Research Article

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Multifunctional nanoparticles for targeted delivery of apoptin plasmid in cancer treatment

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Abstract: The systemic toxicity and low efficacy of traditional chemotherapy for hepatocellular carcinoma (HCC) result in poor clinical outcomes. This study was designed to achieve targeted delivery of apoptin plasmid (AP) to liver tumors and killing of cancer cells using multifunctional nanoparticles (MFNPs) having sustained-release properties. The MFNPs featuring a distinct core-shell structure were prepared using poly(lactic-glycolic acid)-ε-polylysine copolymer and loaded with AP by adsorption. Specific targeting of liver tumor cells was achieved by biotinylation of the nanoparticles (NPs), while an improvement in lysosomal escape and nuclear localization enhanced the tumor cell killing capability of AP. Blank MFNPs exhibited good biocompatibility while AP-loaded NPs were found to exert strong inhibitory effects on both tumor cells in vitro and solid tumors in vivo. Taken together, these findings demonstrate a promising route for the development of tumor-targeted NPs which may lead to improved therapeutic strategies for treating HCC.

Keywords: multifunctional nanoparticles, targeted delivery, apoptin plasmid, hepatocellular carcinoma

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers, with approximately 800,000 cases per year worldwide (1). Although the incidence and mortality of HCC are still increasing (2), conventional clinical treatments such as surgical resection and loco-regional ablation are only suitable for early-stage HCC (3). Advanced cases featuring malignant proliferation and metastasis are still treated mainly by chemotherapy at present (1,3). However, systemic toxicity and drug resistance severely limit the therapeutic outcomes with a little patient survival benefit (4,5). Therefore, novel anti-cancer agents with improved efficacy and tumor-targeted drug delivery systems, to reduce the administered dose and side effects, are urgently needed to improve HCC treatment.

A wide variety of nanoparticle (NP) carriers have been developed and investigated extensively for the delivery of anti-cancer therapeutics including liposomes, dendrimers, biodegradable polymers including poly(lactide-co-glycolide) (PLGA) and polysaccharides, and inorganic particles based on silica, for example (6–9).

Intravenously injected “monofunctional” NPs, in general, passively accumulate at tumor sites due to the enhanced permeation and retention effect. The subsequent interaction of NPs with cells in vivo is controlled by various factors, including particle size, surface charge, and morphology (10,11), which are difficult to control accurately to enhance the internalization of NPs in the tumor (10). As a result, multifunctional nanoparticles (MFNPs) displaying “active” tumor-targeting ability and controlled drug release have attracted significant interest for improving cancer treatment. Active targeting strategies often involve incorporating ligands in the (drug-loaded) NP surface which engage with surface receptors on cancer cells (12,13). Drug release rates can be controlled by manipulation of the particle size, morphology, polymer degradation, and pH-responsive behaviors among other factors (14–16).

Biotin (BT) has attracted much attention in this respect due to its high tumor specificity. Since BT is an essential
nutrient for cell growth and function, malignant growth and proliferation of tumor cells result in much higher demand than that of normal cells, causing over-expression of BT receptors in various tumor cells (17,18). In comparison, ASGPR receptors are abundant and specifically expressed in both normal and tumor liver cells and can bind specifically to lactose and galactose residues (5,10,19,20). Thus, NP carriers modified by lactose or galactose residues accumulate in both sites, showing a lack of targeting specificity.

Apoptin plasmid (AP) is a 121-amino acid proline-rich protein derived from the chicken anemia virus, which can specifically induce tumor cell apoptosis without harming normal cells (21,22). AP contains a bipartite nuclear localization signal (amino acids 82–88 and 111–121, NLS) and a nuclear output signal (amino acid 33–46, NES). Apoptosis is associated with its intracellular distribution; AP predominantly accumulates in the nucleus of tumor cells, whereas in normal cells, AP remains in the cytoplasm. Apoptosis induced by AP is mitochondrial-dependent and involves three key proteins, apoptotic protease-activating factor 1 (apaf-1), cytochrome C (Cyt C), and caspase9 (21–24).

