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 xenograph 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-1 alpha (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-1α expression by CST is correlated with ROS-initiated Akt activation, which enhances HIF-1α translation. As an important transcriptional factor, the accumulation of HIF-1α 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 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 chemotherapy agents [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-productivity, 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 antitumor 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 (NH4F), aminopropyltriethoxysilane (APS), cholesterol B, and 1-palmitoyl-2-(6-[7-nitro-2-1,3-benzoxa diazol-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 NH4F. 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/v) of AXT was added to the lipid mixture before the thin-film was hydrated. The PEGylated lipid bilayers were prepared using DSPE-PEG2000 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.
2.3. Particle size and analysis of ζ-potential

The Z-averaged hydrodynamic particle size (nm), polydispersity index (PDI), and ζ-potential (mV) of the nanoparticles were determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) using the dynamic light scattering (DLS) method. The mean value was calculated from three individual measurements.

2.4. Drug loading

To calculate drug-loading efficiency (LE %), the drug-loaded nanoparticles were separated from the unbound drugs using ultrafiltration with an Amicon centrifugal filter device (molecular weight cut-off, MWCO, 10,000 Da, Millipore). The formulations were centrifuged at 5000 rpm for 10 min, and the unbound drugs in the filtrate were analyzed using high-performance liquid chromatography (HPLC). A Hitachi L-2400 equipped with a C18 column (5 µm, 4.6 × 150 mm) was used. A mobile phase mixture of acetonitrile:ethanol:water:trifluoroacetic acid (3/5/2/0.1, v/v/v/v) was used at a flow rate of 1 mL/min.

The drug loading capacity (LC wt%) and LE% were calculated using the following equations:

\[
\text{LC } (\%) = \left( \frac{W_{\text{drug in NP}}}{W_{\text{NP}}} \right) \times 100
\]

\[
\text{LE } (\%) = \left( \frac{W_{\text{drug in NP}}}{W_{\text{total drug}}} \right) \times 100
\]

2.5. Morphological analysis

The morphology of ACML was determined using transmission electron microscope (TEM) (CM 200 UT, Philips, MA, USA) operated at 100 kV. Briefly, the ACML dispersions were applied onto a carbon-coated copper grid (300-mesh) and allowed to adhere to the carbon substrate. A drop of 2% phosphotungstic acid solution was applied as a negative stain, followed by air-drying, and observation using a TEM.

2.6. X-ray diffraction analysis

The X-ray diffraction (XRD) patterns of the samples were recorded using a vertical goniometer and X-ray diffractometer (X’Pert PRO MPD diffractometer, Almelo, The Netherlands) to measure the Ni-filtered CuKα radiation (voltage, 40 kV; current, 30 mA) for 24 h. After harvesting, the cells were lysed using M-per buffer (Thermo Scientific, USA) with proteinase inhibitors and shaking (thrice), followed by a 40-min incubation on ice. The mixture was then centrifuged at 13,000 rpm in a microcentrifuge for 20 min at 4 °C. The proteins were quantitatively analyzed using the bicinchoninic acid (BCA) method (Thermo Scientific, USA). Proteins in the cell lysate extracts were electrophoretically separated using a 12% bis-Tris-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (210 mA for 90 min). After blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing Tween 20 (TBST, pH 7.2), the membranes were incubated with antibodies against hydroxy-HIF-1α, cytochrome-C, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and cleaved-caspase-9 (Cell Signaling Technology, Beverly, MA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, UK) in 5% BSA in TBST, overnight. Membranes were further incubated with goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP antibodies (Santacruz, USA) in 5% BSA in TBST.

