controls for RT-PCR and western blot, respectively. (B) HUVEC were treated with either purpurin or siRNA for A-LAP and tube formation assay was conducted. Arrows indicate broken tube networks, an indicative of angiogenesis inhibition. (C) HUVEC were grown in 12-well plates until confluent and the bottom of each well was scratched using the tip of a micropipette. Each wound edge is indicated by white dotted lines. The cells were then treated with either purpurin or siRNA for A-LAP for indicated time periods. The cells were fixed and processed for A-LAP immunofluorescence and observed under fluorescent (*top panels*) and phase contrast microscopes (*bottom panels*). IgG alone was used as a negative control for the immunofluorescence analysis. (D) The cell migration was quantified by counting the migrated cells from each wound edge. $^{*}P < 0.05$ versus control.

functional inhibition of A-LAP in endothelial cells is sufficient to block angiogenesis, we knocked down the expression of A-LAP in HUVEC using a specific siRNA and analyzed the HUVEC angiogenesis in comparison with purpurin treatment. The transfection of siRNA specific for A-LAP markedly reduced the mRNA and protein levels of A-LAP in HUVEC (Fig. 4A). The treatment of purpurin did not significantly affect the expression level of A-LAP in HUVEC. Knock-down of A-LAP by the siRNA was further confirmed by immunofluorescence analysis in HUVEC (data not shown). Like purpurin, siRNA knock-down of A-LAP significantly inhibited the tube formation of HUVEC, implying that the depletion of A-LAP level is sufficient to inhibit angiogenesis in HUVEC (Fig. 4B). We next tested the effect of siRNA knock-down of A-LAP on the scratch-induced HUVEC migration through the parallel observation of immunofluorescent and phase-contrast microscopy. As a negative control, immunofluorescence analysis with IgG alone (without primary antibody against A-LAP) did not show any fluorescence in HUVEC (Fig. 4C, *left panel*). Control and purpurin treated groups showed high fluorescence, whereas siRNA for A-LAP treated one showed significant decrease in fluorescence intensity (Fig. 4C, *right panels*). At 6 h after scratching, there were no significant differences in HUVEC migration between control and treatment groups. However, at 12 h after scratching, both purpurin and siRNA for A-LAP significantly delayed the HUVEC migration compared to control. Overall, these in vitro results demonstrate that purpurin inhibits angiogenesis, at least in part, through inhibition of A-LAP in endothelial cells.

4. Discussion

A-LAP belongs to the M1 subfamily of aminopeptidase, which contains a consensus HEXXH(X)18E motif and a central Zn^{2+} ion essential for enzymatic activity [16,24]. A crucial role of A-LAP in angiogenesis has been recognized since the A-LAP gene was known to be differentially expressed upon VEGF treatment to murine embryonic stem cells (ECs) [16]. By the PCR-coupled subtractive hybridization, A-LAP was identified as one of the genes exclusively overexpressed by VEGF in a murine EC line MSS31. Specific inhibition of A-LAP expression in the murine ECs inhibited the VEGF-induced proliferation, migration and tube formation of the ECs in vitro. It also inhibited angiogenesis in vivo. These results led us to hypothesize that specific inhibition of A-LAP by small molecules could be a promising strategy to suppress angiogenesis. In this study, we screened a library of synthetic compounds and natural products using a well-established spectrofluorometric enzyme assay and identified purpurin as a novel small molecule inhibitor of A-LAP. A closely related M1 aminopeptidase, CD13/aminopeptidase N, was not significantly inhibited by purpurin, demonstrating a specificity of purpurin toward A-LAP. We further showed that purpurin attenuated VEGF-induced angiogenesis in vitro and inhibited angiogenesis in a zebrafish model. In an attempt to validate causal relationship between A-LAP inhibition and angiogenesis inhibition by purpurin, we found that purpurin phenocopied A-LAP knock-down in endothelial cells as demonstrated by inhibition of tube formation and cell migration by both treatments. These results demonstrate that purpurin is a novel small molecule inhibitor of A-LAP with potent anti-angiogenic activity.

Purpurin is an anthraquinone pigment from madder root, which has been traditionally used for food coloring, textile staining, color paints and medicinal treatments. Very little is known about the biological activity of purpurin. In 1999, Marczylo et al., has shown that purpurin inhibited the mutagenicity of heterocyclic amines in bacteria [25]. They subsequently found a strong anti-genotoxic activity of purpurin in Drosophila by inhibiting DNA damage induced by a number of carcinogens [26]. These studies suggest that there are beneficial effects of this natural product on cancer prevention. From the mechanistic view, purpurin is known to form a complex with various metal ions [27,28]. Since A-LAP is a zinc-binding metalloenzyme, it is intriguing to postulate that purpurin may form a complex with Zn^{2+} in the active site, thereby sequestering the metal ion from the enzyme active site. But, this hypothesis needs to be further validated at a molecular level.

In conclusion, we here report for the first time that a natural product purpurin is a novel inhibitor of A-LAP and is a potent angiogenesis inhibitor in vivo. Purpurin can be used as a lead to develop more potent and selective inhibitor of A-LAP and applied to the preclinical/clinical testing of anticancer activity.

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