In this study, AP-loaded MFNPs were prepared using a copolymer of PLGA and e-polylysine (EPL). PLGA has been used extensively for controlled drug delivery due to its properties of biodegradability and biocompatibility and has been approved by the US Food and Drug Administration (FDA) and European Medicine Agency (25–27). EPL has also been exploited for controlled drug delivery. The polymer exhibits favorable biocompatibility and has been approved by the FDA (28,29). Multifunctional tumor-targeted NPs incorporating BT as the targeting ligand were constructed using a “layer-by-layer” approach. PEGylated BT was adsorbed on the surface of MFNPs through electrostatic interaction, to reduce the clearance of MFNPs by the reticuloendothelial system and prolong the blood circulation time (18,30). The cell-targeting ligand was anticipated to greatly enhance tumor-specificity and receptor-mediated endocytosis (20). MFNP escape from lysosomes was envisaged due to the pH response mechanisms of functional materials (30) or the “proton sponge effect” (31). Tumor cell apoptosis follows AP release from the NPs and subsequent localization in the nucleus under the action of NLS. The synthesis and apoptosis pathway of MFNPs is shown in Scheme 1.

The production and physicochemical characterization of the AP-loaded MFNPs are reported here along with in vitro and in vivo studies of liver tumor-targeting capacity to evaluate their potential utility for the treatment of HCC.

2 Materials and methods

2.1 Materials

PLGA, polyethylene glycol (PEG), N-hydroxysuccinimide-polyethylene glycol-maleimide (NHS-PEG-MAL), N-hydroxysuccinimide (NHS), 4-dimethylaminoppyridine (DMAP), 1-(3-dimethylaminopropyl)-3-ethylcarboxydiimide hydrochloride (EDC), BT, and lactose acid (LA) were all purchased from Carbon Water Technology Co., Ltd. (Hangzhou, China). EPL was purchased from Ron Reagent Co., Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC), deuterated water (D2O), and deuterated dimethyl sulfoxide (DMSO–D6) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Cell navigator lysosome staining kit and NLS (MW 1.0 kDa) were all purchased from Applied Biosystems Trading Co., Ltd. (Shanghai, China). High glucose Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), 4,6-diamino-2-phenyl indole (DAPI), and Hematoxylin and Eosin (H&E) Staining Kit were all purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Cell Counting Kit 8 (CCK8) was purchased from AbMole BioScience Co., Ltd. (Houston, USA). Apaf-1, cyt C, and anti-caspase9 antibody were purchased from Zen BioScience Co., Ltd. (Chengdu, China). All reagents were of analytical grade, unless otherwise stated.

2.2 The synthesis of functional material

PLGA (MW 10,000, 50/50) and EPL (MW 4080) were dissolved in DMSO. DMAP was used as catalyst, with NHS and EDC as coupling agents to synthesize poly(lactic-glycolic acid)-e-polylysine (PLGA-EPL) copolymer. NHS-PEG-MAL (MW 400) conjugated NLS was attached to PLGA-EPL copolymer to produce PLGA-EPL-PEG-NLS (PEPN) macromolecular entity.

PEGylation of BT and LA was based on the amide reaction scheme. BT, NHS, and EDC were dissolved separately in DMSO. NH2-MFNPs were added to the mixture and allowed to react at 45°C for 12 h, resulting in synthesis of BT-Peg. BT-Peg was amidated with EDC and NHS and reacted with EPL at 45°C for 48 h to synthesize EPL-PePEG-BT (EPBT). The synthesis process of EPL-PePEG-LA (EPLA) was similar to EPBT and is detailed in the supplementary information.

2.3 Production of MFNPs

PEPN NPs were prepared initially using the nanoprecipitation method. PEPN was dissolved in DMSO and the solution was slowly added dropwise to a distilled aqueous solution in
a v/v ratio of 1:9 while stirring at high speed. The PEPN copolymers readily form NPs in aqueous solutions due to the presence of PLGA hydrophobic segments and EPL hydrophilic segments in the copolymer chain. The NPs were positively charged due to the effect of the EPL primary amine. MFNPs were constructed by adsorbing AP on the surface of the PEPN NPs through electrostatic interaction. EPBT or EPLA was subsequently adsorbed on the AP-modified NPs. AP was replaced by Glypicans-3 plasmid (GPC3) plasmid when preparing blank NPs.

NPs prepared by the above methods included: (i) BT-targeted NPs (MFNPs), (ii) blank NPs (blank MFNPs), (iii) LA-targeted NPs (PEPNL), (iv) untargeted NPs (PEPNP), containing EPL-PEG entity and without targeting ligands in the outer layer. The preparation process of the various NPs is detailed in the supplementary information.

