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# PEGylated lipid bilayer-supported mesoporous silica nanoparticle composite for synergistic co-delivery of axitinib and celastrol in multi-targeted cancer therapy

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ABSTRACT

Small-molecule drug combination therapies are an attractive approach to enhancing cancer chemotherapeutic responses. Therefore, this study aimed to investigate the potential of axitinib (AXT) and celastrol (CST) in targeting angiogenesis and mitochondrial-based apoptosis in cancer. Therefore, we prepared AXT/CST-loaded combination nanoparticles (ACML) with CST loaded in the mesoporous silica nanoparticles (MSN) and AXT in PEGylated lipidic bilayers. We showed that ACML effectively inhibited angiogenesis and mitochondrial function and was efficiently internalized in SCC-7, BT-474, and SH-SY5Y cells. Furthermore, hypoxia-inducible factor (HIF)-1α expression, which increased under hypoxic conditions in all cell lines exposed to ACML, markedly decreased, which may be critical for tumor inhibition. Western blotting showed the superior anticancer effect of combination nanoparticles in different cancer cells. Compared to the cocktail (AXT/CST), ACML induced synergistic cancer cell apoptosis. The AXT/CST-based combination nanoparticle synergism might be mediated by AXT, which controls vascular endothelial growth factor receptors while CST acts on target cell mitochondria. Importantly, ACML-treated mice showed remarkably higher tumor inhibition (64%) than other groups did in tumor xenograft models. Tumor xenograft immunohistochemistry revealed elevated caspase-3 and poly (ADP-ribose) polymerase and reduced CD31 and Ki-67 expression, clearly suggesting tumor apoptosis through mitochondrial and antiangiogenic effects. Overall, our results indicate that ACML potentially inhibited cell proliferation and induced apoptosis by blocking mitochondrial function, leading to enhanced antitumor efficacy.

**Statement of Significance**

In this research, we formulated an anticancer drug combination nanoparticle loaded with axitinib (AXT) in the lipidic bilayer of PEGylated liposomes and celastrol (CST) in mesoporous silica nanoparticles. The anticancer effects of the AXT/CST-loaded combination nanoparticle (ACML) were synergistic and superior to the other formulations and involved more efficient drug delivery to the tumor site with enhanced effects on angiogenesis and mitochondrial function. Therefore, our study demonstrated that the inhibition of cell proliferation and induction of apoptosis by ACML, which was mediated by blockade of mitochondrial function and anti-angiogenesis, led to enhanced antitumor efficacy, which may be potentially useful in the clinical treatment of cancer.



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## 1. Introduction

Cancer development is a complex process that involves the activation of different signaling pathways for cell growth. To meet the high requirements of growing cancer cells, constant blood supply is required. As the tumor enlarges, sufficient supply of oxygen and other nutrients to the innermost cells becomes limited because fewer blood vessels reach the affected area [1]. Furthermore, for the survival of cancer cells, different angiogenesis promoting signaling mechanisms are activated in the tumor area. The process of angiogenesis is mediated by the vascular endothelial growth factor (VEGF) following its interaction with VEGF receptors (VEGFR 1, 2, and 3).

The mitochondrion is an important subcellular organelle and a key mediator of cellular metabolism that plays a critical role in apoptosis [2,3]. Hypoxia-inducible factor (HIF)-1α mediates the suppression of mitochondrial biogenesis in response to hypoxia. The signals that promote biogenesis include starvation-induced peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) activity as well as blocking HIF-1α stabilization and its inhibitory effects on biogenesis [4]. Cell survival requires the control of the redox state by an antioxidant system. Excessive reactive oxygen species (ROS) production can lead to the death or survival of various cell types, and the main sources of ROS are NADPH oxidase (NOX) and the mitochondria [5,6]. The low oxygen level under hypoxic conditions prevents HIF-1 hydroxylation, which subsequently leads to the stabilization of HIF-1. However, several recent studies have shown that the hypoxia-induced production of ROS in mitochondria is both necessary and sufficient for hypoxia-dependent HIF-1 accumulation, suggesting that mitochondria may act as an oxygen sensor for HIF-1 regulation by generating ROS under hypoxic conditions [7,8]. In this aspect, it has been reported that CST targets mitochondrial respiratory chain complex I to induce ROS-dependent cytotoxicity in tumor cells. The enhancement of HIF-1a expression by CST is correlated with ROS-initiated AKT activation, which enhances HIF-1a translation. As an important transcriptional factor, the accumulation of HIF-1a may affect multiple signaling pathways and regulate various biological functions, such as inducing autophagy, promoting tumor cell invasion and metastasis [9,10]. Therefore, CST could provide an alternative or complementary way to treat cancer by targeting multiple dysregulated pathways in cancer cells.