2.12. Apoptosis and necrosis analysis

To determine the apoptosis and necrosis induced by the drug formulations, an Annexin V–fluorescein isothiocyanate (FITC) kit (BD) was used. Briefly, the SCC7, BT474, and SH-SYSY cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, DME/F12 (both containing a high concentration of glucose), and Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Hyclone Laboratories, Logan, UT, USA), respectively. Cells were maintained at 37 °C in an atmosphere of 5% CO₂.
harvested after incubation with the drugs, washed with PBS twice, stained with AnnexinV-FITC and propidium iodide (PI, 2.5 μl each) for 15 min, and then apoptosis and necrosis were evaluated using fluorescence-activated cell sorting (FACS, BD, FACsverse). For each sample, 10,000 cells were analyzed.

2.13. Glutathione (GSH)/glutathione disulfide (GSSG) ratio determination in SCC7, BT-474, and SH-SY5Y cells

To determine the glutathione (GSH)/glutathione disulfide (GSSG) ratio in the three cell lines, we used a GSH/GSSG-Glo™ assay kit (Promega, USA). For each cell line, 4 × 10⁶ cells/well were plated in white 96-well plates, incubated with the drugs, then the media was removed, and cells were lysed and treated with either total (GHS) or oxidized (GSSG) reagent. After a specified period of incubation with shaking, the luciferin-generating reagent was added, and then after a 30-min incubation the luciferin detection reagent was added. Finally, the luminescence was detected.


The care and handling of the mice and the experimental protocols were performed in accordance with the Guidelines of the Institutional Animal Ethical Committee of the Yeungnam University, South Korea. For the antitumor study, the tumor model was established in 7-week-old female nude BALB/c mice by injecting 1 × 10⁶ SCC7 cells (100 μL) subcutaneously into their thighs (flank). The experiments commenced when the tumor volumes attained approximately 50–75 mm³. The mice were randomly divided into five experimental and one control group (7 mice per group) and injected with 1 mg/kg of (1) AXT, (2) CST, (3) AXT/CST cocktail, (4) ACML via the tail vein four times every 3 days, while the control was untreated. The tumor size of each group was measured for 21 days using a digital caliper and final volume calculated using the formula: \[ V = \left( \frac{L \times W^2}{2} \right) \text{mm}^3 \]. In addition, body weight changes were determined to assess the toxicity of each formulation. All xenografted mice in each group were euthanized using the CO₂ inhalation method. Fresh tumor tissue was immediately excised, fixed in formalin solution, and processed for immunohistochemical (IHC) analysis.

2.15. Histopathology and immunohistochemistry

Hematoxylin and eosin (H&E) staining was performed to determine the histopathological and histomorphometric changes in the experimental group tumors. Paraffin-embedded tumor samples were serially sectioned (3–4 μm), stained with H&E, and observed using a light microscope (Nikon, Tokyo, Japan). Apoptosis (anti-caspase-3 and anti-cleaved PARP antibodies), angiogenesis (anti-PECAM-1 antibody), and proliferation (anti-Ki-67 antibody) were analyzed using the indicated antibodies and visualized using an avidin-biotin-peroxidase complex (ABC) method. The sections were incubated with primary antisera overnight at 4 °C and then with detection reagents for 1 h at 25 °C. The tumor sections were then treated with a peroxidase substrate kit for 3 min and rinsed with 0.01 M PBS thrice between each step. The area of each section containing 20% cells that were immunopositive for caspase-3, PARP, CD31, and Ki-67 was regarded as positive. The percentage of each tumor section that contained cells expressing caspase-3, PARP, CD31, and Ki-67 was measured using an automated image analyzer (%/mm² of tumor mass).

2.16. Statistical analysis

A multiple comparison test was performed to analyze the differences between the groups. The homogeneity of variance was determined using the Levene test. If the Levene test indicated no significant deviations from homoscedasticity, the data were analyzed using a one-way analysis of variance (ANOVA) followed by the least-significant differences (LSD) multi-comparison test to determine the pairs of group comparison that were significantly different. Differences were considered significant if \( p < 0.05 \).