2.4 Characterization of EPBT and EPLA targeting moieties and MFNPs

PEPN, EPLA, and EPBT were dispersed in DMSO-D_{6} or D_{2}O, and characterized by Proton nuclear magnetic resonance (^1H NMR) (AVANCEIII, Bruker, Switzerland). The surface charge and size of the various NPs were measured using a dynamic light scatterometer (DLS, Nano ZS90, Malvern, UK). MFNPs were dispersed uniformly on carbon-coated copper grids to observe the morphology and size by transmission electron microscopy (TEM, HT7700, Hitachi, Japan).

2.5 The loading capacity and release of AP from MFNPs

Blank PEPN NPs were suspended in deionized water and slowly added dropwise into AP solution (200 µg·mL^{-1}). The mixture was filtered using a 300 kDa ultrafiltration tube and the filtrate was analyzed using UV spectrophotometry (nano300, Allsheng, China) to determine the concentration of unadsorbed AP. The difference between the initial and unadsorbed AP concentration was used to calculate the weight of AP adsorbed on the NPs. The weight of AP-loaded NPs was obtained by drying the ultrafiltration tube in a vacuum and subtracting the
weight of the empty tube. The AP loading capacity and loading efficiency were determined as follows:

\[
\text{AP loading capacity (\%) \quad = \frac{\text{Weight of adsorbed AP}}{\text{Weight of AP-loaded NPs}} \times 100}\]

\[
\text{AP encapsulation efficiency (\%) \quad = \frac{\text{Weight of adsorbed AP}}{\text{Total weight of AP}} \times 100}\]

AP-loaded MFNPs and AP-loaded PEPN NPs without the EPBT targeting ligand were dispersed in phosphate buffered saline (PBS) with different pH values at 37°C and agitated on a shaker system (250 rpm). The concentration of AP in the release medium was measured at regular intervals over 16 days by UV spectrophotometry, to analyze the release behavior of AP.

### 2.6 Cytotoxicity and biocompatibility studies

Hepa1-6 and aml-12 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were seeded in a 96-well plate at a density of \(3 \times 10^3\) cells well\(^{-1}\) and cultured in a constant temperature incubator (SANYO, Osaka, Japan) at 37°C, 5% CO\(_2\)). After 24 h, the culture medium was removed, 200 μL of fresh medium was added and culture was continued for a further 24 and 48 h. The experimental groups comprised cells, complete medium, functional materials (PEPN, EPLA, EPBT) (200 μg·mL\(^{-1}\)) or NPs (MFNPs, blank MFNPs, PEPNP, PEPNL) (200 μg·mL\(^{-1}\)) or free AP (30 μg·mL\(^{-1}\)). The control group included cells in a complete medium. The blank group only received complete medium. After 24 and 48 h, 10 μL CCK8 solution was added to the 100 μL 96-well culture plates and incubated for 1 h. The absorbance was measured at 450 nm using a microplate reader (Bio Tek, Massachusetts USA) to assess cell viability. The cell viability on exposure to PEPN, EPLA, EPBT, and blank MFNPs was used to analyze biocompatibility. The cell viability following exposure to MFNPs, PEPNP, PEPNL, and free AP was used to evaluate the killing effect of MFNPs on tumor cells.

The biocompatibility of blank MFNPs was tested by evaluation of cytotoxicity and acute toxicity in BALB/c nude mice. Mice were randomly divided into two groups (three mice per group): the control group and the blank MFNPs group. Blank MFNPs (200 μL, 200 μg·mL\(^{-1}\)) were continuously injected through the tail vein with an interval of 1 h through 12 h. The control group was treated with PBS under the same conditions. The health status of the control and blank MFNPs group was monitored regularly up to sacrifice at 14 days when the heart, liver, spleen, lung, and kidney were recovered for histopathological examination.

### 2.7 Tumor targeting of MFNPs

Tumor targeting of MFNPs was investigated using confocal laser scanning microscopy (CLSM; Axio-Imager_LSM-800, Carl Zeiss, Jena, Germany) and flow cytometry (FACSVerse, BD, New Jersey, USA) analysis. Hepa-6 cells were cultured in a laser confocal dish at a density of \(1 \times 10^6\). After 12 h, the culture medium was replaced with a fresh complete medium containing various NP test samples (MFNPs, PEPNP, PEPNL). Free FITC was used as a control group. After 24 h, the cell nuclei were stained with DAPI staining solution (10 μg·mL\(^{-1}\)) for 5 min. Hepa-6 cells were fixed using 4% paraformaldehyde for 10 min and observed by CLSM.

