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Original Article

Pharmacological blockade of cholesterol trafficking by cepharanthine in endothelial cells suppresses angiogenesis and tumor growth

Junfang Lyu [a], Eun Ju Yang [a], Sarah A. Head [b, c], Nana Ai [a], Baoyuan Zhang [a], Changjie Wu [a], Ruo-Jing Li [b, c], Yifan Liu [a], Chen Yang [d], Yongjun Dang [d], Ho Jeong Kwon [e], Wei Ge [a], Jun O. Liu [b, c, f], Joong Sup Shim [a, b, c, *]

[a] Faculty of Health Sciences, University of Macau, Taipa, Macau SAR, China
[b] Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA
[c] The SJ Yan and HJ Mao Laboratory of Chemical Biology, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA
[d] Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China
[e] Chemical Genomics Global Research Laboratory, Department of Biotechnology, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[f] Department of Oncology, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA

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ABSTRACT

Cholesterol is an important modulator of membrane protein function and signaling in endothelial cells, thus making it an emerging target for anti-angiogenic agents. In this study, we employed a phenotypic screen that detects intracellular cholesterol distribution in endothelial cells (HUVEC) and identified 13 existing drugs as cholesterol trafficking inhibitors. Cepharanthine, an approved drug for anti-inflammatory and cancer management use, was amongst the candidates, which was selected for in-depth mechanistic studies to link cholesterol trafficking and angiogenesis. Cepharanthine inhibited the endolysosomal trafficking of free-cholesterol and low-density lipoprotein in HUVEC by binding to Niemann-Pick disease, type C1 (NPC1) protein and increasing the lysosomal pH. The blockade of cholesterol trafficking led to a cholesterol-dependent dissociation of mTOR from the lysosomes and inhibition of its downstream signaling. Cepharanthine inhibited angiogenesis in HUVEC and in zebrafish in a cholesterol-dependent manner. Furthermore, cepharanthine suppressed tumor growth in vivo by inhibiting angiogenesis and it enhanced the antitumor activity of the standard chemotherapy cisplatin in lung and breast cancer xenografts in mice. Altogether, these results strongly support the idea that cholesterol trafficking is a viable drug target for anti-angiogenesis and that the inhibitors identified among existing drugs, such as cepharanthine, could be potential anti-angiogenic and antitumor agents.

Introduction

The tumor microenvironment, consisting of non-cancer cells, blood vessels and secreted proteins produced by those surrounding cells, has been recognized as a key factor influencing the growth of cancer [1]. One of the key events shaping the tumor microenvironment is the formation of new blood vessels into tumor tissues, termed tumor angiogenesis. The role of angiogenesis in cancer growth was first suggested by Judah Folkman in 1971 [2]. Since then, several angiogenesis inhibitors, including monoclonal antibody drugs and small molecule kinase inhibitors, have been developed and successfully introduced into the clinic for the treatment of cancer [3]. However, anticancer effects of the angiogenesis inhibitors varied across different cancer types and not all the treatment trials were successful. The difference in cancer sensitivity to angiogenesis inhibitors is likely to be attributable to development of resistance in cancer cells to angiogenesis inhibitors and tumor vascular heterogeneity [4]. Therefore, it is necessary to diversify drug targets and anti-angiogenic strategies to overcome such clinical challenges of current angiogenesis inhibitors.

Cholesterol is a fundamental component of cellular membranes, which regulates membrane permeability and fluidity. In addition to

* Corresponding author. Faculty of Health Sciences, University of Macau, Av. Universidade, Taipa, Macau SAR, China.
E-mail address: jsshim@umac.mo (J.S. Shim).