3. Results and discussion

3.1. Preparation of ACML

The preparation of the PEGylated lipid bilayer supported MSN composite for co-delivery of AXT and CST (ACML) is illustrated in Fig. 1. First, the MSN with ca. 70 nm particle size was fabricated using the optimal ratio of CST, CTAB, NH₄F, and TEOS at 80 °C in distilled water. Then, the MSN was encapsulated by the PEGylated lipid bilayers with AXT using a thin-film hydration technique. The therapeutic efficacy of anticancer drugs depends on the nanocarriers being able to finally release their drug content near the cancer site. PEGylation of nanocarriers has been reported to prolong their circulation in the bloodstream and reduce their uptake by the reticuloendothelial system (RES) [28]. Therefore, the formulation variables including the weight ratios of the MSN composites and lipid compositions were extensively optimized to obtain suitable nanoparticles. After a capping lipid bilayer was added, the particle

Fig. 1. Preparation of PEGylated lipid bilayer supported mesoporous silica nanoparticle composite for synergistic co-delivery of axitinib (AXT) and celastrol (CST, ACML).
size of ACML became ca. 120 nm and showed a narrow PDI (~0.07). Moreover, uniform coating of the bilayer was confirmed by reversal of the ζ-potential from a positive to a detectable neutral charge (Fig. 2). When the MSN was initially coated, the electrostatic forces induced a physical interaction and at this stage, the ACML formed a stable, protective, and compact nanoparticle. The nanoparticles were prepared using a 1:1 weight ratio of MSN:lipid bilayers. At this optimal ratio, the particle size and ζ-potential were ideal, which prolonged the blood circulation time and the overall anti-cancer effect of the formulation by the preferential extravasation of tumor vessels [29]. Therefore, for further studies, we selected the formulation composed of 213, 30, and 5 mg CTAB, NH₄F, and CST, respectively, and 1.5 mL TEOS, which was coated with a 1:1 weight ratio of MSN:lipid bilayers.

3.2. Morphological analysis

A TEM was used in imaging the particles to evaluate the formation of CST-loaded MSN and ACML. As shown in Fig. 2C and D, both MSN and ACML exhibited a distinguishable spherical particle shape with a uniform size distribution. The particle size of the MSN was approximately 50 nm with smaller mesopores than those of the ACML. When the MSN was covered with lipid bilayers, the particle size increased to approximately 120 nm. Furthermore, the nanoparticle image clearly showed that the pores of the MSNs were surrounded by light gray shell membranes.

The X-ray diffraction (XRD) patterns of the free AXT, CST, blank ACML, and ACML are shown in Fig. S1. The diffraction patterns of free AXT showed numerous sharp and intense peaks at two Theta scattered angles 11.5, 13.8, 18.6, 20.8, 11.5, 21.6, 24.3, and 26.4°, indicating its highly crystalline nature. ACML, however, did not have such characteristics peaks, indicating the presence of either the amorphous or the molecularly dispersed form of the drug. It is worth noting that the peaks at 19.2 and 24.1° correspond to MSN.

3.3. Drug loading and release

A combination of hydration, sonication, and mixing achieved a high LE% of 99.9 ± 2.4% with an LC% > 21%, which was attributable to the possible immersion of CST in the MSN and the deep immersion of AXT in the lipid bilayer. In addition, the added cholesterol acted as a fluidizer or membrane rigidifying agent during the loading of AXT into the lipid bilayer structure [30].

The in vitro drug release study was performed in PBS (pH 7.4) and ABS (pH 5.0, Fig. 3). The release rates of AXT and CST from the ACML were significantly retarded at pH 7.4 (~8.7 and 10.5%
of AXT and CST, respectively) compared with pH 5.0 (15.8% of AXT, ~16.4% of CST) by the end of the 48-h study period. Furthermore, the DSPE-PEG layer controlled the release of the drug from the ACML. Accordingly, a pH-responsive, slower, and continuous release pattern from an optimized ACML formulation may be significant for the adequate delivering of anticancer drugs [31]. The ACML exhibited a controlled release profile at pH 7.4. At pH 7.4 conditions, PEG-b-DSPE exhibits a high stealth layer property thereby preventing the release of the encapsulated compound. The results of present study showed that the drug release rate from ACML accelerated at pH 5.0, indicating the disassembly of the protective shell around the NP surface. In this case, the diffusion was predicted to be the predominant mode of drug release from the carrier system [29]. The pH-sensitive drug release rate, which ensures maximum drug release with in the cancer cells, may improve antitumor efficacy and decrease unwanted toxicity to normal tissues [32].