Hepa-6 cells were cultured in a 6-well plate at a density of \(5 \times 10^5\) cells per well in the presence of various NPs (MFNPs, PEPNP, PEPNL). After 24 h, the medium was removed, the cells were washed three times with PBS and digested with trypsin-ethylene diamine tetraacetic acid (EDTA) solution (0.25%) before centrifuging at 1,000 rpm for 5 min. Hepa-6 cells were resuspended in PBS and filtered with a cell screen to analyze flow cytometry.

### 2.8 Lysosomal escape of MFNPs

The capability of the MFNPs for lysosomal escape was assessed using CLSM. Hepa-6 cells were cultured for 24 h in the presence of MFNP samples as described in Section 2.6. The medium was removed, the lysosome staining solution was added and cell culture was continued for 30 min. FITC-loaded PLGA NPs were used as a control group. Cell nuclei were stained with DAPI (10 μg·mL\(^{-1}\)) for 5 min and Hepa-6 cells were fixed using 4% paraformaldehyde for 10 min, before observation by CLSM.

### 2.9 In vivo distribution of MFNPs

BALB/c tumor-bearing nude mice were randomly divided into four groups: free FICT, FITC-loaded PEPNP, PEPNL, and MFNPs (200 μL, 200 μg·mL\(^{-1}\)) were injected into BALB/c tumor-bearing nude mice through the tail vein respectively.
The \textit{in vivo} distribution of MFNPs was monitored using an \textit{in vivo} imaging system spectral imaging system (IVIS SPEC-TRUM, PE, USA) at 1, 4, 8, and 24 h.

2.10 \textit{In vivo} anti-tumor activity of MFNPs

BALB/c female nude mice were used to establish a subcutaneous tumor model under the guidelines of the Institutional Animal Care Committee. Hepa1-6 cells were inoculated subcutaneously in mice at a density of $1 \times 10^7$ cells/mL. The body weight and tumor volume were recorded daily until the tumor volume reached approximately 100 mm$^3$. At this point 200 $\mu$L of PBS (untreated control), MFNPs (200 $\mu$g.mL$^{-1}$), PEPNP NPs (200 $\mu$g.mL$^{-1}$), PEPNL NPs (200 $\mu$g.mL$^{-1}$), or free AP (30 $\mu$g.mL$^{-1}$) were injected via the tail vein (three mice per group). The body weight and tumor volume were recorded daily. After 45 days, the animals were sacrificed, and the heart, liver, spleen, lung, and kidney were excised for histopathological examination.

2.11 Real-time quantitative Polymerase Chain Reaction (qPCR)

Hepa1-6 cells were cultured with 200 $\mu$L MFNPs (200 $\mu$g.mL$^{-1}$), PEPNP NPs (200 $\mu$g.mL$^{-1}$), PEPNL NPs (200 $\mu$g.mL$^{-1}$), and free AP (30 $\mu$g.mL$^{-1}$) in a 6-well plate for 24 h at a density of $1 \times 10^5$ cells.well$^{-1}$. The cells were washed with PBS and trypsinized and the total ribonucleic acid (RNA) was isolated (RNA kit I, Omega BioTek, Vermont, USA). The quality of RNA samples was assessed by UV spectrophotometry. An aliquot (1 $\mu$L) was used as a template for complementary deoxyribonucleic acid (cDNA) reverse transcription using oligo(dT). The reaction mixture was composed of 50 ng cDNA, 2 $\mu$L FastStart Universal SYBR Green Master (ROX), 2 $\mu$L primer mix (30 $\mu$M), and 5 $\mu$L PCR-grade water. qPCR was performed using real-time PCR (Quantstudio 3, Life Technologies, Carlsbad, USA). The two-step program comprised an initial hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each sample contained three technical replicates and the result was averaged. The melting curve was analyzed to confirm product specificity.

