Novel Dihydroartemisinin Derivative Mito-DHA5 Induces Apoptosis Associated with Mitochondrial Pathway in Bladder Cancer Cells

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Novel dihydroartemisinin derivative Mito-DHA_5 induces apoptosis associated with mitochondrial pathway in bladder cancer cells

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Abstract

Background: Bladder cancer is the second most common genitourinary malignancy and the eleventh most common cancer worldwide. Dihydroartemisinin (DHA), a first-line antimalarial drug, has been found to have potent antitumor activity. In our previous study, a novel dihydroartemisinin derivative Mito-DHA_5 synthesized in our laboratory has a stronger anti-tumor activity than DHA. In this study, we investigated the apoptotic effect of Mito-DHA_5 on bladder cancer T24 cells and molecular mechanisms underlying.

Methods: Antitumor activity *in vitro* was evaluated by MTT and cloning formation assays. Mitochondrial membrane potential (MMP) was detected by JC-1 probe and ROS levels were measured by specific kit. The expression of caspase-3, Bcl-2 and Bax in T24 cells was evaluated by Western blotting.

Results: The results showed that Mito-DHA_5 reduced cell viability with an IC_{50} value of 3.2 μM in a dose-dependent manner, induced T24 cell apoptosis at both early and late stages, increased the production of ROS and decreased MMP. Mito-DHA_5 could down-regulate the expression of Bcl-2 and Caspase-3, and up-regulate the expression of Bax and cleaved Caspase-3.

Conclusion: These data suggested that Mito-DHA_5 had a potent inhibitory effect on T24 bladder cancer cell growth and induced these cells apoptosis associated with mitochondrial pathway.

Keywords: Dihydroartemisinin derivative, ROS, MMP, Apoptosis, Bladder cancer.

Background

Bladder cancer is the second most common genitourinary malignancy and the eleventh most common cancer worldwide [1]. According to the global cancer data, there are 500000 new bladder cancer cases and 200000 deaths in the world every year [2]. Generally, transurethral resection has served as the standard treatment. However, recurrence and metastasis are often seen in clinic after this surgery [3]. Thus, intravesical chemotherapy or immunosuppressive agents are applied to treat these patients to prevent these severe events in the commonest way [4]. However, these agents have considerable side effects such as bone marrow suppression and allergic reactions [5]. Therefore, there is an urgent need to develop anticancer agents with high efficacy and low toxicity to treat bladder carcinoma.

DHA is an active metabolite of artemisinin which is widely used to treat malaria in clinic [6]. Recent years, increasing number of studies reported that DHA exhibited anti-cancer activities in different kind of cancers, such as ovarian cancer [7], lung cancer [8], esophageal cancer [9], prostate cancer [10] and colon cancer [11], etc. In contrast, chemical modification of DHA is becoming a notably research area to find novel small molecules to convey cancers. In our previous study, we found that Mito-DHA$_5$ (**Fig. 1A**), which is a mitochondria-targeted derivative of DHA has more potent anti-tumor activity than DHA [12]. However, the mechanisms of action of Mito-DHA$_5$ remain unknown.

Mitochondria are important bioenergy factories for normal cell function and human health. In tumor cells, mitochondria are dysfunctional and cannot release apoptosis signals in time, leading to indefinite proliferation and apoptotic resistance [13]. The mitochondrial apoptosis pathway takes mitochondrial depolarization as a starting point, which is regulated by members of the Bcl-2 protein family, following the release of apoptosis signal, then activates caspase-3 to trigger apoptosis [14]. Interestingly, Farhad Poupel et al. found that DHA could induce apoptosis through mitochondrial signaling pathway in bladder cancer [15]. In order to clarify the potential mechanisms of action of Mito-DHA$_5$, the effect of Mito-DHA$_5$ on T24, one

of representative bladder cancer cell lines, was investigated in this study.