3.4. Cytotoxicity and cellular uptake

The in vitro cytotoxicity assay was performed to evaluate the toxicity of the anticancer agents against the SCC-7, BT-474, and SH-SY5Y cells. As shown in Fig. 4, combined with a fixed concentration of 5 μM of AXT, the mitochondrial-targeted drug CST, exhibited a concentration-dependent cytotoxicity at 0.001–10 μM. Based on the half-maximal inhibitory concentration (IC50) value of AXT, we used a fixed dose of 5 μM/ml throughout the study. Previously, we showed that AXT is effective in killing cancer cells at concentrations above 5 μM/mL. When CST (5 μM) was used alone, the viability of the SCC-7, BT-474, and SH-SY5Y cell lines was 55, 74, and 56%, respectively, while combination treatment reduced the cell viability to 49, 70, and 44%, respectively. In particular, a significantly higher cytotoxicity was observed at a concentration of 5 μM for both drugs while the ACML-induced cytotoxicity at the same concentration was 27, 66, and 22% in the SCC-7, BT-474, and SH-SY5Y cells, respectively. AXT specifically inhibits VEGFR, which subsequently inhibits tumor cell growth mainly by interfering with angiogenesis, whereas CST plays an important role in the mitochondrial HSP90 of the mitochondrial electron transport chain. The augmented cytotoxicity of the combined drugs was quantitated as synergistic, additive, or antagonistic effects (Fig. S2). The Chou and Talalay method was used to calculate the CI values, where values of 0.9–1.1 indicate additive activity, values less than 0.9 indicate drug synergy, and values...
Fig. 5. Cellular uptake of coumarin 6-loaded AXT/CST-loaded combination nanoparticles (ACML) by SCC7, SH-SY5Y, and BT-474 cells. Cells were incubated with coumarin 6-loaded ACML for 60 min and observed using a confocal microscope.

Fig. 6. Apoptotic effects of free drugs and nanoparticle formulation in cancer cell lines. Flow cytometric analysis of apoptosis with free drugs and AXT/CST-loaded combination nanoparticles ACML using fluorescence-activated cell sorting (FACS) analysis in (A) SCC7, (B) SH-SY5Y, and (C) BT-474 cells.
more than 1.1 indicate antagonism. Although the level varied from cell to cell, the combination (AXT/CST) indicated synergism in the cell lines tested. It is noteworthy that for all the drug combinations, the concentration of AXT was fixed at 5 μM while that of CST was varied from 0.01 to 100 μM. These two drugs delivered by PEGylated lipid bilayer-coated MSN acted synergistically by blocking the signal transduction pathways of the inner vessel walls and the apoptotic pathway of the mitochondria and, thereby, maximized the tumor suppressant effect.

The subcellular localization of ACML in each of the three cell lines was investigated using confocal microscopy (Fig. 5). ACML was mainly colocalized in the peripheral cytosol of all the cell lines tested, suggesting that this system was bound to the cell surface and then taken up via endocytosis. The colocalization of ACML and the Lysotracker-red fluorescence revealed the presence of combination particles in the lysosomes. Studies have shown that dual drugs from ACML can be eluted in the low pH environment of the endosomes and lysosomes on reaching the desired target [33,34]. Furthermore, the efficiency of the cellular uptake was similar with the three cell lines. In addition, the neutrally charged surface of the ACML decreased the nonspecific binding to opsonins and inhibited the RES effect, which can prolong the circulation of this carrier in the bloodstream. Therefore, ACML could be accumulated at the tumor site by the EPR effect [35].

