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# Lipid-modified cell-penetrating peptide-based self-assembly micelles for co-delivery of narciclasine and siULK1 in hepatocellular carcinoma therapy

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer, and one therapeutic approach is to target both the AMPK and autophagy pathways in order to synergistically promote programmed cell death. Here, a series of amphiphilic, lipid-modified cell-penetrating peptides were synthesized and allowed to self-assemble into micelles loaded with the AMPK activator narciclasine (Narc) and short interfering RNA targeting the unc-51-like kinase 1 (siULK1). The size of these micelles, their efficiency of transfection into cells, and their ability to release drug or siRNA cargo *in vitro* were pH-sensitive, such that drug release was facilitated in the acidic microenvironment of the tumor. Transfecting the micelles into HCC cells significantly inhibited protective autophagy within tumor cells, and delivering the micelles into mice carrying HCC xenografts induced apoptosis, slowed tumor growth, and inhibited autophagy. Our results indicate that co-delivering Narc and siULK1 in biocompatible micelles can safely inhibit tumor growth and protective autophagy, justifying further studies into this promising therapeutic approach against HCC.

**Statement of Significance**

We have focused on the targeted therapy of HCC via synergistically inhibiting the autophagy and inducing apoptosis. The lipid-modified cell-penetrating peptide can not only aggregate into micelles to load natural product narciclasine and ULK1 siRNA simultaneously, but also facilitate uptake and endosome escape with a pH-sensitive manner in HepG2 cells. HepG2 cell treated with siULK1-M-Narc has increased apoptotic levels and declined autophagy via the targeted regulation of AMPK-ULK1 signaling axis. The *in vivo* studies have confirmed that siULK1-M-Narc efficiently reduce the growth of tumor on HCC xenograft models with good safety. Thus, we suppose the lipid-modified cell-penetrating peptide has good application prospects in the targeted combinational therapy of HCC.

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## 1. Introduction

Liver cancer ranks among the top 10 causes of new cancers and of cancer-related deaths, and it is the second most frequent cause of new cancers and cancer-related mortality in men [1,2]. The most frequent type of liver cancer is hepatocellular carcinoma (HCC), which accounts for more than 80% of primary liver cancers [1]. HCC is often treated using transcatheter arterial chemoembolization (TACE) with cytotoxic drugs, which can lead to multidrug resistance, dose-limiting toxicities, and other unfavorable side-effects [3]. Research into HCC biomarkers and signaling pathways has led researchers to generate several small-molecule kinase inhibitors and monoclonal-antibodies, which have been approved for

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clinical use or are being tested in clinical trials [4]. The multi-kinase inhibitor sorafinib, the first approved kinase inhibitor against HCC, has been shown to prolong median survival time by three months among patients with advanced HCC [5]. Several kinase inhibitors have entered phase III clinical trials for advanced HCC: regorafenib, sunitinib, tivantinib, erlotinib and linifanib [6].

The combination of TACE and monoclonal antibodies (mAbs) can prolong survival of HCC patients. For example, the combination of TACE and $^{131}$I-labeled CD147 mAbs (metuximab) can be used to treat patients with inoperable HCC [7]. Phase II/III clinical trials suggest that the combination of the mAbs bevacizumab or ramucirumab, which target vascular endothelial growth factor receptors [8], with sorafinib or the mTOR inhibitor temsirolimus can benefit patients with advanced HCC [9]. Other promising therapeutic mAbs against HCC include antibodies targeting glypican-3 [10], programmed cell death protein 1 (PD-1), ligand of PD-1 (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) [11]. Novel HCC chemotherapies are still urgently needed, because many patients suffer poor outcomes because of the high risk of HCC metastasis and recurrence [12–14].

One potential drug target is dysregulation of metabolism: inhibition of the intracellular serine/threonine kinase called adenosine monophosphate-activated protein kinase (AMPK), which operates as a metabolic "checkpoint", facilitates HCC onset and progression [15]. Activating AMPK by triggering phosphorylation of Thr172 suppresses HCC by regulating protein synthesis and signaling pathways involving mTOR and Wnt/β-catenin [16–19].

Another potential drug target against HCC is autophagy [20–23]. On one hand, autophagy suppresses carcinogenesis by preventing inflammatory processes that occur during viral infection, alcohol steatohepatitis and non-alcohol steatohepatitis and that transform tissue into cancer [24]. On the other hand, autophagy can protect solid tumors by rendering cancer cells resistant to apoptosis and chemotherapy and tolerant to hypoxia [25–27]. It may be possible to harness the anti-cancer activities of autophagy and combine them synergistically with those of AMPK activators as a novel dual therapy against HCC.