2.12 Western blot

Hepa1-6 cell culture was carried out as described above for the qPCR experiments. After the cells were trypsinized, 100 $\mu$L lysis buffer radio immunoprecipitation assay (10% phenylmethanesulfonyl fluoride) was added for 30 min on ice. The cell mixture was centrifuged at 12,000 rpm at 4°C for 5 min to collect protein. The protein concentration was determined using a bicinchoninic acid protein assay kit (Solarbio, Beijing, China). Samples were mixed with Laemmli buffer and heated at 95°C for 5 min. The total protein samples (50 $\mu$L) were separated using 12% (cyt C, caspase9) or 8% (apaf-1, $\beta$-actin) sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Gels were transferred to a 0.22 mm polyvinylidene fluoride membrane and blocked using tris buffered saline with tween (TBST) containing 5% milk. The gels were incubated with the following rabbit monoclonal antibodies: anti-cyt C (1 $\mu$g), anti-caspase9 (1 $\mu$g), and anti-apaf-1 (1 $\mu$g), $\beta$-actin (1 $\mu$g) served as the loading control. After incubating overnight at 4°C, membranes were incubated with rabbit-anti-mouse-horseradish peroxidase (HRP) and the individual protein bands were detected with western chemiluminescence substrate using a fluorescence luminescence imaging analyzer (SYNGENE GBOXiChemix XT, SYNGENE, UK).

2.13 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using a Student’s \textit{t}-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3 Results and discussion

3.1 Characterization of material and MFNPs

The $^1$H NMR spectra of the constituent polymers used to prepare the MFNPs are shown in Figure S1 (in Supplementary Material). PLGA, EPL, and PEG are commonly used to produce nanocarriers. PLGA is a hydrophobic polymer, with poor loading capacity for AP and is rapidly eliminated from the bloodstream following intravenous injection (22). However, modification of PLGA by EPL greatly increased the hydrophilicity of the resulting NPs and the loading capacity of APs (15%, loading efficiency 91%) compared with PLGA (5%) (32).

The layer-by-layer construction process of MFNPs is shown in Figure 1a. PEPN was expected to form NPs with a core-shell structure through the nanoprecipitation method, comprising a hydrophobic PLGA core and a hydrophilic EPL shell. AP was adsorbed on the surface through electrostatic interaction, and EPBT was subsequently attached to provide the tumor-targeting capability. The
TEM results (Figure 1b–d) clearly show the core-shell structure of the PEPN NPs and MFNPs. The PEPN-AP NPs appeared as solid spheres surrounded by a corona (Figure 1c) which might partly reflect the weak electron scattering ability or low density of adsorbed AP and PEGylated BT at the MFNP surface. The mean diameter of PEPN NPs, PEPN-AP NPs, and MFNPs (Figure 1e) increased as the layer-by-layer assembly of MFNPs progressed. Positive zeta potential of 29.1 ± 3.06 mV was measured for PEPN NPs (Figure 1f), which was reversed to −26.1 ± 2.05 mV for PEPN-AP NPs suggesting adsorption of AP. However, in the case of the MFNPs, the surface charge approached neutrality, indicating a compensatory shielding effect of the EPBT targeting molecules.
3.2 Release behavior of AP from MFNPs

The cumulative release of AP from MFNPs at various pH levels over 16 days is shown in Figure 1g. The release profile revealed an initial rapid increase within the first 24 h followed by a plateau. The total release of AP in 16 days was less than 40%. AP release increased with a reduction in pH of the release medium but was generally less than 20% in 48 h. In contrast, AP release from PEPN-AP NPs (produced without the EPBT targeting ligand) at neutral pH was significantly higher (50% in 16 days). This behavior suggests that AP loss from MFNPs would be restricted in the blood circulation and tumor environment but would increase significantly in tumor cytoplasm (pH = 7.4) after escaping from the lysosome. The mechanism of AP release was predicted using the Ritger–Peppas kinetic model (33).

\[ Y = \frac{M_t}{M_\infty} = Kt^n \]  

where \( Y \) is a cumulative release amounts rate of AP; \( M_t \) and \( M_\infty \) are cumulative release amounts of AP at time \( t \) and at infinite time; \( K \) is a constant related to the properties of the nanocarrier. The release exponent \( n \) may be used to assess the release mechanism. When \( n \leq 0.45 \), drug release is dominated by Fickian diffusion. When \( 0.45 < n < 0.89 \), non-Fickian diffusion predominates (drug diffusion and nanocarrier degradation), while \( n > 0.89 \), indicates degradation control. In this study, all release exponent \( n \) is less than 0.45, indicating that AP release was controlled by Fickian diffusion (Figure 1h). The analysis of the kinetic curve is detailed in the supplementary information.