3.5. In vitro apoptosis effect and GSH/GSSG ratio

Growth factors, cytokines, and DNA damage appear to signal cell death through the mitochondria and, therefore, we used Annexin-V/PI to confirm the apoptotic effect of AXT, CST, and ACML. In Fig. 6, we showed that the free drug-treated group exhibited a strong apoptosis (SCC7 and SH-SY5Y) and necrosis rates (BT-
However, the ACML-treated group improved the drug combination activity by minimizing the necrosis region and enhancing the apoptosis region in all three cell lines. The controlled release of the drugs from the formulation might have contributed to this result. Additionally, we expect that the persistent release of drugs from the two kinds of carriers would further enhance the apoptosis of cancer cells.

Apoptosis occurs in the cell though two pathways, which are the extrinsic and intrinsic pathways. The depletion of mGSH by drugs can compromise ROS detoxification, leading to its accumulation, which ultimately results in the oxidation of cardiolipin, which plays a key role in mitochondrial physiology and cell-death regulation [36]. Therefore, we also sought to confirm the effect of AXT, CST, and ACML on the GSH/GSSG ratio of the mitochondria. Fig. 7 shows that the groups treated with drugs exhibited a reduced GSH/GSSG ratio compared with that of the control group. Specifically, the CST-treated group showed a more significant reduction in the GSH/GSSG ratio than the AXT-treated group did. Because AXT mainly targets the VEGF to inhibit angiogenesis, it does not significantly affect the mitochondrial GSH. The dual drug (AXT/CST)-treated group exhibited a synergistic effect against the target cell. Therefore, the dual drug-treated group decreased the GSH/GSSG ratio more than the single drug-treated groups did. Importantly and noteworthy is the observation that the ACML group showed similar effects with the AXT/CST cocktail group. Based on the results, the change in the intracellular GSH levels and GSH/GSSG ratio can be considered as evidence of the influence of the drug on apoptosis of the cells (Fig. 7).

3.6. Angiogenesis and mitochondrial targeting

Angiogenesis is one of the major factors that control cancer cell growth and is modulated by HIF-1α [2,20]. Therefore, angiogenesis has emerged as an attractive target for the development of new anticancer therapeutics [37]. In addition, the mitochondria are involved in the regulation of cellular proliferation. Thus, the effects of AXT, CST, and ACML on HIF-1α and mitochondrial function were determined using western blot analyses (Fig. 8). Under hypoxia, ACML markedly increased hydroxy-HIF-1α expression in the SCC.

![Fig. 9. In vivo antitumor efficacy of AXT/CST-loaded combination nanoparticles (ACML). Effects on (A) tumor volume and (B) body weight following intravenous administration of AXT, CST, AXT/CST, and ACML (1 mg/kg each) to SCC7 xenografted mice. Each formulation was administered four times at 4-day intervals. Data are presented as mean ± standard deviation (SD, n = 7). *p < 0.05 and **p < 0.01.](image)

![Fig. 10. Histopathological and immunohistochemical analyses of changes in antitumor activities of AXT and CST after treatment with AXT/CST-loaded combination nanoparticles (ACML). (A) Representative images of histopathological changes in tumor xenografts of nude mice. Immunoreactivities of (B) caspase-3 and poly (ADP-ribose) polymerase (PARP) and (C) CD-31 and Ki-67 in tumor masses of nude mice following treatment with indicated formulations. Caspase-3 and PARP, markers for apoptosis; CD-31 and Ki-67, markers for angiogenesis; scale bars = 120 μm.](image)
7 cell line and decreased HIF-1α expression in the BT-474 and SH-SY5Y cell lines compared with the control. However, we observed that BAX expression was similar in all three cell lines. BAX is a key factor in cellular-induced apoptosis through mitochondrial stress [38]. In addition, the BAX protein translocates to the mitochondria in response to death stimuli, including survival factor withdrawal. Through interactions with pore proteins on the mitochondrial membrane, BAX increases the membrane permeability, which releases cytochrome C from the mitochondria and, thereby, activates caspase-9, initiating the caspase activation pathway of apoptosis [39,40].

