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A pH-sensitive methenamine mandelate-loaded nanoparticle induces DNA damage and apoptosis of cancer cells

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ABSTRACT

Methenamine mandelate is a urinary antibacterial agent, which can be converted to formaldehyde in urine that has a relatively low pH of average 5.5–6.8. Here, we prepare a pH-sensitive PLGA-based nanoparticle containing both methenamine mandelate and $NaHCO_3$. Methenamine mandelate/$NaHCO_3$-coloaded nanoparticle could enter cells via endosome/lysosome pathway. The pH in lysosomes and endo-lysosomes is approximately 5.0. In the acidic environment, $NaHCO_3$ reacts with proton and produce CO_2 bubbles, which burst nanoparticles and lead to the rapidly release of methenamine mandelate. Meanwhile, methenamine mandelate was then quickly converted to a sufficient amount of formaldehyde in this acidic environment, which induced DNA damage and DNA damage response (DDR). Consequently, methenamine mandelate/$NaHCO_3$-coloaded nanoparticles caused cell cycle arrest, cell growth inhibition and apoptosis of cancer cells. Moreover, methenamine mandelate/$NaHCO_3$-coloaded nanoparticles also show intensive inhibitory effect on the growth of MCF-7 xenograft tumor *in vivo*. Therefore, methenamine mandelate/$NaHCO_3$-coloaded nanoparticle is a promising type of formulation for the treatment of cancer, which could give the "old drug" methenamine mandelate a new anti-cancer function in clinical.

Statement of Significance

Methenamine mandelate is a urinary antibacterial agent, which can be converted to formaldehyde in urine that has a relatively low pH of average 5.5–6.8. Here, we prepare a pH-sensitive PLGA-based nanoparticle containing both methenamine mandelate and $NaHCO_3$. Methenamine mandelate/$NaHCO_3$-coloaded nanoparticle could enter cells via endosome/lysosome pathway. The pH in lysosomes and endo-lysosomes is approximately 5.0. In the acidic environment, $NaHCO_3$ reacts with proton and produce CO_2 bubbles, which burst nanoparticles and lead to the rapidly release of methenamine mandelate. Meanwhile, methenamine mandelate was then quickly converted to a sufficient amount of formaldehyde in this acidic environment, which induced DNA damage and DNA damage response (DDR). Methenamine mandelate/$NaHCO_3$-coloaded nanoparticle is a promising type formulation for the treatment of cancer, which could give the "old drug" methenamine mandelate a new anti-cancer function in clinical.

1. Introduction

Methenamine has been prescribed as a urinary antibacterial agent for almost 100 years, which has two FDA-approved preparations: methenamine hippurate and methenamine mandelate [1,2]. Methenamine is stable in alkaline conditions, and can be converted to formaldehyde and ammonia when the pH falls below 6 [3]. The range of normal human blood pH is 7.35–7.45, however urine has a

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relatively low pH of average 5.5–6.8 [4]. Thus, when the pH of urine can be kept in an acidic environment (pH < 6), the equation ($(N_4[CH_2])_6 + 6H_2O + 4H^+ \leftrightarrow 4NH_4^+ + 6HCHO$) is shifted to the right, and formaldehyde is produced. Formaldehyde is shown to inhibit various gram-negative, gram-positive and anaerobic bacteria by denaturing proteins and nucleic acid of bacteria [5]. The bacteria are commonly responsible for urinary tract infection and the characteristics of methenamine and its salts make them highly suitable for treating infections of the lower urinary tract [6,7].

Formaldehyde has been considered as a probable human carcinogen owing to its ability to covalently bind and induce DNA monoadducts, DNA-DNA crosslinks, DNA-protein crosslinks and DNA-glutathione crosslinks [8,9]. Epidemiologic association between formaldehyde exposure and leukemia has been reported, although this remains debatable due to inconsistencies in human and animal studies [10,11]. In fact, the genotoxicity of formaldehyde can also inhibit the proliferation, induce apoptosis of cancer cells [12]. For example, damage to DNA caused by formaldehyde exposure can induce a DNA damage response (DDR) in cells. DDR responses involves signaling cascades that include sensing of DNA lesions, activating appropriate damage repair pathways, arresting the cell cycle, and ultimately determining cell survival or programmed cell death [13]. Previous research have shown that low doses of formaldehyde triggered DDR responses, caused cell cycle arrest, reduced mitotic activity and enhanced apoptosis [14,15].