Materials and Methods

DHA was purchased from Energy Chemical Reagent Company (Shanghai, China). Mito-DHA_5 was synthesized in our laboratory. Compounds were dissolved in Dimethyl sulfoxide (DMSO). During the experiment the concentration of DMSO did not exceed 0.1%. Dulbecco's modified Eagle's medium (DMEM, U.S.), fetal bovine serum (Hyclone, Logan, UT, USA), penicillin-streptomycin solution, 0.25% trypsin and phosphate buffer (Hyclone, USA) were bought from Hyclone company. Mitochondrial Membrane Potential Detection Kit was bought from Solarbio company (Beijing, China). AnnexinV- FITC/PI Apoptosis Detection Kit was purchased from Vazyme (Nanjing, China). ROS Detection Kit, Hochest33258 Kit and Bax (AF0057) protein antibodies were all from Beyotime company (Shanghai, China). Bcl-2 (CAS7511) protein antibody is from Bioworld. Caspase-3 (#9662) antibody was purchased from Cell Signaling Technology company.

Cell culture

Human bladder cancer cell T24 was obtained from Dr. P Guo (Xi'an Jiaotong University). The cells were grew in Dulbecco's modified Eagle's medium (DMEM) (HyClone) contained with 10% of FBS (HyClone) and 1% of penicillin–streptomycin at 37°C, in a constant temperature incubator containing 5% of CO_2.

MTT assay

T24 cells were planted in 96 well plates (8.0×10^3 per well) and added different concentrations (0, 3, 10, 30 and 100 μM) of Mito-DHA_5 or DHA after sticking to the wall in the DMEM for 24, 48 or 72 h. Then MTT (Solarbio 2 mg/ml, 50 μL) was mixed in the medium. Then removing the medium after 4 h incubation at 37℃ and mixing in DMSO (150 μL), then measuring OD values at 490 nm thourgh a microplate reader (Biotek).

Clonogenic assay

In brief, seeding 3×10^3 cells per well in 24 well plates. After 12 h, using different concentrations (0, 1, 2, 4 μM) of Mito-DHA_5 or DHA treated the cells for about 7 days. Fixing T24 cells with 10% formaldehyde and then adding 0.1% crystal violet to

stain cells. Then measuring the OD values through area scanning at 550 nm by using microplate reader.

Hoechst 33258 staining

Plating T24 cells in 24 well plates (2.0×10^5 cells per well) and after overnight incubating in the presence of Mito-DHA_5 at 30 μM or DHA at 30 μM for 48 h or pretreated by 5 mM NAC (Beyotime, China) for 2 h then incubated in presence of Mito-DHA_5 (0, 10 μM) at various concentrations for 48 h to induce T24 cell apoptosis. Then T24 cells were washed three times with PBS, stained with Hoechst 33258 according to instructions. After this processing, observed by fluorescence microscope (DFC450C; Leica, Wetzlar, Germany).

Cell apoptosis determination

In brief, T24 cells (5×10^5 cells per well) were plated in 6-well plates. After 12h, treating T24 cells with Mito-DHA_5 (0, 3, 10, 30 μM) for 48 h. Then washing cells use cold PBS for three times and resuspending the cells in binding buffer. Adding annexin V-FITC and PI solution according to the instructions. Incubating at room temperature for 10 minutes in the dark, and then testing the sample with a flow cytometry (Becton Dickinson).

Measurement of Intracellular ROS Generation

Planting T24 cells in 12-well plates (3×10^5 cells per well). After overnight, adding Mito-DHA_5 (0, 3 and 10 μM) or pretreated by 5 mM NAC (Beyotime, China) and then cultured by Mito-DHA_5 (0, 3 and 10 μM) for 12 h, removed the culture medium, and then incubated with 10 μM DCFH-DA at 37°C for 30 min. After that, washing the cells three times with serum-free medium and then photographed by a fluorescence microscopy (DFC450C; Leica, Wetzlar, Germany).

JC-1 assay

MMP was detected through using JC−1 (Solarbio, Beijing, China) staining. In brief, T24 cells (2.0×10^5 cells per well) were plated in 12-well plates. After 12 h, treating cells with different concentrations (0, 3, 10 and 30 μM) of Mito-DHA_5 or DHA (30 μM) for 48 h. First of all, incubating with JC−1 stain working solution (0.5 mL) at 37°C for 20 minutes. Then washing the cells with JC−1 staining buffer (1×) for twice and resuspending the cells in JC−1 staining buffer (1×). Fluorescence

microscopy was used to measure MMP.

