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Original Article

Identification of a novel autophagic inhibitor cepharanthine to enhance the anti-cancer property of dacomitinib in non-small cell lung cancer

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ABSTRACT

Inhibition of autophagy is a promising strategy for non-small cell lung cancer (NSCLC) treatment, which is in the clinical trials. However, only chloroquine is used in clinic as an autophagic inhibitor and the inhibitory effect of chloroquine on autophagy is finite. Therefore, the development of an alternative autophagic inhibitor for NSCLC therapy becomes necessary. In the present study, cepharanthine (CEP), an alkaloid extracted from *Stephania cepharantha* Hayata, was identified as a novel autophagic inhibitor in NSCLC cells. The potential mechanism of the CEP-inhibited autophagy was by blockage of autophagosome-lysosome fusion and inhibition of lysosomal cathepsin B and cathepsin D maturation. Furthermore, we found for the first time that dacomitinib (DAC), a second-generation epidermal growth factor receptor inhibitor that in the phase III clinical trials for NSCLC treatment, induced a protective autophagy to decrease its anti-cancer effect. Combined treatment with CEP increased the anti-proliferative and apoptotic effects of DAC *in vitro* and enhanced the anti-cancer effect of DAC in NCI-H1975 xenograft mice. Collectively, CEP might be further developed as an autophagic inhibitor, and combined treatment of CEP and DAC could offer an effective strategy for NSCLC treatment.

Introduction

Autophagy is a cellular degradation system, in which unnecessary cytoplasmic contents are sequestered, degraded, released and recycled for cell survival in a lysosome-dependent manner [1]. During autophagy, several autophagy-related proteins are engulfed to form autophagosomes; after fusion with lysosomes to form autolysosomes, the cargos in the autolysosomes are degraded by various lysosomal hydrolytic enzymes, for example cathepsins [1]. Increasing evidence has indicated that autophagy plays an important effect on a variety of diseases, including cancer [1,2]. It is becoming clear that autophagy provides survival advantages to resist cell death under stressful conditions [3,4]. Moreover, some anti-cancer drugs induce a protective autophagy to resist their anti-cancer effects [5–7]. For instance, erlotinib, a first generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that has been approved for the treatment of non-small cell lung cancer (NSCLC) harboring *EGFR* sensitive mutation, induced a protective autophagy and a combined treatment with an autophagic inhibitor increased the therapeutic effect of erlotinib in *EGFR* sensitive mutant NSCLC cells [5,6]. Therefore, inhibition of autophagy during drug therapy is proposed to be an effective therapeutic approach to sensitize cancer cells to anti-cancer drugs [8,9].

Given the efficiency of autophagy inhibitor in clinical trials is finite, the development of a novel autophagic inhibitor for cancer therapy becomes necessary [10]. Cepharanthine (CEP, Fig. 1A), a benzylisoquinoline alkaloid extracted from *Stephania cepharantha* Hayata [12], possesses a wide range of pharmacological activities, such as anti-cancer, anti-inflammation and anti-virus [11–13]. The anti-cancer effect of CEP has been investigated in diverse types of cancer cells, such as nasopharyngeal carcinoma cells, cholangio carcinoma cells and oral squamous cell carcinoma cells [14–16]. In nude mouse xenografts of osteosarcoma Saos-2 cells,