3.3 Biocompatibility and cytotoxicity of MFNPs

The biocompatibility of the functional polymers used to produce MFNPs and blank MFNPs was investigated using a combination of in vitro and in vivo cytotoxicity tests. PLGA, EPL, and PEG are acknowledged to have good biocompatibility. Hepa1-6 cells and Aml-12 cells were exposed to blank MFNPs and their constituent copolymers (PEPN, EPBT, EPLA) for 24 and 48 h respectively. The cell viability was above 90% (Figure 2a and b) and no significant difference was recorded between 24 and 48 h indicating very low cytotoxic activity.

Intravenous injection of BALB/c mice with blank MFNPs (24 mg·kg\(^{-1}\)) resulted in no morbidity, abnormal activity, or significant weight loss within 15 days. The mice were sacrificed after 15 days and the heart, liver, spleen, lung, and kidney were collected for pathological analysis. The myocardial cells of the MFNPs group and the control group have normal morphology, clear nuclei, and no bleeding or necrosis (Figure 2e). The liver tissue structure is complete, the morphology and structure of the liver lobules are normal, the central vein and the surrounding hepatocyte cords are clear, there are no vacuoles in the cells and no cholestasis. The structure of the splenic corpuscle and splenic trabecula is normal, and the cell structure is clear. In the lung tissue sections, the lung lobules were normal, the alveoli were dilated normally, there was no inflammatory exudate and no obvious congestion. The renal cortex and medulla are demarcated, and the morphology of nephrons is not abnormal. The glomeruli and tubules are structured. There is no congestion and edema in the renal interstitium and no inflammatory cell infiltration. The in vitro cytotoxicity assay and mouse acute toxicity test jointly confirmed the good biocompatibility of blank MFNPs and their constituent polymeric materials.

3.4 Tumor targeting of MFNPs

The activity of Aml-12 cells exposed to free AP, PEPNP NPs, PEPNL NPs, and MFNPs was above 90% at 24 h (Figure 2c) and was not significantly reduced when the exposure time was extended to 48 h (Figure 2d). The result indicated that free AP and AP-loaded NPs were nontoxic toward normal cells. In contrast, free AP, PEPNP NPs, PEPNL NPs, and MFNPs all showed varying degrees of cytotoxicity toward Hepa1-6 tumor cells.

The Hepa1-6 cell-killing activity of free AP and PEPNP NPs was around 70% and 65%, respectively in 24 h. The cytotoxic activity of free AP increased slightly to 75% after 48 h, while the cytotoxic effect of PEPNP NPs decreased to 60%. The poor performance of PEPNP NPs may result from the lack of active targeting capability. Free AP may show poor accumulation in tumor cells. The killing effect of PEPNL NPs was similar to that of MFNPs. The viability of cells exposed to MFNPs was slightly lower than that of PEPLB, indicating that the BT-targeted NPs may promote tumor cell internalization and kill tumor cells more effectively than LA-targeted NPs. The cell activity following exposure to PEPNL and MFNPs remained about 43% and 38%, respectively. After 48 h, the Hepa1-6 cell activity due to exposure to PEPNL and MFNPs decreased to about 40% and 30%, respectively. Cytotoxicity assay illustrated MFNPs enhanced killing capacity of Hepa1-6 cells indicating more efficient delivery of AP closer to the intracellular site of action.
3.5 Endocytosis of MFNPs and lysosomal escape

The targeting effect of MFNPs on tumor cells was investigated using CLSM and flow cytometry. CLSM images (Figure 3a) revealed internalized nontargeted and targeted NPs as green fluorescence spots, located in the perinuclear and nuclear region. Compared with the control group (free FITC), the endocytosis of FITC-loaded NPs was significantly higher. Analysis of targeted NPs (PEPNL NPs and MFNPs) and nontargeted NPs (PEPNP NPs), it can be seen that active targeting did greatly enhance the ability of the nanocarrier to internalize in tumor cells. Compared with PEPNL NPs, the fluorescence intensity of the MFNPs was stronger (Figure 3d), indicating that endocytosis of BT-targeted MFNPs was enhanced. The targeting effect of BT
Figure 3: (a) Hepa1-6 endocytosis of control group, PEPNP NPs group, PEPNL NPs group, and MFNPs group at 24 h. (b) Lysosomal escape of the MFNPs and PLGA NPs at 24 h. (c) Flow cytometry image of the control group, PEPNP NPs group, loaded PEPNL NPs group, and MFNPs group at 24 h. (d) Quantitative analysis of endocytosis.
significantly improved the uptake of MFNPs by tumor cells, and most of MFNPs were located in the nuclear area with a concentration in the nucleus, forming a highly fluorescent region (Figure 3a).