Lysosomes are membrane-bound spherical organelles which contain more than 50 different hydrolytic enzymes including nucleases, proteases, lipases, glycosidases, phosphatases, phospholipases and sulfatases in order to break down the biomolecules engulfed by the cell. All of these enzymes require an acidic environment for optimal activity, and lysosome provides an acidic environment (pH ~ 5.0) within its interior via an H^+ pump located in the lysosomal membrane compared to the slightly alkaline cytosol (pH 7.2–7.4) [16]. Previous researches showed that some copolymer-based particles that carry chemical drugs and biological macromolecules could be absorbed via an endocytosis pathway to form endosome in cells, and then the late endosomes fuse with lysosome [17–19]. Poly(D,L-lactic-co-glycolic acid) (PLGA), a FDA-approved polymer, has been widely used as a drug delivery carrier owing to its good biodegradability and biocompatibility. The drug release from PLGA carriers is mainly through diffusion and/or polymer degradation [20]. However, these types of release process are slow, so that therapeutic drug levels will not be reached promptly in target areas. Here we developed a pH-triggered PLGA-based nanoparticle through a double emulsion method that can deliver methenamine mandelate together with $NaHCO_3$ into tumor cells via endocytosis pathway and fuse with lysosome. Then we tested the hypothesis that in the acidic environment of lysosome, Proton (H^+) can penetrate into nanoparticles and react with $NaHCO_3$ to quickly generate CO_2, which results in the burst of nanoparticles and the rapid release of methenamine mandelate to lysosome. Thus, methenamine mandelate can also react with proton to generate adequate concentrations of formaldehyde in the acidic environment of lysosome. These formaldehyde diffuses into the nucleus and causes DNA damage, DDR response, cell cycle arrest and programmed cell death.

2. Materials and methods

2.1. Materials

MCF-7, MCF10a and HeLa cells were purchased from the American Type Culture Collection (ATCC). PLGA (LA:GA = 75: 25, Mn20 000), methenamine mandelate, Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet and 3,3′-dioctadecyloxacarbocyanine perchlorate (Dio) were purchased from Sigma-Aldrich (Saint Louis, MO). Coumarin-6 was from Aladdin (New York, NY). Sodium bicarbonate ($NaHCO_3$) was bought from Sangon Biotech (Shanghai, China). Methanol and acetonitrile were purchased from EM Science (HPLC grade). Antibodies against Chk1, Chk2, phospho-Chk1 (S345), phospho-Chk2 (T98) and phospho-H2AX (S139) were purchased from Cell Signaling Technology (Danvers, MA). Anti-beta-actin was obtained from Santa Cruz (Santa Cruz, CA) and anti-H2A.X antibody is from Abcam (Cambridge, MA). An alexa® fluor 488 annexin V/PI dead cell apoptosis kit was obtained from Invitrogen (Carlsbad, CA, USA). All other high quality chemicals were of analytical grade and commercially available.

2.2. Formulation of drug-loaded PLGA nanoparticles

To observe the burst and drug release *in vitro*, the particles were prepared by using a water-in-oil-in-water (W/O/W) double-emulsion, solvent-diffusion-evaporation technique [18]. In brief, 1 mL aqueous PVA (10 mg/mL) containing 2.5 mg $NaHCO_3$ and 1 mg doxorubicin (DOX, red fluorescence) was emulsified with 2 mL PLGA (5 mg/mL in CH_2Cl_2) containing DiO (0.05 mg/mL, a green lipophilic dye, which can doped to the PLGA shells) to generate the primary W/O emulsion. The emulsification was gave a 35 W ultrasonic for 2 min in an ice bath. The emulsion was then added to a 6 mL PVA solution and homogenized at 6000 rpm for 30 min in an ice bath to produce the W/O/W double emulsion. The double emulsion was then transferred into 30 mL deionized water and stirred overnight to evaporate CH_2Cl_2. The particles were collected by centrifugation at 2000 rpm for 30 min.

To investigate the *in vivo* effects of NPs, Drug-free, coumarin-6-loaded, methenamine mandelate-loaded or methenamine mandelate/$NaHCO_3$-coloaded PLGA nanoparticles were prepared through a modified nano-precipitation method using an acetone-water system according to the previous report [21]. Briefly, for methenamine mandelate/$NaHCO_3$-coloadedPLGA nanoparticles, 100 mg PLGA were dissolved in 10 mL acetone through vortexing. Then 5 mg $NaHCO_3$ together with 10 mg methenamine mandelate were dissolved in 1 mL deionized water, and added dropwise into the 10 mL PLGA solutions. Subsequently, the mixture was added dropwise into 100 mL of solution with 0.03% TPGS under stirring. The resulting suspensions were stirred for 24 h to remove the acetone and then centrifuged at 20000 rpm for 20 min to harvest nanoparticles.

2.3. Plasmids and transfection

The DsRed-Rab5 and DsRed-Rab7 plasmids were purchased from Addgene (Cambridge, MA) and were confirmed through DNA sequencing. DsRed-Rab5 and DsRed-Rab7 plasmids were transfected into MCF-7 and HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Characterization of nanoparticles

Particle size distributions were measured by Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). Surface morphology of nanoparticles was observed by field emission scanning electron microscopy (FESEM) (JEOL JSM-6301F, Tokyo, Japan). Before detecting, PLGA-based nanoparticles were fixed on a stub and coated with platinum layer using a JFC-1300 automatic fine platinum coater (JEOL, Tokyo, Japan).

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