Tmpyp4 Induces Autophagy in Cancer Cells By Activating AMPK

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Keywords: Tmpyp4; Cancer Cells; Autophagy; AMPK

Abstract: Objective: To investigate the effect of porphyrin compound TMPyP4 on autophagy of tumor cells and the underlying molecular mechanism. Methods: the effect of TMPyP4 on autophagy related protein expression and autophagy body formation was detected by immunoblotting and fluorescence. Results: TMPyP4 activate autophagy flow and up-regulate autophagy level in tumor cells; TMPyP4 induced autophagy dependents on AMPK signal pathway; inhibition of autophagy enhances the cytotoxic effect of TMPyP4 on tumor cells. Conclusion: the above results indicate that TMPyP4 induce protective autophagy in tumor cell by activating AMPK signal pathway, suggesting that combination with autophagy inhibitors can improve the antitumor effect of TMPyP4 and its mediated photodynamic therapy.

Introduction

About Autophagy: (Autophagy) is a mass degradation mechanism that widely exists in eukaryotic cells [1]. Autophagy is a multi-step dynamic process that forms autophagosomes that form a double-layered membrane, encapsulates senescence or loss of organelles, protein complexes and other components in the cell, transports to lysosomes to form autophagolysosomes and degrades the contents Cell physiological process. Autophagy plays an important role in maintaining the normal growth of eukaryotic cells. On the one hand, autophagy can maintain the homeostasis and normal growth of cells; on the other hand, autophagy helps cells cope with nutritional deficiencies caused by various stress conditions and maintain cell survival. [2]. Excessive autophagy can also lead to cell death, that is, autophagic cell death. Studies have shown that abnormal autophagy is closely related to the occurrence of various diseases such as neurodegenerative diseases, viral infections and tumors [3, 4]. Studies have found that autophagy may have a dual role in the occurrence and treatment of tumors. It may not only maintain the survival of tumor cells by providing nutrition, but also play an anti-tumor effect through autophagic cell death [5]. Studies have found that many anti-cancer drugs can induce autophagy in tumor cells. Our previous research found that triptolide can induce protective autophagy in prostate cancer cells by activating CaMKKβ-AMPK signaling pathway [6]. Therefore, the relationship between autophagy and tumors as a current research hotspot may provide new targets and ways for the treatment of tumors.

TMPyP4 is a cationic porphyrin compound, which has the characteristics of high water solubility, high membrane permeability and preferential aggregation in tumor cells, and has good application prospects in tumor treatment. In addition to being a PDT photosensitizer [7], in recent years, studies have reported that TMPyP4 itself also has good antitumor efficacy. TMPyP4 has a good inhibitory effect on various tumor cells such as ovarian cancer [8], lung cancer [9] and cervical cancer [10]. At present, the research on the mechanism of TMPyP4's anti-tumor effect mainly focuses on the strong affinity of TMPyP4 for nuclear DNA. As a G4 ligand, TMPyP4 can specifically bind and stabilize a special four-stranded nucleic acid structure-G-quadruplex (G-quadruplex, G4), which inhibits telomerase activity and oncogene expression [11].

Studies have shown that TMPyP4 has good anti-tumor efficacy, but the molecular mechanism of TMPyP4's own anti-tumor effect is not fully understood. Autophagy plays an important role in tumorigenesis and treatment, and the relationship between TMPyP4's antitumor activity and

autophagy has not been reported. Therefore, this paper uses laryngeal cancer cells Hep-2 and breast cancer cells MCF-7 as models to detect whether TMPyP4 induces autophagy, as well as its molecular mechanism and role to induce autophagy. New test basis.

1. Materials

1.1 Cells

293T, Hep-2 and MCF-7 cells were purchased from the National Experimental Cell Resource Sharing Service Platform (Beijing Headquarters), all using high-sugar DMEM medium supplemented with 10% FBS (Fetal Bovine Serum) and 100U/mL penicillin-streptomycin Place in 5% CO2, 37 ℃ incubator.

1. 2 Reagents

The siRNA was synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. Lipofectamine 2000 (11668) was purchased from Invitrogen. β-actin antibody (AF003), caspase-3 antibody (AC030), PARP antibody (AP102) were purchased from Biyuntian Biotechnology Company. LC3 antibody (12741), ATG7 antibody (2631S), ATG5 antibody (2630S), AMPKα antibody (5832P), Phospho-AMPKα (Thr172) antibody (2870), mTOR antibody (2983), Phospho-mTOR (Ser2448) antibody (5536) Purchased from CST. SQSTM1/P62 antibody (sc-28359), caspase-8 p18 (C-20) antibody (sc-6136) and caspase-9 p10 (H-83) antibody (sc-7885) were purchased from Santa Cruz Biotechnology. HRP labeled goat anti-mouse (CW0102) and goat anti-rabbit secondary antibody (CW0103M) were purchased from Beijing Kangwei Century Biotechnology Co., Ltd.

