Biomaterials 141 (2017) 188–198

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Liposomal honokiol induced lysosomal degradation of Hsp90 client proteins and protective autophagy in both gefitinib-sensitive and gefitinib-resistant NSCLC cells

Jianhong Yang [a, 1], Wenshuang Wu [b, 1], Jiaolin Wen [a], Haoyu Ye [a], Hong Luo [c], Peng Bai [a], Minghai Tang [a], Fang Wang [a], Li Zheng [a], Shengyong Yang [a], Weimin Li [d], Aihua Peng [a], Li Yang [a], Li Wan [e, **], Lijuan Chen [a, *]

[a] State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, China
[b] Department of Thyroid and Breast Surgery, West China Hospital of Sichuan University, Chengdu, China
[c] Department of Ultrasonic Medicine, West China Second Hospital, Sichuan University, Chengdu, China
[d] Department of Respiratory Medicine, West China Hospital, Sichuan University, Chengdu, China
[e] School of Pharmacy, Chengdu University of TCM, The Ministry of Education Key Laboratory of Standardization of Chinese Herbal Medicine, State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu, China

ARTICLE INFO

*Article history:*
Received 1 December 2016
Received in revised form
30 June 2017
Accepted 2 July 2017
Available online 4 July 2017

*Keywords:*
Honokiol
HSP90
Autophagy
EGFR
Gefitinib-resistance

ABSTRACT

Honokiol (HK), a natural chemical isolated from *Mangnolia officinalis*, has shown antitumorigenic activities when used to treat a variety of tumor cell lines. The mechanism of honokiol activity when used to treat gefitinib-sensitive and gefitinib-resistant non-small cell lung cancer (NSCLC) requires elucidation. Here, the presence of liposomal honokiol (LHK) induced apoptotic and antitumor activities in four xenograft models generated using NSCLC cell lines such as HCC827 (gefitinib-sensitive) and H1975 (gefitinib-resistant). Mechanistic studies revealed that LHK inhibited the Akt and Erk1/2, both EGFR signaling cascades effectors, by promoting degradation of HSP90 client proteins (HCP), including wild-type or mutant EGFR, Akt and C-Raf. Molecular biology assays showed that LHK induced HCP degradation through a lysosomal pathway, rather than the canonical proteasome protein degradation pathway. As a result of misfolded protein accumulation, LHK induced endoplasmic reticulum (ER) stress and autophagy. Inhibition of ER stress (with 4-phenylbutyrate) or autophagy (with small interfering RNA) reduced LHK-induced HCP degradations. Additionally, LHK induced autophagy showed a protective role for cancer cell as inhibition of autophagy *in vitro* and *in vivo* by autophagosome degradation inhibitors could promote the anticancer activity of LHK. LHK has been approved by the China Food and Drug Administration for first-in-human clinical trials in NSCLC. The current study will guide the design of future LHK clinical trials.



## 1. Introduction

Non-small cell lung cancer (NSCLC) patients with L858R or exon 19 deletion mutations in epidermal growth factor receptor (EGFR) benefit from treatment with EGFR tyrosine kinase inhibitors (EGFR-TKI), such as gefintinib and erlotinib. Unfortunately, a second EGFR mutation, T790 M, often develops in patients after EGFR-TKI treatments, eventually resulting in resistant disease [1]. Therefore, it is of great importance to find other strategies to treat patients with EGFR (T790 M) mutations.

Heat shock protein 90 (HSP90) is a molecular chaperone that

* Corresponding author. State Key Laboratory of Biotherapy and Cancer Center, West China Hospital of Sichuan University, Chengdu, China.
** Corresponding author. School of Pharmacy, Chengdu University of TCM, The Ministry of Education Key Laboratory of Standardization of Chinese Herbal Medicine, State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu, China.
*E-mail addresses:* wanli8801@163.com (L. Wan), chenlijuan125@163.com, ljchen@scu.edu.cn (L. Chen).
[1] These authors equally contribute to this paper.

http://dx.doi.org/10.1016/j.biomaterials.2017.07.002
0142-9612/

facilitates protein folding and conformational stability, including proteins required for tumor survival (e.g., EGFR, C-Raf and Akt). HSP90 is a viable target for cancer treatment [2]. Acetylation mediates HSP90 client protein (HCP) binding; targeting HSP90 acetylation may be an effective cancer treatment [3,4]. In addition, mutant EGFR proteins require HSP90 for folding, and inhibiting HSP90 has induced mutant EGFR degradation, making this a potential cancer treatment in first-generation EGFR-TKI-resistant lung cancers [5,6]. These collective data support the hypothesis that altering HSP90 acetylation to enhance mutant EGFR protein degradation could have antitumor effects on EGFR (T790 M) mutant human cancers.

Autophagy is a physiological protein degradation phenomenon in healthy eukaryotic cells. Autophagy maintains cellular homeostasis by mediating abnormal protein and subcellular organelle degradations in a lysosome-dependent pathway [7]. Excessive accumulations of abnormal proteins within endoplasmic reticulum (ER) structures cause severe ER-stress events, which result in autophagy and degradation of abnormal proteins [8,9]. In addition to the autophagy-lysosome pathway (ALP), the proteasomal pathway is critical for the degradation of abnormal proteins; usually, this pathway requires ubiquitination of target proteins [10]. Previously, both the ALP and the proteasomal pathway mediated the degradation of HCP [11–13]. To promote drug development of HSP90 inhibitors, elucidating the molecular mechanism of HCP degradation was required.