Western blotting analysis

Western blotting experiment was carried out with regular procedure. In brief, the protein bands incubated in first antibody for 15-18 h at 4℃， washed them with PBST for 3 times (10 minutes per time), then incubated in secondary antibody for 1h at room temperature. After this, washed 3 times again. After added Pierce Super Signal chemiluminescent substrate (Rockford 1L), imaging on Chemi Doc (Bio-Rad, USA).

Data analysis

We used the GraphPad Prism software to analyze our data and the mean ± SD to present results. The statistical significance of the data is indicated as: *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Results

Mito-DHA$_5$ inhibits the proliferation of T24 cancer cell line

The anti-proliferation activity of Mito-DHA$_5$ and DHA in T24 cells were assessed by MTT assay. As shown in **Fig. 1B**, incubation of T24 cells with different concentrations of Mito-DHA$_5$ for 24 h, 48 h and 72 h resulted in reduction of the cell viability as compared with DHA in a dose-dependent manner and time-dependent manner. At the 3 μM, the cell viabilities of T24 were 87.94±1.97% for 24 h, 54,40±1.47% for 48 h and 50.44±3.79% for 72 h, respectively. At the 10 μM, the cell viabilities of T24 were 59.91±0.29% for 24 h, 30.02±0.43% for 48 h and 23.47±0.52% for 72 h, respectively. At the 30 μM, the cell viabilities of T24 were 56.27±0.25% for 24 h, 23.95± 0.83% for 48 h and 17.00±1.08% for 72 h, respectively. At the 100 μM, the cell viabilities of T24 were 3.89±3.02% for 24 h, 3.12±1.69% for 48 h and 0.97±1.01 for 72 h, respectively. In order to evaluate the potency, half maximal inhibitory concentration (IC_{50}) value of 72 h for T24 cell lines were calculated for Mito-DHA$_5$ (3.2±0.74 μM) and for DHA (71.5±5.24 μM), respectively (**Table 1**). Mito-DHA$_5$ displayed 22 times higher inhibitory effects than DHA in the T24 cancer cell line.

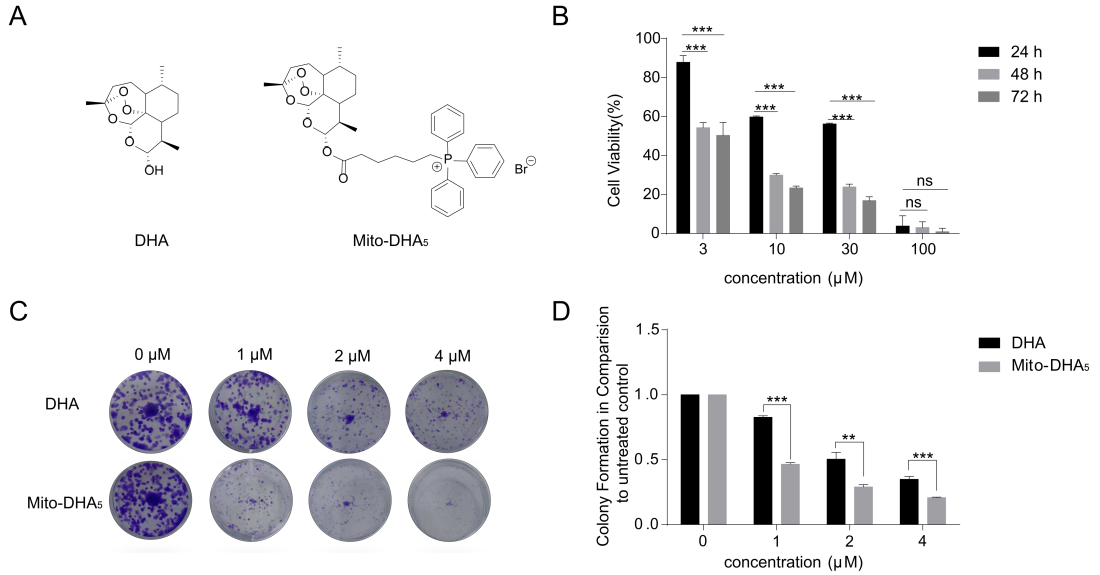
Table 1. The anti-proliferation activity of Mito-DHA$_5$ and DHA against T24 cell line