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the structural support, it also functions in intracellular transport and cell signaling. Cholesterol can be synthesized in the endoplasmic reticulum (ER) or absorbed from extracellular space via low-density lipoprotein (LDL) receptor-mediated endocytosis [5]. Cellular cholesterol absorption and distribution requires appropriate intracellular transport of LDL. Upon internalization, LDL is delivered to early sorting endosomes and then to late endolysosomes where LDL and cholesteryl esters are hydrolyzed, after which the LDL receptor can be recycled back to the plasma membrane [6]. The Niemann-Pick type C (NPC) proteins, NPC1 and NPC2, in the late endolysosomes play an important role in hydrolyzing the cholesterol esters and delivering free cholesterol out of the endolysosomes [7]. Inhibition of NPC1 or NPC2 causes accumulation of cholesterol and glycolipids in the endolysosomes, a phenotype named NPC after the genetic disease of the same name [8]. Circulating cholesterol, in the form of LDL, has to enter or pass through the endothelium that lines blood vessels to be distributed to the whole body. Therefore, endothelial cells play an important role in whole body LDL penetration, accumulation and metabolism [9]. Conversely, a role of cholesterol in endothelial cell functions and angiogenesis has been increasingly suggested. In the early 2000s, several groups reported functional links between plasma cholesterol levels and angiogenesis based on the observation that cholesterol-lowering agents, statins (HMG-CoA reductase inhibitors) could modulate angiogenesis [10–12]. More direct evidence between endothelial cell cholesterol content and angiogenic signaling pathways has emerged recently. Depletion of plasma membrane cholesterol in endothelial cells by liver X receptor agonists or an over-expression of apolipoprotein A-I binding protein (AIBP) that promotes the efflux of cellular cholesterol to high-density lipoprotein (HDL) could lead to a reduction of lipid rafts and impaired vascular endothelial growth factor receptor-2 (VEGFR2) signaling pathways [13,14]. In addition, ezetimibe, an approved cholesterol-uptake blocker that works by inhibiting NPC1-like 1 (NPCL1), significantly inhibited tumor angiogenesis in animal models [15].

In addition to the cholesterol contents, proper intracellular cholesterol trafficking was also suggested to play a critical for endothelial cell signaling and angiogenesis. We have previously shown that small molecule inhibitors of cholesterol trafficking, such as itraconazole and tamoxifen suppressed mTOR signaling and angiogenesis in vitro and in preclinical models [16–21]. Based on the promising preclinical data, itraconazole has entered Phase II clinical trials for the treatment of various types of cancer, including non-small cell lung cancer (NSCLC), basal cell carcinoma (BCC) and metastatic prostate cancer. Recently, positive clinical outcomes have been reported from the NSCLC, BCC and prostate cancer trials [22–24]. These results suggest that cholesterol trafficking is a viable drug target and that inhibitors of endothelial cell cholesterol trafficking could be a new class of anti-angiogenic agents. This notion prompted us to screen the Johns Hopkins Drug Library (JHDL) [25,26] to identify existing drugs that can modulate endothelial cell cholesterol trafficking. In this study, we successfully identified cholesterol trafficking inhibitors among existing drugs, including an anti-inflammatory drug cepharanthine (CEP). CEP is a natural product derived from a plant *Stephania cepharantha* Hayata and was approved in Japan for the use in various disease conditions, such as inflammation and cancer management [27,28]. Recent studies suggested potential effects of CEP on angiogenesis and cancer metastasis [29,30]. However, precise molecular mechanisms behind the pharmacological effects of CEP have not been fully addressed. Our study shows that CEP inhibits angiogenesis by blocking cholesterol trafficking and provides a strong evidence that cholesterol trafficking inhibitors could be potential anti-angiogenic and anticancer agents.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Thermo Fisher Scientific and grown in Medium 200 supplemented with low serum growth supplement (LSGS) (Thermo Fisher Scientific, Waltham, MA). A549, MDA-MB-231 and HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). A549 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). MDA-MB-231 and HEK293T cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. All the cells were maintained in a humidified incubator at 37 °C adjusted to 5% CO_2.

