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Research paper

Antitumor activity of transferrin-modified- artemether lipid nanospheres in cancer cell lines

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ABSTRACT

Diverse lines of research show that the cellular response to artemether (ART) is multi-factorial in nature. The cytotoxicity of ART is specific to cancer cells because most of them over expressed; transferrin receptors and have high level of intracellular iron and ART is mainly toxic after; interaction with iron ion. Our aim is to investigate the effect of some formulation characteristics on; the cytotoxicity of ART in C6 and MCF-7 cells lines. In this study, the cytotoxicity of ART-loaded; anionic, cationic or neutral (transferrin modified) lipid nanospheres was studied by MTT and; apoptosis tests. Characterizations of apoptosis were done. The effect on the mitochondria and the; nucleus was qualitatively characterized after using different types of cell tracker dyes. The cellular; uptake, accumulation and distribution of the formulations were characterized after loading; different hydrophobic fluorescence probes instead of ART. The relation between the; accumulated amount of ART and its cytotoxicity was defined. Our study shows that ART can be highly toxic to the tumor cells if accumulated in a large amount in the cell. Lipid nanosphere containing tween80 and transferrin can highly accumulate ART in brain cells and can be; formulated as a promising potent, safe and inexpensive drug carriers for brain tumors.

1. Introduction

Artemether (ART), a potent and well tolerated antimalarial drug, has also been studied for other therapeutic uses such as schistosoma, viral and leishmenia infections. The potent antiproliferative activity in various tumor cell lines, established record of safety and no development of resistance in children and adults with malaria for a long time, recently published clinical trial of short-term use in lung cancer and the permissive cost are considerable reasons to study the contribution of artemesinin and its derivatives in the management of tumors that require adjuvant and neo-adjuvant therapies [1,2] (Table 1).

Considerable progress has been made during the past years towards understanding the molecular mode of action of artemisinin and its derivatives against tumor cells. Diverse lines of research show that the cellular response to artemisinin and its derivatives is multi-factorial in nature. This may be beneficial in treating otherwise drug-resistant tumors and may explain why the development of artemisinin resistance has not yet been encountered in cancer cells or malaria patients. Artemesinin and its derivatives exerted antiproliferative, antiangiogenic and apoptotic activities [1,3,4].

The term reactive oxygen species (ROS) is used for short-lived diffusible entities such as hydroxyl (•OH), alkoxyl (RO•) or peroxyl (ROO•) radicals and for some radical species of medium lifetime such as superoxide (O2•) or nitroxyl radical (NO•). It also includes the non-radicals hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and hypochlorous acid (HOCl). ROS has destructive actions of both DNA and proteins [5].

Artemisinin and its derivatives are capable of inducing the

Abbreviations: ART, artemether; TR, transferrin; SA, stearylamine; C6, coumarin-6; NIRD-15, near infra red dye-15; MOM, the mitochondrial outer membrane; PTP, permeability transition pores; ROS, reactive oxygen species; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM-HG, High glucose-Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffer saline; DMSO, dimethyl sulphoxide; PI, propidium iodide; ART-LNSs, artemether unmodified lipid nanospheres; SA-LNSs, artemether lipid nanospheres containing stearylamine; TR-LNSs, transferrin modified artemether lipid nanospheres; U-C6-LNSs, coumarin-6 unmodified lipid nanospheres; SA-C6-LNSs, coumarin-6 lipid nanospheres containing stearylamine; TR-C6-LNSs, transferrin modified coumarin-6 lipid nanospheres; U-NIRD-LNSs, NIRD-15 unmodified lipid nanospheres; SA- NIRD-LNSs, NIRD-15 lipid nanospheres containing stearylamine; TR-NIRD-LNSs, transferrin modified NIRD-15 lipid nanospheres.

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Table 1
The composition of ART-LNSs, SA-LNSs and TR-LNSs.

Ingredient	ART-LNSs	SA-LNSs	TR-LNSs
Artemether	1.5%	1.5%	1.5%
Mixture of soya oil and Crodamol GTCC, in ratio of 1: 2	5%	5%	5%
Mixture of Epikuron200 and Tween 80, in ratio of 1:1	5%	5%	5%
Stearylamine	NA	0.075% w/v of oil phase	0.075% w/v of oil phase
Transferrin	NA	NA	0.8 mg/ml of SA-LNSs
Glycerinated water(2.25%w/v)	Q.S	Q.S	Q.S

production of carbon-centered radicals and ROS. Their molecule contains an endoperoxide bridge (−C−O−O−C−) that interacts with Fe (II) to form free radicals. They are initially activated by the cleavage of the endoperoxide with intracellular-iron. Subsequent biochemical events and cellular target (s) of artemisinin, however, remain unclear. It has been proposed that the transfer of an oxygen atom from the peroxide group of artemisinins to the chelated iron generates Fe (IV) = O species. The resulting free radical intermediate may kill the cell by alkylating and damaging essential and target proteins and by lipid peroxidation. The cytotoxicity of ART is specific for cancer cells because most of cancer cells over expressed transferrin receptors and have a higher level of intracellular iron than normal cells [1,3,4].