Compounds	IC_{50} (μM)
	T24
Mito-DHA$_5$	3.2±0.74
DHA	71.5±5.24

Mito-DHA$_5$ inhibits colony formation of T24 cells

The effectiveness of Mito-DHA$_5$ to inhibit the clonogenicity of T24 cells was evaluated. The results showed that Mito-DHA$_5$ exerted potent inhibitory effects on T24 cells (**Fig. 1C**). At 4 μM of Mito-DHA$_5$, almost no colony formation was observed with T24 cells. The effect was dose-dependent and much more potent than the DHA treatment group at the same concentration.

Fig. 1



Proliferation inhibition of Mito-DHA_5 on bladder cancer T24 cells. (A). The structure of DHA and Mito-DHA_5. (B). Treat T24 cells with 3, 10, 30, 100 μM of Mito-DHA_5 for 24 h, 48 h, and 72 h, then observe the survival rate of the cells. (C). Evaluation of colony suppression by Mito-DHA_5. T24 cells were treated for 7 days with Mito-DHA_5 and then stained with crystal violet to detect colony formation. (D). Quantification of the experiments conducted in panels. Wells were scanned at a wavelength of 550 nm. Data are presented as mean±SD. *P<0.5, **P<0.01, ***P<0.001.

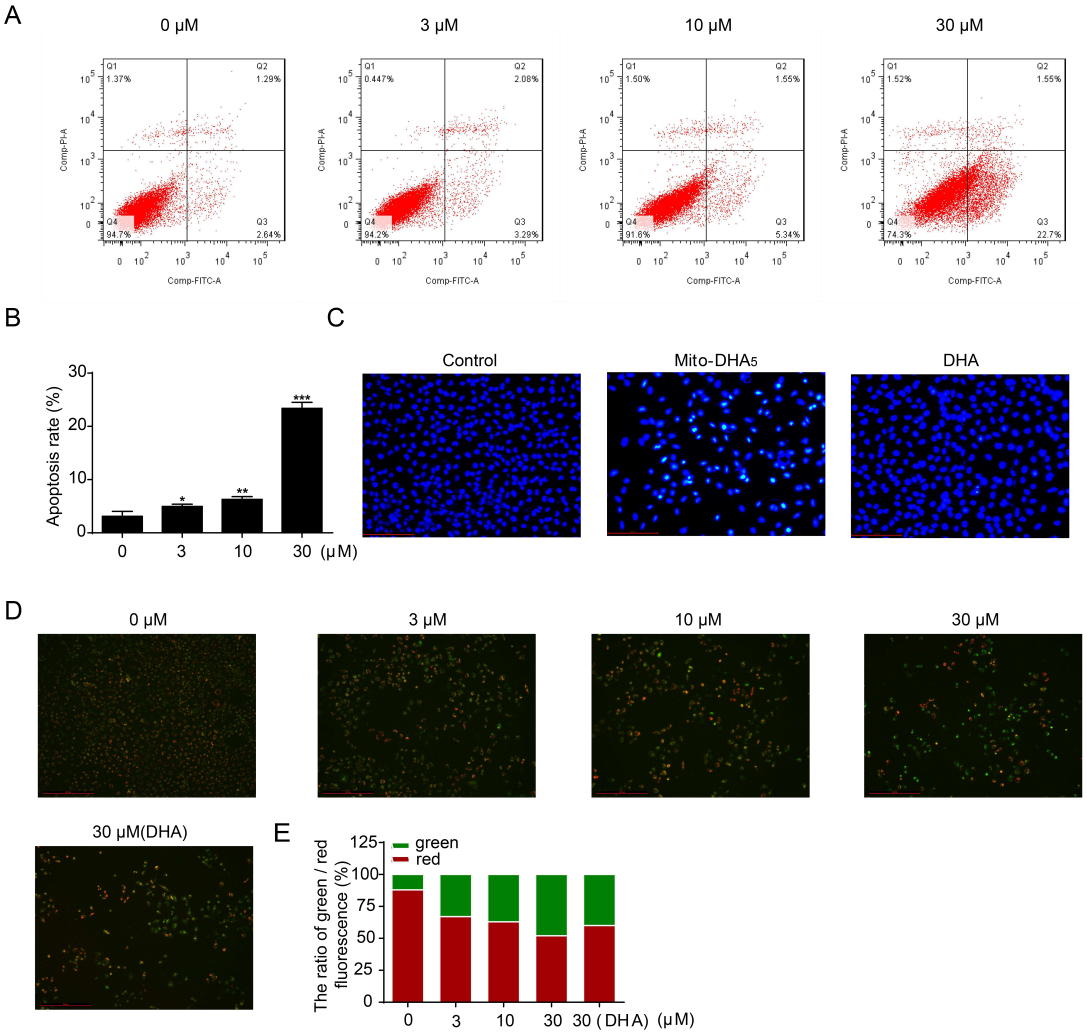
Mito-DHA_5 induces cell apoptosis in T24 cells

