Biomedicine & Pharmacotherapy 96 (2017) 396–403

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Mitochondrial dysfunction is responsible for fatty acid synthase inhibition-induced apoptosis in breast cancer cells by PdpaMn

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ARTICLE INFO

Keywords:
Manganese complex
Anti-breast cancer
Mitochondria dysfunction
Fatty acid synthase
De novo fatty acid synthesis

ABSTRACT

Targeting cellular metabolism is becoming a hallmark to overcome drug resistance in breast cancer treatment. Activation of fatty acid synthase (FASN) has been shown to promote breast cancer cell growth. However, there is no concrete report underlying the mechanism associated with mitochondrial dysfunction in relation to fatty acid synthase inhibition-induced apoptosis in breast cancer cells. The current study is aimed at exploring the effect of the novel manganese (Mn) complex, labeled as PdpaMn, on lipid metabolism and mitochondrial function in breast cancer cells. Herein, we observed that PdpaMn displayed strong cytotoxicity on breast cancer cell lines and selectively targeted the tumor without affecting the normal organs or cells in vivo. We also observed that PdpaMn could bind to TE domain of FASN and decrease the activity and the level of expression of FASN, which is an indication that FASN could serve as a target of PdpaMn. In addition, we demonstrated that PdpaMn increased intrinsic apoptosis in breast cancer cells relayed by a suppressed the level of expression of FASN, followed by the release of mitochondrial cytochrome c and the activation of caspases-9. Instigated by the above observations, we hypothesized that PdpaMn-induced apoptosis events are dependent on mitochondrial dysfunction. Indeed, we found that mitochondrial membrane potential (MMP) collapse, mitochondrial oxygen consumption reduction and adenosine triphosphate (ATP) release were deeply repressed. Furthermore, our results showed that PdpaMn significantly increased the reactive oxygen species (ROS) production, and the protection conferred by the free radical scavenger N-acetyl-cysteine (NAC) indicates that PdpaMn-induced apoptosis through an oxidative stress-associated mechanism. More so, the above results have demonstrated that mitochondrial dysfunction participated in FASN inhibition-induce apoptosis in breast cancer cells by PdpaMn. Therefore, PdpaMn may be considered as a good candidate for anti-breast cancer therapeutic option.

1. Introduction

Breast cancer continues to be the highest incidence of cancer in women worldwide and as the leading cause of death by cancer among women [1]. Adjuvant therapies for breast cancer have achieved great success in recent years. However, there is still a certain proportion of breast cancer patients with drug resistance to endocrine therapy, which makes it difficult to control the progress of the disease [2]. Therefore, finding novel treatments or agents to overcome drug resistance and then to improve the therapeutic effect is rewarding towards the diagnosis of breast cancer.

As an essential metal, manganese (Mn) exists in a variety of biological tissues that can influence cellular metabolism associated with the mitochondria [3]. It is also a co-factor for many enzymatic reactions, including those involved in neurotransmitter synthesis and metabolism in the brain, and as the main co-activator of superoxide dismutase in mitochondria [4,5]. In addition, manganese (Mn) plays a central role in a variety of physiological processes including amino acid, lipid, proteins and carbohydrate metabolism, which makes manganese compounds less harmful to the patients and are therefore safer [6,7]. Moreover, Mn (II) ions are primarily transported via divalent metal transporter 1(DMT-1) and the transferrin–transferrin receptor (Tf-TfR) system [8].

Recently, altered energy metabolism has become one of the hallmarks of breast cancer [9]. Some critical metabolic enzymes serving as targets for cancer therapy and or reverse drug resistance and have been widely recognized [10,11]. Fatty acid synthase (FASN) is a key enzyme involved in de novo fatty acid synthesis. In most normal tissues, the expression and activity of FASN are low or absent, however, a variety of human cancers overexpress FASN, one such cancer is breast cancer. FASN synthesizes long-chain fatty acids palmitate by using acetyl-CoA and malonyl-CoA [12,13]. Up-regulated de novo fatty acid synthesis

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http://dx.doi.org/10.1016/j.biopha.2017.10.008
Received 6 July 2017; Received in revised form 14 September 2017; Accepted 2 October 2017

serve as a major metabolic alteration of breast cancer cells and is associated with a poor prognosis [14]. FASN inhibition reduces cell proliferation and induces apoptosis in vitro and decreases the size of prostate, ovarian and breast cancer xenografts [15,16]. This suggests that inhibition of FASN may be considered as an attractive target for breast cancer. However, the detailed mechanism by which FASN inhibition induces apoptosis still remains to be explored.

Mitochondria are the primary site of ROS generation within cells and are also sensitive targets for ROS [17]. The contribution of mitochondria in the suppression of tumor growth is determine by the aberrant energetic metabolism of malignant cells and regulation of cell death by apoptosis [18]. Mitochondrial involvement in apoptosis is manifested by the increased expression of the level of caspase-9 and the release of cytochrome c. During mitochondrial-mediated apoptosis, mitochondrial dysfunction is induced, including the mitochondrial membrane potential (MMP) dissipation, ROS production, oxygen consumption inhibition and adenosine triphosphate (ATP) reduction [19]. Perturbation of energy metabolism or mitochondrial dysfunction may be considered as a promising therapeutic approach to breast cancer [20]. Therefore, in the current study we sought to explore whether mitochondrial dysfunction is involved in FASN inhibition.