Here we devise a self-assembling, biocompatible micelle system to deliver the AMPK activator narciclasine (Narc) together with short interfering RNA targeting the autophagy regulator unc-51-like kinase 1 (ULK1) into HCC cells. The micelles are based on novel, lipid-modified cell-penetrating peptides. We found that the pH sensitivity of the micelles led to efficient delivery of siULK1 into tumor cells and escape from lysosomes. The combination of inhibition of ULK1-dependent autophagy and AMPK activation synergistically enhanced the anti-tumor activity of Narc [28], likely because AMPK activation induced greater autophagy levels. Our results present a novel paradigm for translational research in nanomedicines involving the rational design and preparation of multi-functional biocompatible carriers.

## 2. Materials and methods

### 2.1. Materials

Lipofectamine 2000 was purchased from Thermo Fisher Scientific Co., Ltd (Waltham, USA). ULK1 siRNA and scrambled control siRNA were purchased from Guangzhou Ribobio Co. Ltd. (Guangzhou, China) and used according to our previous report [29]. Narciclasine (Narc) was purchased from Shanghai Aladdin Co., Ltd. (Shanghai, China). The other chemicals were purchased and used as analytical grade without additional purification. Narc was formulated for intravenous (i.v.) injection by adding to a 1:1 (v/v) mixture of Cremophor-EL/ethanol and diluting with normal saline (NS) [30].

### 2.2. Cell culture and animals

The normal human liver cell line L02 as well as human hepatocellular carcinoma cell lines SMMC-7721, Bel-7402, HepG2 and HuH-7 were obtained from the American Type Culture Collection (ATCC) and maintained in our laboratory. HepG2 and Huh-7 cells were cultured in DMEM with 10% fetal bovine serum. Bel-7402, SMMC-7721 and L02 cells were cultured in RPMI-1640 with 10% fetal bovine serum. BALB/c nude mice 6–8 weeks old were purchased from the Beijing Huafukang Co. Ltd (Beijing, China). All animal experiments were approved by the Animal Care and Treatment Committee of West China Hospital of Sichuan University.

### 2.3. TCGA database and tissue microarray (TMA) analysis

Gene expression profiles were extracted from the Cancer Genome Atlas (TCGA) database [31–33]. The hepatocellular cohort of TCGA contained 421 clinical samples (371 HCC tissues and 50 adjacent normal tissues). Levels of mRNA expression were determined by mRNA sequencing, quantification and normalization using the EdgeR method as described before [34]. A human hepatocellular carcinoma TMA was purchased from Shanghai Outdo Biotech (HLivH160CS01). This TMA contained tissue from 80 tumors and matched adjacent normal tissue from 80 patients. Immunohistochemistry (IHC) staining was performed using rabbit monoclonal antibodies against ULK1 (Abcam, USA, 1:50 dilution), AMPKα (Abcam, USA, 1:200 dilution) or EEF1A1 (Abcam, USA, 1:200 dilution) as in our previously described studies [29], unless otherwise stated. Expression of ULK1, AMPKα and EEF1A1 was semi-quantitated independently by three histopathologists as the percentage of positive tumor cells.

### 2.4. Synthesis and characterization of lipid-modified cell-penetrating peptides

Lipid-modified cell-penetrating peptides were synthesized using standard solid-phase peptide synthesis as described [35]. The crude peptide products were cleaved from the RINK-MBHA resin and purified by preparative HPLC (LC-50A, Novasep, France) using acetonitrile and 0.1% TFA as mobile phase. The purity and structure of the final products were determined by high-resolution LC-MS (Waters Xevo-G2 Q-TOF MS, USA).

### 2.5. Preparation and of characterization of pH-sensitive co-delivery micelles

The siULK1/Narc co-delivery or single delivery micelles were prepared by a simple self-assembly method. In brief, the organic solution (typically a mixture of dichloromethane and acetone) of Narc and/or lipid-modified cell-penetrating peptides were evaporated under vacuum at room temperature, and the aqueous phase containing ULK1 siRNA was added. Then the mixture was subjected to probe sonication, typically at 20 W for 20 s, yielding self-assembled micelles. Similar procedures were used to prepare various control samples, including micelles loaded only with siULK1 (siULK1-M), loaded only with Narc (M-Narc), or loaded only with scrambled control siRNA (siNC-M). Micelles were stored at 4 °C before use and used without further purification. All measurements were made in triplicate.

The size distribution and zeta-potential of siULK1/Narc co-delivery micelles were measured using a Nano ZS instrument (Malvern Instruments, Ltd., Worcestershire, UK) at room temperature. The morphology of siULK1/Narc co-delivery micelles was analyzed by transmission electron microscopy (TEM, H-600, Hitachi, Japan). For TEM images, the siULK1/Narc co-delivery micelles dispersed in phosphate-buffered saline (PBS) were added to formvar-stabilized

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