Anti-breast cancer and anti-angiogenic potential of a lichen-derived small-molecule: barbatolic acid

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Abstract Natural products have been used for centuries as the most potent remedies to cure many diseases including cancer diseases. Angiogenesis is defined as the formation of new capillaries from existing vessels and plays a key role in the tumorigenesis process. Barbatolic acid is a little known lichen-derived small-molecule. In the present study, barbatolic acid was isolated from the acetone extract of *Bryoria capillaris*, and its anti-breast cancer and anti-angiogenic potential was investigated using human umbilical vein endothelial cells (HUVECs), human breast ductal carcinoma (T-47D) and cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma (HCC1428) cells. AlamarBlue™ cell viability, lactate dehydrogenase cellular membrane degradation and PicoGreen™ dsDNA quantitation assays were performed to determine the cytotoxic potential of barbatolic acid. Anti-angiogenic

and anti-migratory activities were investigated using endothelial tube formation assay and scratch wound healing assay, respectively. Half maximal inhibitory concentration of barbatolic acid was found to be higher than 100 μM for HUVEC, HCC1428 and T-47D cells. The sub-cytotoxic concentrations such as 25 μM , 50 μM and 100 μM were applied to determine anti-angiogenic and anti-migratory activities. Although the sub-cytotoxic concentrations inhibited endothelial tube formation and cellular migration in a concentration depended manner, barbatolic acid was more effective on the migration of HCC1428 and T-47D breast cancer cells than the migration of HUVECs. Consequently, the findings suggest that barbatolic acid is a promising anti-angiogenic and anti-migratory agent and the underlying activity mechanisms should be investigated by further in vitro and in vivo experiments.

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Introduction

Drug design and discovery studies still constitute a considerable amount of scientific research because of the lack of convenient medical cures available for many diseases such as cancer, retinopathies, psoriasis, atherosclerosis, etc., and screening of natural products is considered to be the most rational way in the search

for potentially functional and promising drug candidates (Atanasov et al. 2015). Lichens that are defined as the complex symbiotic organisms composed by photobiont (algae and/or cyanobacteria) and mycobiont (fungus) organisms, are considered to be not completely discovered, but promising natural sources of pharmacologically and biologically active compounds because they can produce more than thousands of exclusive secondary metabolites as a result of the tight metabolic relationships between the symbiotic partners (Elix and Stocker-Wörgötter 2008; Ranković and Kosanić 2015).

On the other hand, cancer still preserves its importance as the major health problem and second lead death factor for human being and many scientists consume a great effort and financial support to find new molecular targets, treatment strategies and anti-cancer drug candidates (Siegiel et al. 2017). Taking aim at the cancer development processes or the common features of different cancer types is considered as a rational strategy in anticancer drug design and discovery studies (Carmeliet and Jain 2000). Angiogenesis, the formation of new capillaries from existing blood vessels, regarded as the most important and common target in anticancer approaches because almost all cancer types require the formation of new blood vessels due to the irresistible need for removing metabolic wastes and acquiring nutrients, and the limited diffusion capability of oxygen in tissue since the maximal distance from the blood vessel for allowing a certain oxygen availability is approximately 100–200 μm (Grimes et al. 2014; Weis and Cheresch 2011; Wenger et al. 2015). Although many studies have been published on the anticancer activities of lichen-derived small molecules, very few studies have been conducted on the anti-angiogenic potential of lichen-derived small molecules, which can be listed as emodin, secalonic acid-D, vulpinic acid, olivetoric acid and usnic acid enantiomers, and only the last three of them are unique to the lichens (Varol 2015). However, these limited sources have shown that lichen-derived small-molecules have a promising potential to be anti-angiogenic agents because of their low cytotoxic potential and significant anti-angiogenic capacities (Foden et al. 1975; Guru et al. 2015; Koparal 2015; Koparal et al. 2010; Song et al. 2012). In the present study, barbatolic acid (Fig. 1a, b) was isolated from *Bryoria capillaris* and its cytotoxicity was investigated using cell viability, cellular

Fig. 1 Chemical structure of barbatolic acid. **a** 2D structure and **b** 3D structure

membrane degradation and dsDNA quantitation assays on human umbilical vein endothelial cells (HUVEC), human breast ductal carcinoma cell line (T-47D) and cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma cancer cell line (HCC1428). Additionally, anti-angiogenic and anti-migratory activities of the sub-cytotoxic concentrations of barbatolic acid were determined using endothelial tube formation assay, scratch wound healing assay, respectively.