Quantitative analysis revealed that the endocytosis efficiency of Hepa1-6 cells toward targeted NPs (MFNPs and PEPNL NPs) was significantly higher than that of nontargeted NPs (PEPNP NPs) and the control group. In addition, endocytosis of BT-targeted MFNPs was higher than LA-targeted PEPNL NPs.

Flow cytometry (Figure 3c) was performed to compare the cellular uptake of nontargeted and targeted NPs using an LA and BT receptor-expressing liver tumor cell line (Hepa1-6). The fluorescence intensity of the NP group was significantly higher than that of the control group, indicating the involvement of endocytosis. The targeted NPs (PEPNL NPs and MFNPs) resulted in fluorescence intensity approximately 10 times higher than that of nontargeted NPs, indicating the advantages of receptor-mediated endocytosis for promoting cellular uptake of NPs. Moreover, BT-targeted MFNPs resulted in higher endocytosis than LA-targeted PEPNL NPs. Flow cytometry results were consistent with the findings of the CLSM study and confirmed that BT receptor-mediated endocytosis could provide a major pathway for uptake and targeted delivery of AP vector to liver tumor cells.

The failure of antitumor therapeutics to reach intracellular targets, due to lysosomal degradation, presents a major barrier in cancer treatment (34). Sequestration of protons by pH-sensitive groups, termed “the proton-sponge” effect, is known to facilitate lysosomal disruption, thereby releasing therapeutic agents into the cytoplasm. The lysosomal escape of MFNPs is shown in Figure 3b, where the lysosomes of Hepa1-6 cells are stained using dye. The green fluorescence and red fluorescence of the MFNPs did not significantly overlap in the merged images and were mostly located in the nucleus. In contrast, the PLGA control group showed almost no lysosomal escape and no bright green fluorescence. These findings demonstrate that MFNPs could escape from the lysosome after internalization by causing an osmotic rupture and accumulation in the nuclear area of target cells.

3.6 In vivo distribution of MFNPs

MFNPs were designed to protect AP from degradation in the bloodstream and deliver AP specifically to tumor sites for uptake by tumor cells. The in vivo distribution of intravenous injected FITC-loaded PEPNP NPs, PEPNL NPs, and MFNPs, respectively, in BALB/c tumor-bearing nude mice is shown in Figure 4. In the PEPNP group (Figure 4a), fluorescence was mainly distributed in the abdomen of mice at 4 h, with no localization in the tumor at 24 h, providing evidence that the nontargeted NPs were cleared quickly in vivo. The fluorescence of the PEPNL group was also mainly distributed in the abdomen with a small amount detected in the tumor. The fluorescence signal was still strong after 24 h. The whole-body fluorescence produced by the MFNPs group decreased at 24 h, but the fluorescence at the tumor site increased markedly indicating significant tumor-targeting behavior.

The BALB/c tumor-bearing nude mice were sacrificed after 24 h, and the fluorescence distribution of the organs is shown in Figure 4b. The fluorescence associated with the PEPNP group accumulated mainly in the liver and intestine, with a small amount in the tumor. In contrast, the fluorescence of the MFNPs group was specifically concentrated in the tumor, and the fluorescence of liver and intestine sections was much lower than that of the other groups. ASGPR receptors are highly expressed in the liver. However, the limited accumulation of PEPNL NPs in the tumor suggests that LA receptors are more prevalent in the liver than in the liver tumor. Overall, the in vivo distribution studies indicated that MFNPs displayed tumor-targeting capacity and reduced accumulation at nonspecific body sites.