Reagents and antibodies

Cepharanthine (CEP) and cholesterol were purchased from Santa Cruz Biotechnology (Dallas, Texas). Methyl-β-cyclodextrin, filipin, itraconazole and cisplatin were bought from Sigma-Aldrich (St. Louis, MO). Primary antibodies for S6 kinase (S6K) (sc-8418, mouse monoclonal, 1:200), phospho-S6K (Thr421/Ser424) (sc-7984-R, rabbit polyclonal, 1:100), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-365062, mouse monoclonal, 1:1000) and lysosomal-associated membrane protein 1 (LAMP1) (sc-20011, mouse monoclonal, 1:100), and horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2005, goat anti-mouse IgG-HRP, 1:2500; sc-2004, goat anti-rabbit IgG-HRP, 1:2500) were purchased from Santa Cruz Biotechnology. Antibodies for eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (9452S, rabbit monoclonal, 1:1000), protein disulfide isomerase (PDI) (3501S, rabbit monoclonal, 1:100) and mammalian target of rapamycin (mTOR) (2983S, rabbit monoclonal, 1:100) were from Cell Signaling Technology (Danvers, MA), for GM130 (610823, mouse monoclonal, 1:100), CD31 (550274, rat monoclonal, 1:50) and Ki67 (550609, mouse monoclonal, 1:100) were from BD Biosciences (San Jose, CA), and for NPC1 (13926-1-AP, rabbit polyclonal, 1:500) was from Proteintech (Chicago, IL). Secondary antibodies conjugated with Alexa Fluor 488 (A21202, donkey anti-mouse IgG conjugate, 1:1000; A21206, donkey anti-rabbit IgG conjugate, 1:1000; A11006, goat anti-rat IgG conjugate, 1:1000) and Alexa Fluor 647 (A21235, goat anti-mouse IgG conjugate, 1:1000; A21244, goat anti-rabbit IgG conjugate, 1:1000) were from Thermo Fisher Scientific.

Screening of cholesterol trafficking inhibitors

Total 3131 drugs of the Johns Hopkins Drug Library (JHDL) arrayed in 96-well plates were diluted in sterile PBS at 100 μM (working dilution) and used to screen in HUVEC. The final drug concentration of 5 μM was used for the screening since screening assays for hit discovery are typically run at 1–10 μM compound concentration [31]. After 24 h incubation with the drugs, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with filipin (50 μg/ml) for 2 h at room temperature. Cells were washed with phosphate buffered saline (PBS) and the fluorescent cholesterol images in each well were obtained using the Olympus IX81 fully automated fluorescence microscope (Olympus, Tokyo, Japan) equipped with Prior motorized stage. Each screening plate contains negative (dimethyl sulfoxide, DMSO) and positive (itraconazole, U18666A and imipramine) control compounds. All the captured images were manually assessed and hits were identified by comparing the cholesterol distribution patterns from each well with those treated with positive control compounds. The primary hits were further validated by confocal microscope analyses of intracellular cholesterol distribution. Briefly, HUVEC were seeded in a Nunc Lab-Tek II 8-Chamber Slide (Thermo Fisher Scientific) and treated with hit compounds for 8 h. The concentrations of each hit compound used for confocal microscope analysis were chosen based on their IC_{50} values in HUVEC. After fixation and staining with filipin, cells were washed with PBS, mounted with Immu-mount (Thermo Fisher Scientific), and observed under the Carl Zeiss LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY).

Immunofluorescence imaging of endothelial cells

HUVEC were seeded in a Nunc Lab-Tek II 8-Chamber Slide and treated with compounds for indicated time points. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then permeabilized with 0.5% Triton X-100 (for protein immunostaining) or 0.2% saponin (for co-staining with filipin) for 10 min prior to blocking in a blocking buffer (3% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20) for 1 h. Cells were incubated with primary antibodies, including anti-LAMP1, anti-PDI, anti-mTOR and anti-GM130 in the blocking buffer overnight at 4 °C, followed by the incubation with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 647 for 1 h at room temperature. The cellular nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific). Cells were washed with PBS, mounted with Immu-mount, and observed under a Carl Zeiss LSM 710 confocal microscope. For the analysis of LDL trafficking, HUVEC were pretreated with DMSO or CEP for 8 h, and then 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labeled LDL (DiI-LDL) (Thermo Fisher Scientific) was added to the cells. The incubation was continued for 1 h or 6 h before cells were fixed and co-stained with filipin or primary antibodies against LAMP1, PDI and mTOR.