Materials and methods

Lichen material and isolation of barbatolic acid

Bryoria capillaris (Ach.) Brodo and Hawksw was collected from *Pinus sylvestris* forest located in Mihaliçcik-Catacik, Eskişehir province on August 16, 2015. A voucher specimen was stored at the Herbarium of Anadolu University in the Department of Biology (ANES). Isolation of barbatolic acid from the acetone extract was achieved as previously described (Sariözlü et al. 2016; Varol et al. 2015, 2016). Briefly, 10 g grounded lichen sample was added to 100 ml acetone, sonicated for 60 min, left at room temperature overnight and filtered. Barbatolic acid was isolated from the extract residues using thin layer chromatography (TLC) with the standardized three solvent systems: A (Toluene: Dioxane: Acetic Acid, 180:45:5), C (Toluene: Acetic

Acid, 170:30) and G (Toluene: Ethyl Acetate: Formic Acid, 139:83:8), and the melting points and IR spectra were assessed as described in literature (Culbertson et al. 1977; Edwards et al. 2003; Huneck and Yoshimura 1996; Orange et al. 2010). Norstictic acid was used as an internal control for the calculation of Rf values. Stock solutions were initially prepared in dimethyl sulfoxide (DMSO) at 0.05–0.1 M concentration and further diluted with fresh complete medium.

Cell culture condition

Roswell Park Memorial Institute Medium (RMPI) 1640, nutrient mixture Ham's F-12 K medium, Fetal Bovine Serum (FBS), Endothelial Cell Growth Supplement (ECGS), penicillin–streptomycin, insulin solution and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). AlamarBlue™ cell viability kit and Quant-iT™ PicoGreen™ dsDNA assay kit were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Cytotoxicity detection kit was purchased from Roche (Mannheim, Germany). Tissue culture plates were purchased from TPP (Trasadingen, Switzerland). HUVEC (CRL-1730™), T-47D (HTB-133™) and HCC1428 (CRL-2327™) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained following the instructions of ATCC in a humidified atmosphere containing 5% CO₂ at 37 °C.

AlamarBlue™ cell viability assay

Growth inhibitory activities of barbatolic acid towards HUVEC (10×10^3 cells/well), T-47D (15×10^3 cells/well) and HCC1428 (15×10^3 cells/well) were measured using AlamarBlue™ (resazurin) cell viability assay as previously described (Hamid et al. 2004). Briefly, the seeded cells in 96-well cell culture plates were incubated for 24 h before they were treated with 25, 50, 100, 200 and 400 µM concentrations of barbatolic acid for 24 h and 48 h. After the incubation period, AlamarBlue™ solution (10 µl/well) was added to the cells and incubated for further 4 h. Fluorescence of the produced resorufin was monitored at 560 nm excitation wavelength and 590 nm emission wavelength using SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA, USA).

Membrane degradation assay

Lactate dehydrogenase (LDH) membrane degradation assay was performed as previously described (Varol et al. 2016). Briefly, the cells were plated and treated as in the AlamarBlue™ cell viability assay. After treatment, aliquots (100 µl) of the media were transferred to corresponding wells in 96 new well plates. The reaction mixtures that include the lactate molecules and the tetrazolium salts were added to the transferred media, incubated for 30 min at 25 °C, and 50 µl/well stop solutions were added to each well. Absorbance of the produced formazan salts was measured at 490 nm using the ELX808 IU microplate reader (Winooski, VT, USA).

PicoGreen™ dsDNA quantitation assay

PicoGreen™ dsDNA measurements were performed by following the instruction of Thermo Fisher Scientific Inc. The treatment processes were achieved as described in AlamarBlue™ assay. After that, the cells were washed thrice with phosphate buffered saline solution (PBS; Life Technologies, Carlsbad, CA, USA). The cells adhering to the plate surface were lysed using lysis solution, which was obtained from the ingredient of the LDH assay kit (Roche Applied Science, Mannheim, Germany). Then, PicoGreen™ solution was added to the cells and the culture plates were incubated on ice for 30 min. After the incubation period, fluorescence was measured at 480 nm excitation wavelength and 520 nm emission wavelength by using SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA, USA).