We used annexin-V and PI double staining to determine whether the growth-inhibiting effects of Mito-DHA_5 in T24 cells was related to cell apoptosis. After the treatment of Mito-DHA_5 with different concentration for 48 h, we analyzed the results by flow cytometry. As shown in **Fig. 2A**, Mito-DHA_5 induced significantly cell apoptosis in T24 cells in a dose-dependent manner. Apoptosis ratio at 30 μM was 24.2% (**Fig. 2B**). In order to explore whether the effect of Mito-DHA_5 in inducing cell apoptosis was stronger than DHA, the nuclei change in T24 was observed under a fluorescence microscope by Hoechst 33258. As shown in **Fig. 2C**, treatment with Mito-DHA_5 at 30 μM induced more significant bright blue nuclei blebbing, nuclear rounding and shrinkage than treatment with DHA at 30 μM in T24 cells. And the cell density of treatment with Mito-DHA_5 under the same concentration was much lower than that of treatment with DHA.

Mito-DHA_5 decreases MMP in T24 cells

To explore the effect of Mito-DHA_5 in MMP in T24 cells, we used the JC-1 staining method. After the treatment of Mito-DHA_5 with different concentration for 48 h, the red fluorescence and green fluorescence were analyzed. An increase in green fluorescence intensity represents a decrease in MMP, while the red fluorescence intensity represents an increase in MMP. As shown in **Fig. 2D** and **E**, Mito-DHA_5 caused a decrease in MMP of bladder cancer T24 cells with a dose-dependent fashion. Compared with the ratio of green fluorescence/red fluorescence of DHA at 30 μM, Mito-DHA_5 increased the ratio at the same concentration, indicating the stronger effect of Mito-DHA_5 on decreasing capacity of MMP.