Morphologically, apoptosis and necrosis, and non-morphologically paraptosis are the main mechanisms of the anti-cancer drugs to kill the proliferative cells. Apoptosis is characterized by a change in the refractive index of the cell followed by cytoplasmic shrinkage, nuclear condensation and formation of apoptotic bodies. Apoptotic cells also cease to maintain phospholipid asymmetry in the cell membrane, and phosphatidyl serine appears on the outer leaflet [6]. The mitochondrial outer membrane (MOM) also undergoes changes that include loss of its electrochemical gradient, possibly by the formation of pores in the MOM, and substances such as cytochrome c leak from the MOM into the cytoplasm. Necrosis is first marked by a loss of cell membrane integrity. The cytoplasm and mitochondria of the necrotic cell swell, and ultimately the cell and many of its internal organelles lyse. There is no vesicle or apoptotic body formation, and often necrosis affects groups of adjacent cells. Paraptosis describes a cell death that requires gene expression but morphologically does not resemble either apoptosis or necrosis [7]. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium [8]. Therefore, cells that have initiated apoptosis may exhibit some of the morphological phenotypes associated with necrosis.

Mitochondria play a central role in energy-generating processes within the cell and involved in such complex processes as apoptosis and cardio-protection. The number of mitochondria per cell is roughly related to cell energy demands. Organs that are very active metabolically, such as muscles, liver, brain, and cardiac and skeletal muscles, contain the largest number of mitochondria and are most susceptible to drugs acting on mitochondria and to mitochondrial pathologies. A subtle balance between the pro-apoptotic and anti-apoptotic proteins and their interaction with the mitochondrial permeability transition pores (PTP) are decisive for the survival or apoptotic death of the cell. This balance can be affected by a number of mitochondria-targeted drugs. ROS action on mitochondria results in the opening of PTP and thus triggers the mitochondria-related apoptotic pathway. It is also possible that peroxidative attack may directly damage the outer mitochondrial membrane resulting in the unspecific liberation of intermembrane proteins, including a fraction of cytochrome *c*. Mitochondria are unique cellular organelles that can build up a trans-membrane electric potential of up to 180 mV, negative inside, and whose internal milieu maintains a pH value of about 8. As a consequence, they can not only accumulate membrane permeable compounds of cationic character, but also trap weak acids in their anionic form. Both properties may be of importance in targeting specific drugs into mitochondria. Many lipophilic compounds penetrate the inner mitochondrial membrane freely [9]. Therefore, the characteristics of surface charge of nanoparticles will determine their accumulation in mitochondria.

In this study, we loaded ART in anionic, cationic and neutral, transferrin modified, lipid nanospheres to study their cytotoxicity in MCF-7 and C6-cell lines. The cytotoxicity was studied by MTT assay and apoptosis. The apoptotic effect of the formulations was quantitatively and qualitatively characterized. The effect on mitochondria was qualitatively characterized by using different types of cell tracker dyes and inspected under a fluorescence microscope. Cellular uptake, accumulation and distribution of the formulations were characterized after loading a hydrophobic fluorescence probe, coumarin-6 or NIRD-15, instead of ART. The amount of coumarin-6 accumulated in mitochondria, nucleus or cytoplasm was quantified by flow cytometer after isolation of cell parts through a defined protocol. The relationship between the accumulated amount of ART and its cytotoxicity toward cancer cells was defined.

2. Materials and methods

2.1. Materials

Artemether was purchased from Nanjing Chemlin Chemical Industrial Co.Ltd. (Nanjing, China). Coumarin-6 and transferrin (TR) was from Sigma. (Shanghai, China). Near infrared dye (NIRD) was provided by Huahai-Lanfan Chemical Technology Co., Ltd. (Liaoning, China). Stearylamine (SA) was from Nanjing Aoduofuni Biological Technology (Nanjing, China). Crodamol gtcc was from Bejing Fengli Jingqiu Commerce & Trade Co.Ltd (Beijing, China). Tween80 was from China Pharmaceutical Group Shanghai Chemical Reagent Company (Shanghai, China). Glycerol was from J&K chemical Ltd. (Shanghai, China). Soya oil was from Aladdin Chemistry Co.Ltd. (Shanghai, China). Epikuron200 was from Cargil texturizing solutionsdeuschland GmbH& Co.KG (Hamburg, Germany). Hoechst 33342 and Mito-tracker green were purchased from Beyotime Biotechnology (Jiangsu, China). Dimethyl sulfoxide (DMSO) was obtained from Shanghai lingfeng chemical reagent Co.Ltd (Shanghai, China). 3-(4,5-methylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, Ohio, USA). The cell culture flasks and plates were purchased from Corning (USA). Cell culture medium Dulbecco's modified Eagle's medium−high glucose (DMEM-HG), fetal bovine serum (FBS), penicillin with streptomycin solution, trypsin 0.25% and phosphate buffer saline (PBS) were purchased from National HyClone Bio-Engineering Co., Ltd. (Beijing, China).