Matrigel tube formation assay

To identify the anti-angiogenic activity of barbatolic acid, endothelial tube formation assay was applied as previously described (Koparal 2015; Ouchi et al. 2004). Briefly, HUVECs were serum starved in endothelial growth basal medium (EBM)-2 for 4 h. Then, HUVECs (4×10^4 cells/well) were seeded on the matrigel-coated wells in 96-well culture plates, and were equilibrated with EBM-2 medium containing the test substance. After 12 h, 24 h and 48 h incubation periods, tube formations were observed under an Olympus IX71 inverted microscope (Tokyo, Japan)

and photographed with an Olympus DP71 camera at 10 × magnification.

Scratch wound healing assay

To observe the activity of barbatolic acid on the migration potential of cells, scratch wound healing assays were performed as previously described (Jonkman et al. 2014; Lampugnani 1999). Briefly, HUVEC (6×10^4 cells/well), T-47D (9×10^4 cells/well) and HCC1428 (9×10^4 cells/well) were plated in 24-well plates and incubated for 24 h. After incubation, the scratches were formed using a 1 ml pipette tip across the center of the wells. After scratching, the cells were gently washed twice with medium and the fresh medium that contained the sub-cytotoxic concentrations of barbatolic acid were added to the wells. The scratches were photographed at 0 h, 12 h, 24 h and 48 h time points using Olympus IX71 inverted microscope and photographed with an Olympus DP71 camera at 10 × magnification.

Statistical data analysis

SPSS (Statistical Package for Social Science) software was performed for the statistical analysis of the data, which was evaluated using a one-way ANOVA (Analysis of Variance) followed by the Tukey test. The experimental study results given in the figures were expressed according to the percentages of control as the mean $\pm$ SD. Asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$). Gaussian 03 software with HF theory at the B3LYP/3-21G level was performed to determine the geometric and electronic structure of barbatolic acid by ab initio/DFT quantum mechanical calculations, and ChemOffice Professional 15.1 was used to create the chemical structure of barbatolic acid (Fig. 1a, b). The figures were created using Microsoft® Excel and organized by using Photoshop CS6 software. ImageJ 1.50i was employed to evaluate the microscope photos of the wound healing and endothelial tube formation assays using the MRI Wound Healing Tool and Angiogenesis Analyzer, respectively. The parameters evaluated for the endothelial tube formation assays were number of junctions, total meshes area, number of segments, total branching length and total segment length.

Results

Cytotoxicity

The cytotoxic activity of barbatolic acid (Fig. 2) has been precisely determined using different assays in order to assess mitochondrial metabolic activity (AlamarBlue™ cell viability, Fig. 2a), cellular membrane degradation activity (LDH assay, Fig. 2b) and dsDNA degradation activity (PicoGreen™ assay, Fig. 2c). The results were observed in a correlation with each other and barbatolic acid showed a cytotoxic activity on HUVEC, HCC1428 and T-47D cells in a time- and concentration-dependent manner. Although T-47D breast cancer cells were the most affected cells, cisplatin-resistant HCC1428 cells were defined as the least affected cells. Although 200 and 400 μ M concentrations dramatically reduced proliferation of the cells, half maximal inhibitory concentration (IC_{50}) of barbatolic acid was observed to be higher than 100 μ M for HUVEC, HCC1428 and T-47D.

Angiogenesis

Anti-angiogenic activities of the sub-cytotoxic concentrations of barbatolic acid were investigated using endothelial tube formation assay and documented at 12, 24 and 48 h time points (Suppl. Fig. 1). Endothelial tube formation parameters such as number of junctions, total meshes area, number of segments, total branching length and total segment length were statistically analyzed using Angiogenesis Analyzer plugin, as can be seen at Fig. 3a. The results showed that barbatolic acid has a significant anti-angiogenic activity. Although 50 and 100 μ M concentrations completely suppressed the tube formation, it was observed that 25 μ M concentration decreased the branch sites and number of tubes (Fig. 3a, b).