Bcl-xl, Bcl-2, and BAX may influence the voltage-dependent anion channel, which likely plays a role in regulating cytochrome C release. Based on the function of these BAX proteins, we confirmed that there was a decrease in Bcl-2 and increase in cleaved caspase-9 protein expressions. However, in the AXT and AXT/CST groups, we observed that the cleaved caspase-9 expression was deceased compared with control in the SH-SY5Y cell line and this was likely because the effect of AXT is specific to the SH-SY5Y cell line model. In addition, we confirmed the association of these proteins with mitochondrial apoptosis under normoxic conditions (data not shown). The protein level in each treated groups did not change compared with the control group. Therefore, we concluded that inhibition of the mitochondrial survival and HIF-1α expression in the cancer cells, as well as the enhanced accumulation of the drug in the cytoplasm, promoted the antiangiogenic and mitochondrial apoptotic effects of the drug against the tumor cells.

3.7. In vivo antitumor efficacy

We evaluated the anticancer effect of different formulations in an in vivo xenograft mouse model. After inducing tumor growth for 1 week (SCC7 cell line), we administered the free drug and the different formulations via tail vein injections. Compared with the control and free drug groups, the ACML group remarkably inhibited the tumor growth. Tumor regression on day 18 in all the groups was in the order ACML > AXT/CST cocktail > CST > AXT compared with the untreated group (Fig. 9A). We also determined the weight of the mice to evaluate the safety profile of individual formulations (Fig. 9B). The CST- and AXT/CST cocktail-treatment groups showed a 20% body weight loss. This result suggests that the high level of cytotoxicity of CST resulted in body weight loss while the dosage forms of ACML likely protected the mice from the side effects of the anticancer drug. The enhanced antitumor effect and fewer side effects of ACML were attributable to the characteristics of the combination nanoparticle drug delivery vehicles. These characteristics include its dual site targeting and sustained drug release, prolonged circulation in the bloodstream, and the EPR effect that facilitated the accumulation and intracellular delivery of the drug to the tumor interstitial space [41–43].

Angiogenesis is one of the critical steps in tumor growth and metastasis. Inhibition of HIF-1α transcription by AXT inhibits the expression of angiogenic factors and their binding (e.g., VEGF), tumor metastasis, invasion, and growth [44,45]. Here, we demonstrated that HIF-1α is rapidly degraded by a family of hypoxia-inducible factor prolyl hydroxylases, which may further control the neovascularization and regeneration of tumor tissue after degradation. Based on the results, we are highly convinced that the systemic co-delivery of AXT/CST by the PEGylated lipid bilayer-supported MSN composite effectively inhibited the HIF-1α expression in the xenograft model.

3.8. Histopathological and immunohistochemical (IHC) analyses

Histopathological and IHC examinations were performed after treatment with AXT, CST, and ACML. A significant (p < 0.01) decrease in tumor cell volumes as well as CD31- and Ki-67-positive cells and increase in caspase-3 and PARP-immunoreactive cells was demonstrated with all the drugs or formulations. The anticancer activity was in the order of ACML > AXT/CST > CST > AXT, compared with the tumor-bearing vehicle control (Fig. 10A). Especially, the AXT/CST cocktail- and ACML-treated tumors showed significant (p < 0.01) decreases in tumor cell volume as well as CD31 and Ki67 immunolabeled cells, and increases in caspase-3 and PARP-immunopositive cells compared with the AXT- and CST-treated tumor masses. More specifically, the tumor cell volumes and CD31 and Ki-67 immunoreactive cells in the ACML-treated tumor masses were significantly (p < 0.01 or p < 0.05) decreased compared with the AXT/CST cocktail-treated tumor masses and showed significant (p < 0.01) increases of caspase-3 and PARP-positive cells (Fig. 10B). These results indicate that the antitumor activities of AXT and CST were noticeably increased following modification by the ACML system as evidenced by the increased tumor cell apoptosis likely mediated by inhibition of angiogenesis and tumor cell proliferation. The decreases in tumor cell volumes, angiogenesis and cell proliferating markers, CD31 and Ki-67 immunolabeled cells, as well as increases in the immunoreactivities of apoptotic markers, caspase-3, and PARP were demonstrated in the order of ACML > AXT/CST > CST > AXT compared with the tumor-bearing vehicle control in the histopathological and IHC analyses (Fig. 10C). In particular, the ACML-treated tumor masses showed a significant increase in immunoreactivities of the apoptotic markers compared with tumor masses of the AXT-, CST-, and AXT/CST-treated groups in addition to significant decreases of tumor cell masses and CD31 and Ki67 immunolabeled cells, in this study. Therefore, these results indicate that the complete arrest of angiogenesis, proliferation, and mitochondrial apoptosis was corroborated by the H&E