3.7 Anti-tumor effect of MFNPs

The antitumor activity of intravenous injected MFNPs was investigated in BALB/c tumor-bearing nude mice. During administration, the weight of the tumor-bearing mice in the control group, free AP group, and PEPNP group decreased rapidly, while the PEPNL and MFNPs groups decreased slowly (Figure 5a). Compared with other groups, the bodyweight of tumor-bearing mice in the MFNPs group remained stable (Figure 5a). Based on tumor volume (Figure 5b), the tumor inhibition effect of the free AP group was limited, and tumor volume increased rapidly within 30 days of administration. The tumor suppression effect of the PEPNP group was slightly improved. Tumor growth was inhibited in PEPNL and MFNP groups, and tumor suppression by MFNPs (BT-targeted) was higher than that of the PEPNL group (LA-targeted). The tumor volume of the control group was about 3 times and 6 times that of group PEPNL and MFNPs, respectively. After 30 days of administration, tumor
suppression in the PEPNL and MFNPs group was slightly reduced, possibly due to a resistance to AP treatment or decreased AP concentrations at the target nuclear site. The solid tumor is shown in Figure 6d. After 45 days, the tumor-bearing mice were sacrificed. The organs and tumor sections are shown in Figure 5c. Compared with the control group, the tumor cells in the PEPNL and MFNPs groups died in large numbers, and the nuclei shrank. Additionally, immune cell infiltration was evident in the liver of the PEPNL group, suggesting that PEPNL NPs undergo nonspecific absorption in the liver. There were no obvious abnormalities in the other organs examined.

3.8 Western blot and qPCR

AP can promote tumor cell apoptosis with high specificity without harming normal cells, due to its NLS and NES. To evaluate whether AP delivered by MFNPs remains functional following uptake by cells and release from lysosomes, we used MFNPs to transfect Hepa1-6 cells and assessed AP mRNA expression. The results (Figure 6a and b) showed that AP was overexpressed in Hepa1-6 cells, but had almost no expression in Aml-12 cells. In addition, expression of AP was greatly increased in Hepa1-6 cells transfected with targeted NPs (PEPNL NPs and MFNPs) compared with free AP and nontargeted PEPNP NPs (Figure 6b). The significant
increase in AP expression confirmed the effectiveness of targeted NPs to deliver AP into Hepa1-6 cells. The AP expression level of MFNPs (BT-targeted) was higher than that of PEPNL NPs (LA-targeted), and about twice that of PEPNL NPs, clearly demonstrating the targeting advantage of BT over LA for Hepa1-6 cells.

In Aml-12 cells (Figure 6a), there was no statistically significant difference in AP expression between the NP groups and the controls apart from (nontargeted) PEPNL NPs. The expression level of AP in the PEPNL group was about 4 times than that of the other groups, which may be explained by the overexpression of ASGPR cell surface receptors. Overall, these findings indicate that MFNPs offer an effective vehicle for delivering functional AP to liver tumor cells.

To understand the apoptotic pathway that was activated following MFNPs uptake by Hepa1-6 cells, the levels of apaf-1, caspase9, and cyt C proteins were measured using Western blot analysis. Compared with the control group, caspase9, apaf-1, and cyt C proteins were overexpressed in the NP groups. Expression of caspase9, apaf-1, and cyt C protein in the PEPNP NPs and free AP groups was similar and slightly lower than that in the PEPNL NPs group. Protein expression was highest in the MFNPs group, indicating the mitochondria-dependent apoptosis pathway of AP.

Combining the Western blot and qPCR results, we believe that MFNPs efficiently delivered AP to tumor cells, which was subsequently released and internalized in the nucleus, resulting in the formation of caspase9 and...
These proteins acted on mitochondria to upregulate the expression of Cyt C, forming apoptotic bodies to promote tumor cell apoptosis. AP had a weaker effect on the expression of apaf-1, which may be an important factor limiting apoptosis efficiency.

4 Conclusion

AP-loaded MFNPs presenting BT targeting moieties for binding with tumor cell surface receptors greatly enhance endocytosis, lysosomal escape, and nuclear localization of NPs in liver tumor cells in vitro. MFNPs loaded with AP significantly suppress liver tumor cell activity in vitro but are nontoxic to normal cells. Tumor growth was inhibited in BALB/c mice for 30 days following intravenous injection of AP-loaded MFNPs. Western blotting and qPCR results showed that MFNPs efficiently delivered AP to tumor cells, which subsequently accumulated in the nucleus, resulting in expression of apaf-1, cyt C, and caspase9, and promotion of tumor cell apoptosis. Taken together, these findings demonstrate the potential of MFNPs for targeted delivery of therapeutic agents in the treatment of HCC.

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