Migration

Anti-migratory activity of barbatolic acid on HUVEC (Suppl. Figure 2), HCC1428 (Suppl. Fig. 3) and T-47D (Suppl. Fig. 4) cells were determined by the scratch wound healing assay (Fig. 4a). Although the sub-cytotoxic concentrations of barbatolic acid, except 25 μ M, completely blocked the migration of T-47D cells (Fig. 4b), HUVECs were affected less than the breast cancer cells. Additionally, the

Fig. 2 Antiproliferative activity of barbatolic acid on HUVEC, HCC1428 and T-47D cell lines. **a** AlamarBlue™ cell viability assay, **b** lactate dehydrogenase (LDH) cellular membrane degradation assay and **c** PicoGreen™ dsDNA quantitation

assay. Data are presented as mean $\pm$ standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$)

migration of HCC1428 was completely inhibited by 100 μM of barbatolic acid.

Discussion

Screening natural products is considered as one of the most rational strategies to discover new anticancer agents because there is a traditional knowledge about natural sources that have been employed to treat a broad-spectrum of cancer diseases for centuries (Bačkorová et al. 2012). Lichens have been considered as the exclusive sources of unique natural small-molecules among the other natural sources because of their distinctive biological and chemical structure (Varol 2015). Barbatolic acid is a little known lichen compound and there is only one activity study in the literature, which has investigated the anti-microbial activity of barbatolic acid (Sariözlü et al. 2016). The

Fig. 4 Anti-migratory activity of sub-cytotoxic concentrations of barbatolic acid. **a** Histogram represents wound closure of HUVEC, HCC1428 and T-47D cells at indicated time points during the scratch wound assay. Data are presented as mean $\pm$ standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$). **b** Representative images of HUVEC, HCC1428 and T-47D scratch wound assays are shown for the 0 h and 48 h time points (10 $\times$ magnification, scale bar 200 μm)

present study is the first report about anticancer and anti-angiogenic activity of barbatolic acid. The cytotoxicity assays showed that barbatolic acid has a toxic activity at the high concentrations, e.g., 200 and 400 μM . However, the cytotoxic potential of barbatolic acid is not significant as much as the enantiomers of usnic acid that was evaluated as an anti-angiogenic lichen compound in the previously published reports (Koparal et al. 2006; Koparal 2015; Song et al. 2012). Although our results showed that a concentration of 25

Fig. 3 Anti-angiogenic activity of sub-cytotoxic concentrations of barbatolic acid. **a** Histogram represents quantification of number of junctions, total meshes area, number of segments, total branching length and total segment length in the tube formation assay. Data are presented as mean $\pm$ standard

deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$) and **b** representative images of HUVEC tube formation are shown for the 12 h time point (10 $\times$ magnification, scale bar 200 μm)

μM of barbatolic acid inhibits endothelial tube formation, 50 μM of usnic acid and 150 μM of vulpinic acid were reported as initial concentrations for anti-angiogenic activity (Koparal 2015). Barbatolic acid is therefore more promising anti-angiogenic drug candidate than usnic acid and vulpinic acid. On the other hand, it is well known that angiogenesis as well as metastasis is a complicated process chain including but not limited to the cell proliferation, adhesion, migration, and extracellular matrix (ECM) degradation (Carmeliet and Jain 2000). Thus, identifying of anti-migratory activity of anticancer agent candidates on both the endothelial cells and cancerous cells is very important to evaluate their anti-angiogenic and anti-metastatic potentials. The scratch wound healing assay is widely accepted as a convenient, simple and cheap method to analyze cell motility in vitro (Liang et al. 2007). As a result of the preformed wound healing assays, it was determined that the motilities of HUVEC, HCC1428 and T-47D cells were affected depending on the applied concentrations of barbatolic acid. However, barbatolic acid has not a significant cytotoxic activity as much as the enantiomers of usnic acid that were evaluated as anti-angiogenic lichen compounds in the previously published reports (Koparal et al. 2006; Koparal 2015; Song et al. 2012). Thus, anti-angiogenic activity of barbatolic acid was considered to be depended not only on the inhibition of endothelial cell migration, but also on the inhibition of other angiogenesis related factors, which should be further investigated. Additionally, barbatolic acid would be a potent anti-metastatic agent because it was observed that the anti-migratory activity of barbatolic acid was higher on HCC1428 and T-47D breast cancer cells than on HUVEC cells. Consequently, the present study reveals that barbatolic acid is a promising anti-angiogenic and anti-migratory agent candidate; further studies should be therefore performed to light up the underlying activity mechanisms of barbatolic acid.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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