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ZT-25, a new vacuolar H^+-ATPase inhibitor, induces apoptosis and protective autophagy through ROS generation in HepG2 cells

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3-methyladenine (PubChemCID: 1673)
Chloroquine (PubChemCID: 2719)
N-acetyl-l-cysteine (PubChemCID: 12035)
Propidium iodide (PubChemCID: 104981)
JC-1 (PubChemCID: 5492929)
Dichlorodihydrofluorescein diacetate (PubChemCID: 104913)
MTT (PubChemCID: 64965)
Rapamycin (PubChemCID: 5284616)

ABSTRACT

The vacuolar H^+-ATPase (V-ATPase) has recently been proposed as a key target for new strategies in cancer treatment. Our previous work has proved that diphyllin glycoside is a novel inhibitor of V-ATPase. Here the cytotoxic effects of ZT-25, the most potent diphyllin glycoside derivatives, were studied and some of the underlying mechanisms were elucidated. ZT-25 displayed strong cytotoxicity on several cancer cell lines and relatively low cytotoxicity on human fetal hepatic cells (WRL-68) at submicromolar concentrations. In human hepatoma cells HepG2, ZT-25 induced G_1/G_0 phase arrest and apoptosis, as well as mitochondrial membrane potential (MMP) dissipation and ATP depletion. Furthermore, Bcl-2 protein decreased, while Bax protein and cleaved caspase-3 protein increased upon ZT-25 treatment. Benzyloxycarbony (Cbz)-L-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK), a well-known pan-caspase inhibitor, attenuated ZT-25-induced cell death, suggesting the involvement of caspase-dependent pathway. Intriguingly, ZT-25 induced autophagy in HepG2 cells as characterized by increased the conversion of LC3 I to LC3 II, Beclin-1 expression and autophagosome formation. Meanwhile, p-mTOR expression was decreased which indicated that ZT-25-induced autophagy might be mediated through the suppression of mTOR pathway. Inhibition of autophagy by 3-methyladenine (3-MA) and chloroquine (CQ) obviously promoted ZT-25-induced cell death, suggesting the protective role of autophagy. Increased intracellular ROS level was found to be the early event in ZT-25-treated HepG2 cells. Inhibition of ROS generation by N-acetyl-L-cysteine (NAC) attenuated ZT-25-induced cell death and autophagy. Together, these results provide key insights into the ZT-25-induced cytotoxicity in HepG2 cells, which will have a great impact on the further development of diphyllin derivatives.

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1. Introduction

Tumor cells are usually forced to exist in a hypoxic and acidic microenvironment as a result of low oxygen and nutrient supply leading to increased glycolysis (Forgac, 2007). Radiographic evidence has proved the presence of an acid gradient around the tumor, which has become one of the basic characteristics of solid tumors (Gatenby et al., 2006; Nishisho et al., 2011). Tumor cell survival relies upon adaptation to the acidic conditions of the tumor microenvironment. Conversely, the acidic microenvironment increases tumor malignancy by promoting chemoresistance, invasiveness, and proliferation (Nishisho et al., 2011).

Vacuolar H^+-ATPases (V-ATPases) are ubiquitously expressed ATP-dependent proton pumps (Schempp et al., 2014; Stevens and Forgac, 1997), which play a major role in the regulation of cellular pH conditions and microenvironment acidification (Shang et al., 2012; Villanueva, 2014). Recent studies have shown that V-ATPases play an important role in the development of cancer based on reports of increased expression and activity of the V-ATPase on

Abbreviations: V-ATPases, vacuolar H^+-ATPases; MMP, mitochondrial membrane potential; CQ, chloroquine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 3-MA, 3-methyladenine; NAC, N-acetyl-L-cysteine; DMEM, Dulbecco's modified eagle medium; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; Z-VAD-FMK, benzyloxycarbony (Cbz)-L-Val-Ala-Asp (OMe)-fluoromethylketone

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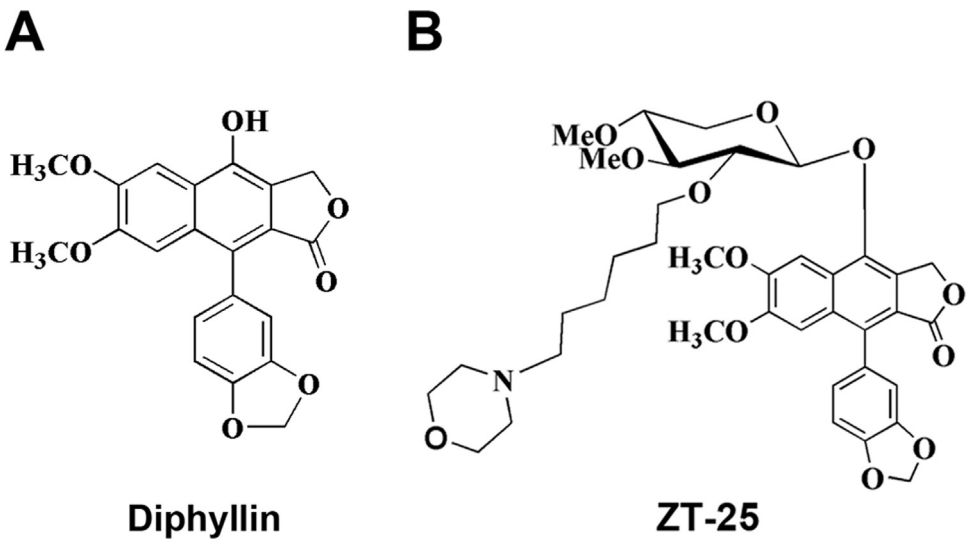