Honokiol (HK) is an easily-isolated lignan harvested from *Mangnolia officinalis* by high-capacity high-speed countercurrent chromatography [14]. Given that HK is a multi-target compound, application of HK has shown anti-inflammatory, anti-oxidative, anti-tumor, anti-carcinogenic and neuroprotective effects [15,16]. In cancer cells, HK induced apoptosis of many malignancies including: myeloma, sarcoma, melanoma, lung, leukemia and others [17–23]. Mechanistic analyses showed that HK modulated the PI3K/Akt, MAPK, nuclear factor kappa B (NF-κB) and mTOR pathways in cancer cell lines [ [20–24]. In addition to apoptosis, autophagy was observed in HK-treated cancer cell lines [18,25], but the mechanistic underpinnings of this phenomenon remain elusive. It is currently unclear whether HK has antitumor activity on NSCLC cells with EGFR-activating and TKI-resistant T790 M mutations.

In the current study, a novel function of liposomal honokiol (LHK) is reported. LHK promoted the degradation of HCP through an ALP in EGFR-mutated and TKI-resistant NSCLC cells. ER-stress caused by accumulation of misfolded proteins contributed to LHK-induced autophagy in NSCLC cell lines. These results indicated that LHK is a promising molecular entity that could be developed as a human antitumor agent, particularly in cases of gefitinib-resistant EGFR-mutant NSCLC. Finally, these results also suggested that combinations of autophagosomal degradation inhibitors could further sensitize NSCLC cells to LHK antitumor activities.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

NSCLC cell lines H1975, HCC827, H460, SPC-A1, H441, H1650, H226, H522 and H1993 were obtained from the American Type Culture Collection (ATCC) in 2012. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% $CO_2$. All the cells used here were annually authenticated with an AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems). The cells were last authenticated in October 2016.

### 2.2. Reagents and antibodies

SPC, cholesterol and $PEG_{2000}$-DSPE were purchased from Avanti Polar Lipids, Inc. DMSO, MTT, gefitinib, bafilomycin A1, chloroquine, $NH_4Cl$, zVAD.fmk, 4-PBA, MG132 and DAPI were obtained from Sigma Aldrich. Antibodies were obtained from the following sources: Atg7 (Santa Cruz), Beclin 1 (Santa Cruz), p-Akt (Cell Signaling Technology), Akt (Cell Signaling Technology), GAPDH (Santa Cruz), SQSTM1/p62 (Santa Cruz), LC3 (Cell Signaling Technology), Caspase 3 (Abcam), EGFR (Abcam), HSP90 (Abcam), HSP70 (Santa Cruz), C-Raf (Cell Signaling Technology), Erk1/2(Santa Cruz), p-Erk1/2 (Santa Cruz), Acetyl-lysine (Abcam), Ubiqutin (Proteintech), eI2Fα (Santa Cruz), p-eI2Fα (Santa Cruz), LAMP1 (Santa Cruz), PDI (Cell Signaling Technology), HRP-labeled anti-rabbit and mouse secondary antibody (Santa Cruz).

### 2.3. Preparation of liposomal honokiol

HK was separated in the laboratory and its purity and structure were analyzed and identified by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance methods [14]. LHK was prepared in our laboratory as previously published [26]. Briefly: SPC, cholesterol, $PEG_{2000}$-DSPE and HK in weight ratios of 1:0.15:0.14:0.22 were dissolved in 100% ethanol. The resulting ethanol-lipid solutions were added drop-wise into 0.5% sucrose solution using injection-sonication methods. Next the solution was evaporated in a vacuum rotary evaporator and the concentrated solution was diluted with 10% sucrose and lyophilized. Empty liposomes lacking HK were also prepared with these methods. LHK and empty liposomes had a size range of 130 ± 20 nm and 80 ± 20 nm, respectively. The liposomal zeta potential ranged from −20.0 to −30.0 mV. Drug loading (DL) and encapsulation efficiency (EE) values of LHK were 14.6% and 85%, respectively. LHK release profiles indicated that HK was released from micelles in a sustained manner *in vitro*.

### 2.4. MTT assay, western blotting, immunofluorescence and immunohistochemistry

These assays were performed by rigorously following established laboratory protocols that were previously published [27].

### 2.5. mRNA silencing

Atg7 trilence-27 siRNA (SR307159) and Beclin1 trilence-27 siRNA (SR305711) were purchased from ORIGENE. Three specific siRNA constructs were tested for each target and the most effective was chosen. The siRNA constructs were transfected with Lipofectamine 2000 reagent (Invitrogen) for 48 h in H1975 and HCC827 cells according to the manufacturer's protocols.

### 2.6. Co-immunoprecipitation

Cells were treated with or without LHK for 24 h. Proteins were extracted using lysis buffer (20 mM Tris (pH = 8), 137 mM NaCl, 10% glycerol, 1% NP-40 and 2 mM EDTA). Protein samples were immunoprecipitated with EGFR, C-Raf, or Akt antibodies at 4 °C with agitation overnight. The immunoprecipitated proteins were pulled down with protein A-agarose beads (Cell Signaling Technology) at 4 °C with rotary agitation overnight. Proteins were eluted from the final supernatant by boiling in 2× SDS loading buffer. Proteins were separated using standard SDS-PAGE electrophoresis techniques and then detected by western blot with methods described previously [26].