Fig 2



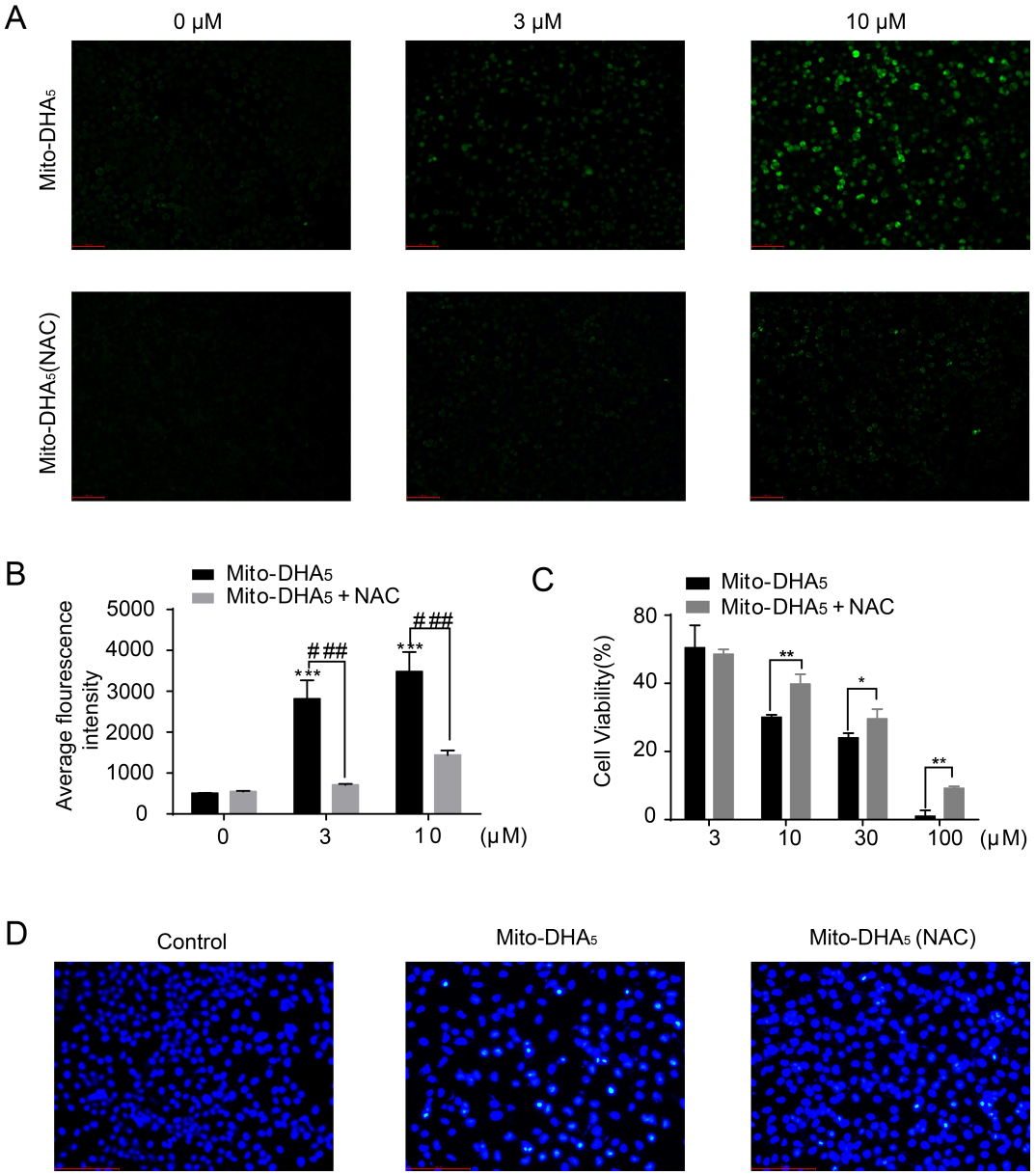
Effect of Mito-DHA$_5$ on cell apoptosis and MMP in T24 cells. (A). T24 cells were treated with Mito-DHA$_5$ (0, 3, 10, 30 μM) for 48 h and then assayed by flow cytometry analysis with Annexin V-FITC staining. (B) Quantification of apoptotic cells. (C). Treat T24 cells with 30 μM of Mito-DHA$_5$ and DHA for 48 h, respectively, stained with Hochest 33258, and viewed by fluorescence microscopy. Representative images were shown. Scale bar 100 μm. (D) T24 cells were treated with Mito-DHA$_5$ (3, 10, 30 μM) or DHA (30 μM) for 48 h and then viewed by fluorescence microscopy after JC-1 staining. Increased green fluorescence represents a decrease in MMP. Scale bar 200 μm. (E). The ratio of green fluorescence to red fluorescence. Results are the mean±SD of three independent experiments. *P<0.5, **P<0.01, ***P<0.001.

Mito-DHA$_5$ increases the ROS level in T24 cells

We evaluated how treatment with Mito-DHA$_5$ in T24 cells influences the production of ROS tracked by DCFH-DA. Mito-DHA$_5$ induced an increase of ROS production in a dose-dependent manner and the pretreatment of NAC significantly

reduced ROS production (**Fig. 3A** and **B**). The cytotoxicity of Mito-DHA$_5$ on T24 cells could be inhibited by NAC (**Fig. 3C**). The cell morphologic change was observed by fluorescence microscopy. The results showed that with the pretreatment of NAC, the induction of cell apoptosis was inhibited in T24 cells (**Fig. 3D**), indicating that Mito-DHA$_5$ induced T24 cell apoptosis dependent on ROS.