Fig. 1. Chemical structures of Diphyllin and ZT-25.

the plasma membrane of tumor cells (Forgac, 2007; Perez-Sayans et al., 2009). Augmented expression of V-ATPase is considered to be a well designed compensatory mechanism allowing tumors to survive in acidic/hypoxic conditions which in fact confers growth advantages of cancer cells (von Schwarzenberg et al., 2013). Thus, inhibitors of V-ATPases could be promising novel therapeutics for cancer and attracted more and more attention (Cotter et al., 2015; Wiedmann et al., 2012).

Most of the known V-ATPases inhibitors are natural compounds of microbial origin such as salicylihalamides and bafilomycin (Huss and Wieczorek, 2009). Diphyllin (Fig. 1A), one of lignans compounds in traditional Chinese medicine astilboides tabularis (Hemsl.) Engler, has been proved to possess good antitumor activity and low toxicity to normal cells (Zhao et al., 2012). However, the exact mechanism of action has not yet been fully understood. It has been reported that diphyllin can inhibit osteoclast V-ATPases activity in a dose dependent manner (Sorensen et al., 2007). Recently, our group successfully synthesized a natural cytotoxic diphyllin glycosides Cleistanthin-A which has been indentified as potent V-ATPase inhibitor (Zhang et al., 2014). In order to find more effective V-ATPase inhibitor, we designed a new class of Cleistanthin-A derivatives by adding various basic side chains to the hydroxyl group with the aim to increase their release rate to acidic tumor position. The most active compound ZT-25 (Fig. 1B) has been shown to inhibit the activity of V-ATPase in HepG2 cells at submicromolar concentration (Zhao et al., 2015). In the present study, the cytotoxic effect of ZT-25 on HepG2 cells was further examined to elucidate some of the underlying mechanisms.

2. Materials and methods

2.1. Materials

Diphyllin and ZT-25 were totally synthesized according to previously reported procedures by Laboratory of Pharmaceutical Chemistry, Institution of Nautical Medicine, Nantong University (Zhao et al., 2015). 3-methyladenine (3-MA), JC-1, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), chloroquine (CQ), rapamycin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) and anti-β-actin monoclonal antibody were purchased from Sigma-Aldrich Corp. (St. Louis, MO, US). The FITC Annexin V Apoptosis Detection Kit I was bought from BD Biosciences (San Jose, CA, US). The ATP assay kit and benzyloxycarbony (Cbz)-L-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK) were purchased from Beyotime Biotechnology Corporation (Shanghai, China). Cleaved Caspase-3 antibody was obtained from Cell Signaling Technology (Beverly, MA, US). The Alexa-Fluor 555 (red)-conjugated secondary antibody was obtained from Invitrogen Corporation (Carlsbad, CA, US). The rabbit and mouse polyclonal secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, US). Cell culture reagents were purchased from Gibco (Carlsbad, CA, US). All chemicals were standard analytical grade or higher.

2.2. Cell culture

Human hepatoma cells (HepG2), colon cancer cells (HCT116), lung carcinoma cells (A549), cervical cancer cells (Hela) and normal hepatic cells (WRL-68) were obtained from cell bank of Chinese Academy of Sciences (Shanghai, China). These adherent cells were grown in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum and 0.5% (v/v) penicillin–streptomycin at 37 °C in a humidified 5% CO_2 incubator (Thermo Forma Electron Co., Marietta, OH, US). Cells were passaged at 70% confluence following trypsinization with 0.05% trypsin/0.02% EDTA. All these cell lines were maintained strictly according to the supplier's instructions and established procedures.

2.3. MTT assay

Cell viability was determined using the colorimetric MTT assay, based on the ability of viable cells to transform MTT to a purple formazan dye (Wang et al., 2008). Cells were plated in 96-well plates and incubated for 24 h prior to treat with ZT-25 at the concentrations of 0, 0.001, 0.01, 0.03, 0.1, 0.3, 1 μM for 72 h. ZT-25 was dissolved in DMSO and the final concentration of DMSO in the medium was 0.1%. Then, 100 μl of MTT (1 mg/ml) was added into each well for 4 h at 37 °C. After treatment, culture fluid was removed and MTT formazan crystals were dissolved in 200 μl lysis buffer (20% SDS in 50% N'N-dimethylformamide, pH 4.7). Optical density was measured at a wavelength of 570 nm and background absorbance was subtracted measuring at 690 nm by the use of a microplate reader (Synergy™ 2, BioTek, US).

2.4. Cell cycle analysis and sub-G1 population

Cells were seeded in 60 mm culture dishes, and after 24 h of

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