Previously, we have shown that [(Pdpa)$MnCl_2$], (labeled as PdpaMn, Pdpa = (4-((bis(pyridine-2-yl-methyl)amino)methyl)-(1H-pyrrol-2-yl)methanone)), exhibited good antitumor activity, but the mechanism remains unclear [21]. In the present studies, we try to access the effect of PdpaMn on FASN in breast cancer cells, and to investigate FASN inhibition-induced apoptosis through impairing mitochondrial function.

2. Methods

2.1. Chemicals, reagents and antibodies

The compound PdpaMn was synthesized according to previously reported method [22]. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was from Amresco (Solon, OH, USA). JC-1, Hoechst 33342 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, US). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA solution were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). The 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) and the Annexin V/propidium iodide (PI) kit were purchased from Beyotime Institute of Biotechnology (Nantong, China)., ATP assay kit and fatty acid synthase (FAS) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against β-actin, caspase-9, cytochrome c, FASN were from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals were of high purity from commercial sources.

2.2. Molecular docking

The initial 3D structures of the ligands shown in Fig. 2A were constructed using chemdraw. All hydrogens of ligands are added. The protein model was constructed based on the crystal structure of hFAS TE domain taken from the Protein Data Bank (PDB code: 1XKT). Water molecules in the crystal structure were removed. All hydrogen atoms were added and Amber all-atom charges were assigned for the whole protein. The AutoDock 4.2 was used for molecular docking simulations.

2.3. Cell lines and cell culture

Human breast cancer cells (MCF-7), mouse breast cancer cells (4T1) were obtained from Cancer Cell Repository (Shanghai cell bank). Cells were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Cell viability assay

Cell viability was assessed by MTT assay. Cells were seeded in 96-well culture plates at a density of approximately 4×10^3 viable cells per well. Increasing concentrations of PdpaMn were added to each well for 24 h. PdpaMn was dissolved in DMSO and the final concentration of DMSO in the medium was 0.1%. Then, 100 μL of MTT solution (1 mg/mL) was added to each well. The plates were incubated for an additional 4 h at 37 °C. MTT formazan crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 570 nm using a plate reader (Spectra MaxGemini, Molecular Devices Corporation). Images of the cell morphology were taken with an inverted microscope (TS100, Nikon) at random fields.

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) reflects the functional state of the mitochondria within cells. Briefly, after different concentrations of PdpaMn treatment for 24 h. Cells were washed with PBS, fluorescence intensity was measured immediately following JC-1 staining (5 mg/mL of JC-1 at 37 °C for 30 min) by fluorescence spectrometry (Molecular Devices Corporation, USA) or observed using a fluorescence microscope (excitation 488 nm/emission 535 nm for JC-1 green and excitation 488 nm/emission 595 nm for JC-1 red).

2.6. Measurement of intracellular ROS production

The intracellular generation of ROS was analyzed with the probe DCFH-DA. Cells were treated with different concentrations of PdpaMn for 24 h. Then the cells were incubated with 10 μM DCFH-DA and protected from light for 30 min. DCF fluorescence intensity was measured with fluorescence spectrometry (Spectra MaxGemini, Molecular Devices Corporation) at excitation wavelength of 488 nm and at an emission wavelength of 535 nm. Morphological changes of cells were observed by fluorescence microscope (ECLIPSE Ti, Nikon).

2.7. Oxygen consumption measurements

After treatment, MCF-7 cells were trypsinized and suspended at 10^4 cells/ml in DMEM + 10% FBS. Oxygen consumption was measured in a 0.5 mL volume using an Oxytherm electrode unit (Hansatech, Norfolk, UK). This system uses the Clark-oxygen electrode to monitor the dissolved oxygen concentration over time. The rate of oxygen consumption was calculated use computerized chart recorder (Oxygraph 1.01, Hansatech, Norfolk, UK).

2.8. Western blot analysis

After treatment, proteins were extracted from the cells with lysis buffer containing protease inhibitors for 30 min on ice and centrifuged at 12000*g* for 10 min. Then total protein was electrophoresed in and 10–15% SDS-PAGE (cytochrome c, Caspase 9 and FASN), transferred to PVDF membrane. Membrane blocking was performed with 1% BSA and 5% (w/v) non-fat dry milk (including TBST buffer) for 1 h and then incubated over night at 4 °C with primary anti-bodies: anti-β-Actin (1:1000), anti-Caspase 9 (1:500), anti-Cytochrome c (1:1000), anti-FASN (1:1000). Then the membranes were incubated for 2 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. Target proteins were detected by the ECL system (Millipore Corporation, Billerica, USA).