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor cell volume (%/mm² of tumor mass)</th>
<th>Immunolabeled cells percentages (%/mm² of tumor mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caspase-3</td>
</tr>
<tr>
<td>Control</td>
<td>83.16 ± 12.16</td>
<td>6.64 ± 2.40</td>
</tr>
<tr>
<td>AXT</td>
<td>65.84 ± 4.58a</td>
<td>28.00 ± 9.76¹</td>
</tr>
<tr>
<td>CST</td>
<td>61.44 ± 8.44a</td>
<td>52.79 ± 13.38</td>
</tr>
<tr>
<td>AXT/CST</td>
<td>47.66 ± 5.38bc</td>
<td>52.79 ± 11.40bc</td>
</tr>
<tr>
<td>ACML</td>
<td>34.28 ± 7.23bd</td>
<td>78.88 ± 10.38</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six tumor mass histological fields.

PARP = Cleaved poly(ADP-ribose) polymerase.

PECAM-1 = Platelet endothelial cell adhesion molecule 1 (CD31).

¹ p < 0.01 as compared with control by LSD test.
² p < 0.01 as compared with AXT by LSD test.
³ p < 0.01 as compared with CST by LSD test.
⁴ p < 0.01 and ⁵ p < 0.05 as compared with AXT/CST by LSD test.
staining analysis, which showed a reduced tumor cell volume (34.28 ± 7.23) in mice treated with ACML (Table 1).

4. Conclusions

In this study, a combination drug study involving the incorporation of two anticancer agents in a novel delivery system, ACML, was achieved successfully. The ACML contained drugs that target angiogenesis and the mitochondria in tumors. Here, we demonstrated that the administration of ACML effectively inhibited angiogenesis and mitochondrial function and was efficiently internalized in the SCC-7, BT-474, and SH-SYSY cell lines. Moreover, we showed a reduced HIF-1α expression level under hypoxic conditions in all three cell lines that were treated with ACML, indicating that this may be a key molecular mechanism of tumor inhibition by this formulation. In addition, ACML blocked tumor cell proliferation and inhibited angiogenesis under hypoxic conditions as well as exhibited a more synergistic apoptotic effect against cancer cells than the AXT/CST cocktail did. Furthermore, this synergistic effect might be attributable to the combination of AXT, which controls VEGFR and CST, which acts on the mitochondria of the target cells. Moreover, the in vivo results confirmed that the tumor inhibitory effect of the dual drug system in a tumor-xenograft mouse model was superior to that of either drug administered alone. This could be attributed to the EPR effect and the effective intracellular delivery of the drug. IHC analysis of tumor xenografts detected elevated levels of caspase-3 and PARP and reduced expression of CD31 and Ki-67, suggesting that apoptosis occurred via the mitochondria and antiangiogenics within the tumor. In conclusion, our results indicate that the AXT/CST drug combination loaded in a nanocarrier inhibited cell proliferation and induced apoptosis by blocking mitochondrial function leading to enhanced antitumor efficacy. These results suggest that this combination drug delivery system has the potential for use as an effective therapeutic strategy for the treatment of cancer, and may be particularly useful in recalcitrant cases.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.05.012.

References