Fig. 3



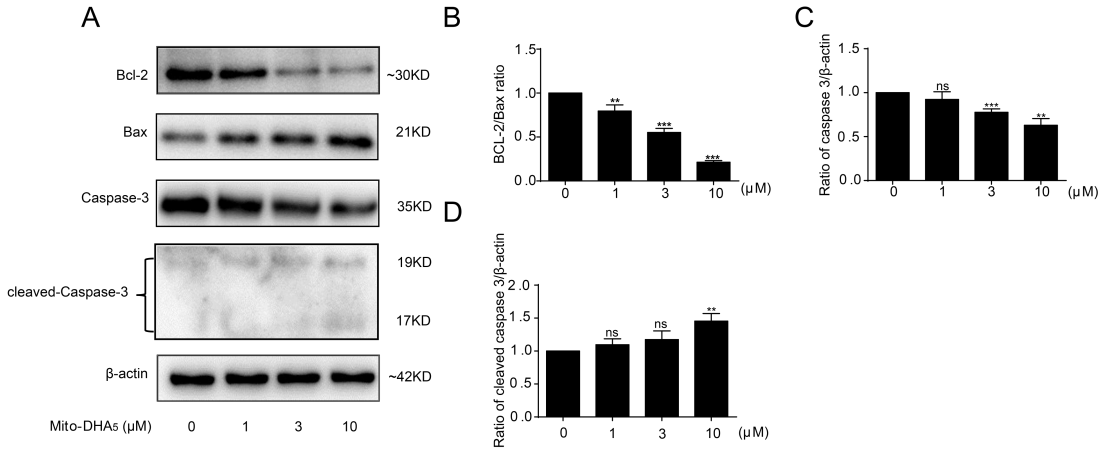
Effects of Mito-DHA$_5$ on ROS production. (A). T24 cells were treated with Mito-DHA$_5$ (0, 3, 10 μM) in the presence or absence of pretreated with 5 mM NAC for 24h, then DCFH-DA (10 μM) was loaded and cells were analyzed by fluorescence microscopy. Scale bar 100μm. (B). Average

fluorescence intensity of different treatment groups. (C). Effects of NAC on Mito-DHA_5-induced cytotoxicity in T24 cells assessed by MTT. Cells were treated with Mito-DHA_5 (3, 10, 30, 100 μM) for 72 h in the presence or absence of pretreated with 5mM NAC. (D). T24 Cells were treated with Mito-DHA_5 (10 μM) in the presence or absence of pretreated with 5 mM NAC for 48 h, stained with Hochest 33258, and viewed by fluorescence microscopy. Scale bar 100 μm. Data are presented as mean ±SD. *P<0.5, **P<0.01, ***P<0.001, #P<0.5, ##P<0.01, ###P<0.001.

Apoptosis effect of Mito-DHA_5 was associated with mitochondrial pathway

To further investigate whether Mito-DHA_5 influences the apoptosis-related protein expression (**Fig. 4**), we used Western blotting to detect the levels of mitochondrial pathway associated proteins. After the treatment of T24 cells with different concentration of Mito-DHA_5 (0, 1, 3 and 10 μM) for 24 h, we found that Mito-DHA_5 treatment could down-regulate the expression of Bcl-2 and up-regulate the expression of Bax in a dose-dependent manner. At the same time, Mito-DHA_5 activated caspase-3 and increased the expression of cleaved caspase-3.

Fig. 4



(A). The expression of cell apoptosis regulatory proteins Bcl-2, Bax and Caspase-3 in T24 cells after treatment Mito-DHA_5 (0, 1, 3, 10 μM). (B). The ratio of BCL-2/Bax protein expression. (C). The expression of Caspase-3 protein. (D). The expression of cleaved-Caspase3 protein. Data are the mean±SD of three independent experiments. *P<0.5, **P<0.01, ***P<0.001.