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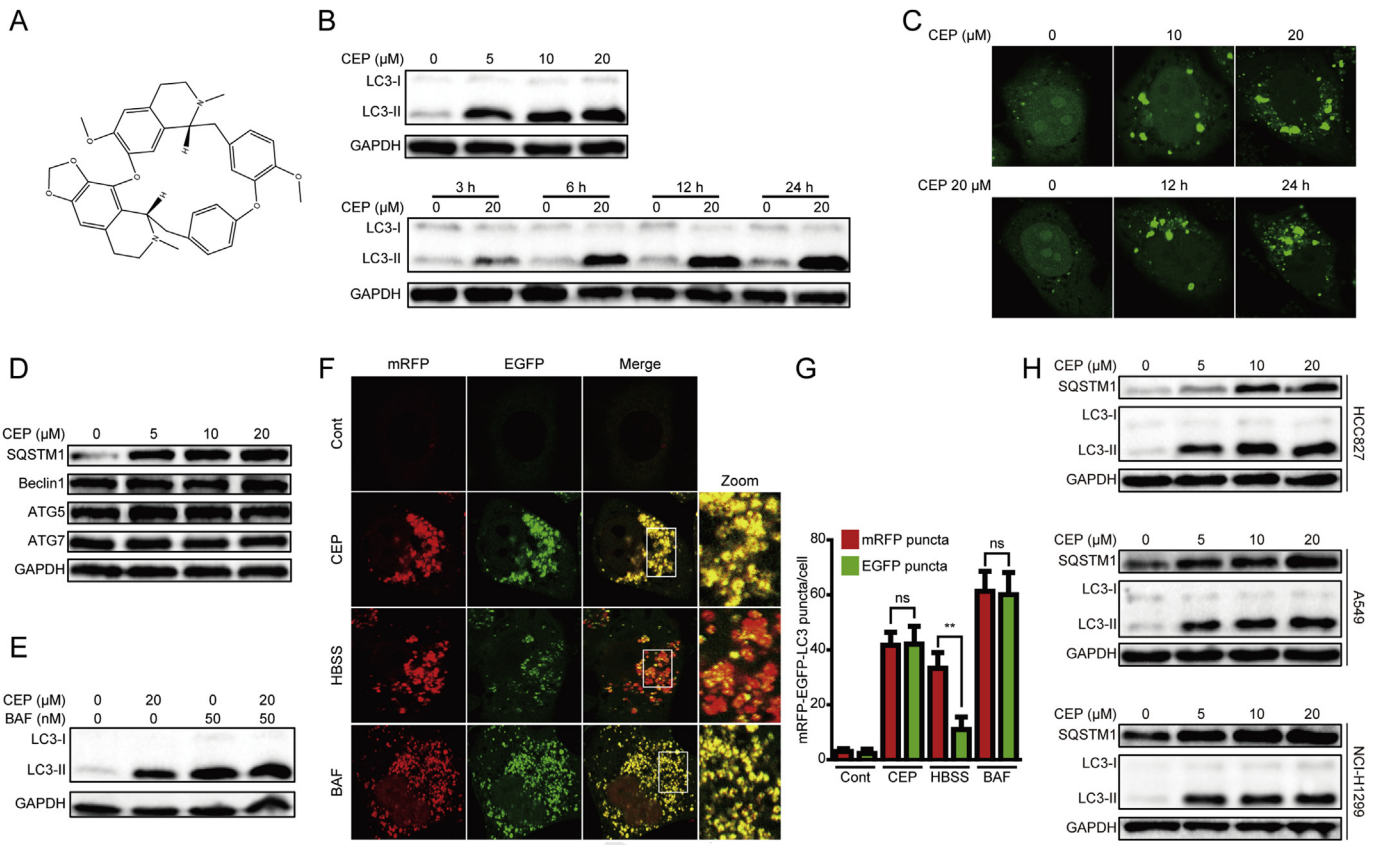