The anticancer drug, axitinib (AXT) is a small molecule tyrosine kinase receptor inhibitor of VEGFR1, 2, and 3 and platelet derived growth factor (PDGF), specifically [11,12]. In addition, celastrol (CST), which is another anticancer drug that induces the sequential inhibition of the HIF-1α and mTOR pathways, leading to the suppression of angiogenesis [13,14], improves the antitumor activity of standard cancer chemotherapeutics [15]. Therefore, the development of a therapeutic strategy including small molecule inhibitors that can effectively exploit the hypoxic tumor microenvironment by suppressing angiogenesis and HIF-1α activity may represent a significant advance in cancer treatment.

Nanoparticles (NPs) as a model for drug delivery have been shown to be a valid approach to achieving co-loading of multiple drugs into a single formulation and their simultaneous delivery to tumor cells *in vitro* and *in vivo* [16–19]. Among them, liposomes are the most widely investigated drug delivery systems for cancer therapy owing to their ability to transport hydrophobic and hydrophilic drugs and excellent *in vivo* performance [20]. Furthermore, mesoporous silica nanoparticles (MSN) are believed to have the potential for use as drug carriers because of their high loading capacity, biocompatibility, and mass-producibility, as well as their high degree of uniformity in size, morphology, and pore diameter [21,22]. For the maximization of therapeutic effectiveness, dual-drug delivery systems have been recommended in clinics. Combinational drug therapy has two important benefits [23–25]. First, the combination of drugs with different molecular targets may delay the development of tumor cell mutations. Second, a combination of drugs may simultaneously target associated signaling pathways and, thereby induce synergistic effects leading to higher therapeutic efficacy and target selectivity [26].

Therefore, we aimed to increase the synergistic efficacy of two drugs by allowing their sequential release from a single delivery system. Therefore, we loaded CST into an MSN carrier and subsequently coated it with a lipid bilayer. Furthermore, AXT was loaded into the lipid bilayer during the self-assembly process. The differential loading of drugs allows the sequential release pattern where AXT is released first to exert its anticancer effect, followed by CST to further induce a synergistic effect. We hypothesized that the combined delivery of AXT and CST would increase their therapeutic efficacy against cancer cells by acting via multiple pathways. The inhibition of angiogenesis and mitochondrial apoptosis by ACML was demonstrated in neuroblastoma (SH-SY5Y), squamous carcinoma (SCC7), and breast cancer (BT474) cell lines. Furthermore, an SCC7-bearing xenograft tumor model was developed to evaluate the synergistic therapeutic efficacy of ACML. Immunohistochemical analysis was performed to characterize the expression of markers of apoptosis (caspase-3 and poly [ADP-ribose] polymerase [PARP]), angiogenesis (platelet/endothelial cell adhesion molecule 1 [PECAM1] and cluster of differentiation 31 [CD31]), and proliferation (Ki-67).

## 2. Materials and methods

### 2.1. Materials

Axitinib was purchased from LC Labs (Woburn, MA, USA). Celastrol was obtained from Aktin Chemical, Inc., (Chengdu, China). Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), ammonium fluoride ($NH_4F$), aminopropyltriethoxysilane (APS), cholesterol B, and 1-palmitoyl-2-(6-[{7-nitro-2-1,3-benzoxadiazol-4-yl}amino]hexanoyl)-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained from NOF America Corporation (White Plains, NY, USA). DSPE-PEG2000 was purchased from Avanti Polar Lipid (Alabaster, AL, USA). All other chemicals were of reagent grade and used without further purification.

### 2.2. Preparation of dual drug-loaded ACML

Briefly, the MSN was prepared by fully dissolving 213 mg of CTAB in 200 mL of water at 80 °C, followed by the addition of CST and then 30 mg of $NH_4F$. This reaction mixture was kept at 80 °C for 1 h with continuous stirring (1200 rpm) [27]. Then, TEOS (1.5 mL) was immediately added dropwise for 20 min, followed by stirring for 2 h until it achieved a semi-transparent colloidal state. MSN was centrifuged for 10 min using a high-speed centrifuge, and the resuspended MSN pellet was then placed on a thin film membrane to form coarse MSN-loaded liposomal nanoparticles, which were immediately probe sonicated to form the monodispersed nanosized ACML. Then, 10% (w/w) of AXT was added to the lipid mixture before the thin-film was hydrated. The PEGylated lipid bilayers were prepared using DSPE-$PEG_{2000}$ by adding an amount that was about 2% (w/v) of the liposomal nanoparticle volume. The exact amount of polymer required to cover the MSN-lipid surface was determined by mixing various ratios of the liposomal nanoparticle (w/v) until nanoparticles with a uniform size distribution were produced.