Discussion

Mito-DHA_5 is a newly synthesized mitochondrial-targeted artemisinin ester derivative. Our previous studies have found that this compound has great anti-tumor activity [12]. In this study, we have provided evidence that Mito-DHA_5 induced mitochondria-associated apoptosis, decreased mitochondrial membrane potential, increased ROS level, and leaded to caspase-3 activation associated with mitochondrial-dependent apoptosis pathway in T24 bladder cancer cells.

Apoptosis is a type of programmed cell death. This programmed cell death process is mediated by a variety of signal pathways (internal and external) triggered by a variety of factors (including cell stress, DNA damage, and immune monitoring) [16, 17]. In this study, we evaluated the ability of Mito-DHA_5 to induce apoptosis of T24 bladder cancer cells. The ratio of apoptosis cells reached 24.2% after incubation with Mito-DHA_5 of 30 μM in T24 cells. At the same concentration, Mito-DHA_5 had a stronger ability to induce cell apoptosis than DHA. Mitochondrial membrane potential is closely related to cell apoptosis, and the decrease of mitochondrial membrane potential indicates that cells may undergo early apoptosis [18]. We treated T24 cells with Mito-DHA_5 of different concentrations, and found that Mito-DHA_5 could significantly reduce the MMP at the concentration of 30 μM. In addition, the mitochondrial membrane potential of the DHA treatment group under the same concentration less than that of the Mito-DHA_5 treatment group. This result was consistent with the apoptosis result, and further told us that Mito-DHA_5 could induce apoptosis of T24 cells with much stronger apoptosis than that of DHA.

Reactive oxygen species (ROS) are a family of short-lived molecules and the production of ROS participates in radiotherapy by affecting downstream cell death signals [19, 20]. Mitochondria, the place where cells breathe, are the main place where endogenous ROS is produced. A sharp increase in ROS in a short period of time will lead to the occurrence of cell apoptosis [21]. Our study showed that Mito-DHA_5 could cause an increase in the level of ROS in T24 cells, and this increase could be inhibited by the antioxidant NAC. At the same time, the killing effect of

Mito-DHA_5 on T24 cells was weakened under the condition of NAC pretreatment. Besides, the results of fluorescence microscopy showed that the apoptosis ratio of the NAC pretreatment group decreased significantly under the same concentration of Mito-DHA_5 treatment. This implied us that Mito-DHA_5-induced apoptosis might be related to the production of ROS.

The Bcl-2 family plays an important role in cell apoptosis [22]. The role of the Bcl-2 family in the regulation of apoptosis is generally described as anti-apoptotic and pro-apoptotic [23]. Bcl-2 is anti-apoptotic protein, while Bax is pro-apoptotic protein and the ratio of them is closely related to cell apoptosis. Mito-DHA_5 treatment resulted in an obvious decrease in Bcl-2 protein expression and a significant increase in Bax protein expression in T24 cells. This indicated that Mito-DHA_5 inhibited the cell through the regulating of the expression of mitochondrial-mediated apoptosis-related protein Bcl-2/Bax. Caspase-3 is the downstream pathway of cell apoptosis and can be activated when cells is apoptosis [24]. Our research showed that after treating T24 cells with Mito-DHA_5, Caspase-3 can be activated and cleaved into 17 and 19KD activators.

Conclusions

In conclusion, this research demonstrated that Mito-DHA_5 could induce apoptosis associated with mitochondrial-mediated pathway, decrease MMP, increased ROS levels and resulting in downregulation of the Bcl-2/Bax ratio and downstream activation the caspase-3. Collectively, these results suggest that Mito-DHA_5 holds promise for further development as a candidate for the treatment of bladder cancer.

Abbreviations

DHA: Dihydroartemisinin; MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; MTT: 3−(4, 5−dimethylthiazol−2−yl)–2, 5− diphenyltetrazolium bromide

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Author's contributions

Linfan Xiao performed the experimental work and analyzed the data. Project administration and supervision were carried out by Xiaoping Yang , Cangcang Xu and Lingli Mu. Peiyu Lin helped to analyze the data. All authors contributed in writing the manuscript and approved it.

Availability of data and materials

All data and materials are contained and described within the manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human or animal subjects performed by the authors.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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