Fig. 1. CEP inhibits autophagic flux in NSCLC cells. (A) The chemical structure of CEP. (B) NCI-H1975 cells were treated with CEP. The expression levels of indicated proteins were detected through the Western blot assay. (C) NCI-H1975 cells were transiently transfected with GFP-LC3 plasmid and subsequently exposed to CEP. The fluorescent images were obtained by using a confocal microscope and typical images were presented. (D) NCI-H1975 cells were treated with CEP for 24 h. The expression levels of indicated proteins were detected through the Western blot assay. (E) Cells were incubated with CEP for 24 h with or without pretreatment with BAF. The expression levels of indicated proteins were studied through the Western blot assay. (F–G) Cells were transiently transfected with mRFP-EGFP-LC3 plasmid and subsequently treated with vehicle, 20 μM CEP, HBSS, or 50 nM BAF for 24 h. The fluorescent images were obtained by using a confocal microscope and typical images were presented. For quantification, more than 20 cells (per experiment) were randomly chosen for counting the numbers of red puncta and green puncta. The "ns" indicates "no statistical difference". $^{*}P < 0.05$, $^{**}P < 0.01$. (H) The NSCLC HCC827, A549 and NCI-H1299 cells were treated with CEP. The expression levels of indicated proteins were detected through the Western blot assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intraperitoneal administration of CEP inhibited the tumor volume and weight by inhibiting the signal transducer and activator of transcription-3, while the body weight of the mice was unaffected [12]. CEP remarkably decreased the growth and infiltration of primary effusion lymphoma cell in mice and no significant side effects of CEP, including for lymphocytes, were observed [17]. Moreover, CEP exhibits the protective effects on the radiotherapy-induced leukopenia down-regulation, acute urethral toxicity and late rectal toxicity [18,19].

Dacomitinib (DAC), one of the second generation EGFR TKIs, is an irreversible TKI of the human epidermal growth factor receptor family receptors (HERs), including HER-1/EGFR, HER-2, and HER-4 [20]. A variety of studies suggested that DAC exhibits a superior anti-cancer activity *versus* erlotinib in erlotinib-sensitive or -resistant NSCLC models [20–22]. In the phase III clinical trial (ARCHER 1009), the progress-free survival of the DAC-treated NSCLC patients harboring *EGFR* sensitive mutation was further improved, when compared with the erlotinib-treated group [23]. Previous studies suggested that EGFR TKIs including erlotinib and gefitinib, the first generation EGFR TKIs, induce a protective autophagy to resist their anti-cancer effects [5,6,24]. Whether DAC also induces a protective autophagy remained unclear.

In the present study, CEP was identified as a new autophagic inhibitor that inhibited autophagic flux by blockage of autophagosome-lysosome fusion and inhibition of lysosomal cathepsin B (CTSB) and cathepsin D (CTSD) maturation in NSCLC cells. DAC induced a protective autophagy with inhibition of Akt/mammalian target of rapamycin (mTOR) pathway. Combined treatment with CEP remarkably increased the anti-cancer effect of DAC through autophagy inhibition in NSCLC cells.

Materials and methods

Reagents and antibodies

CEP was purchased from Shiji-Aoke (Beijing, China), thiazolyl blue tetrazolium bromide (MTT), bafilomycin A1 (BAF) and dimethyl sulfoxide were obtained from Sigma (St. Louis, MO, USA). RPMI 1640 medium, penicillin, streptomycin, fetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) were obtained from Gibco Life Technologies (Grand Island, NY, USA). DAC was obtained from Selleck Chemicals (Houston, TX, USA). Anti-α-Tubulin antibody, cleaved caspase 3 (c-caspase 3) and LysoTracker red probe were purchased from the Beyotime Biotechnology Corporation (Shanghai, China). LC3, Beclin1, ATG5, ATG7, SQSTM1, CTSD, LAMP1 (for Western blot), Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), P70S6K, p-P70S6K (Thr389), 4E-BP1, p-4E-BP1 (Ser65), ULK1, p-ULK1 (Ser757), cleaved poly (ADP-ribose) polymerase (c-PARP), Bax, p21, GAPDH and anti-rabbit IgG HRP-linked were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). CTSB antibody was purchased from the Santa Cruz Biotechnology (Santa Cruz, CA, USA). LAMP1 (for immunofluorescence) was obtained from Biolegend (San Diego, CA, USA).

Cell culture

NCI-H1975, NCI-H1650 and HCC827 cells were purchased from Shanghai Cell Bank (Shanghai, China). A549 and NCI-H1299 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in a 5% CO_2

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