2. Method

2.1 Drug Treatment

Hep-2 and MCF-7 cells in good growth state were taken and inoculated into 6-well plates after trypsin digestion. After night incubation, TMPyP4 (0μM, 25μM, 50μM, 100μM) was added for treatment. After 48h, cell samples were collected for other experiments. For cell transfection, after 24h of siRNA transfection, add 50μM TMPyP4 for 24h, and then collect cell samples for other experiments. DMSO was used as a negative control for drug treatment.

2.2 Western Blot

Cells were collected, lysed with RIPA lysate containing protease inhibitors and phosphatase inhibitors and sonicated to extract total cell protein. Measure the total protein concentration of the sample using the BCA protein quantification kit, adjust the protein concentration of all groups uniformly, add 5 x SDS loading buffer, mix well and boil the water for 5 min to denature the protein. According to the size of the detected protein, choose 8%~15% SDS-PAGE gel for electrophoresis. After electrophoresis, transfer the protein to the NC membrane. The protein-transformed NC membrane was sequentially blocked with 5% skimmed milk powder solution at room temperature for 1 hour, the primary antibody was incubated overnight at 4°C, and the secondary antibody was incubated at room temperature for 1 hour. Finally, the protein was developed with ECL luminescence solution, and β-actin was used as an internal reference.

2.3 Cell Transfusion

Hep-2 and MCF-7 cells in good growth state were taken, inoculated into 6-well plates after trypsin digestion, and cultured overnight. Replace the culture solution in the 6-well plate with Opti-MEM medium, dilute 5μl Lipofectamine 2000 and 100pmol siRNA in 100ul Opti-MEM medium, let stand for 5min, then mix the two dilutions, and let stand for 5min After that, the mixture was added to a 6-well plate, gently mixed, and placed in an incubator for cultivation. After 6 hours, the medium was replaced with a normal culture medium.

Tab 1. Sirna Sequences in This Study

Name	Sequence(5'-3')	Purpose
NC	UUCUCCGAACGUGUCACGUTT	Negative Control
siATG5	CAUCUGAGCUACCCGGAUA	Knockdown ATG5
siATG7	GGA GUCACAGCUCUUCCUU	Knockdown ATG7
siAMPKa	GAGGAGAGCUAUUUGAUUA	Knockdown AMPKa

2.4 Mcherr-LC3B Stable Cell Construction

Inoculate 293T cells with good growth status into 6cm Petri dishes. After overnight cultivation, use Lipofectamine 2000 to co-transform mcherry-LC3B fusion gene expression plasmid and lentiviral packaging plasmid into 293T cells according to the instructions. 48h after transfection, the culture solution was collected, filtered through a 0.45um filter, mixed with DMEM medium supplemented with 1% FBS at 1:1, and added to the Petri dish where Hep-2 was cultured for infection. After 48h of infection, it was replaced with DMEM medium containing puromycin (1ug/mL) for screening. After continuous screening for 2 weeks, the surviving Hep-2 cells were continuously cultured and identified.

2.5 Fluorescence Observation of Autophagosomes

Hep-2 cells stably expressing the mCherry-LC3B fusion gene were inoculated in a 35 mm cell culture dish with sterile coverslips at a rate of 1×105 cells/dish. After the cells adhered, they were treated with 50mM TMPyP4, and DMSO was used as a negative control. After 48 hours of treatment, the cell slide was taken out, inverted on a glass slide, and placed under a BX51+DP70 fluorescence microscope (Olympus) for observation and photographing.

3. Result

3.1 TMPyP4 induces Autophagy in Tumor Cells

Using laryngeal cancer cells Hep-2 and breast cancer cells MCF-7 as research subjects, the changes in the levels of two autophagy marker proteins LC3BⅡ and P62 after TMPyP4 treatment were detected. It was found that treatment of the above two cells with different concentrations of TMPyP4 (0 μM, 25 μM, 50 μM, 100 μM) resulted in increased levels of mature LC3BⅡ protein, while P62 protein levels decreased and showed a concentration-dependent (see Figure 1A); treatment with 50μM TMPyP4 for different durations (0h, 6h, 12h, 24h, 48h) also caused an increase in LC3BⅡ protein levels and a decrease in P62 protein levels, and showed a time dependence (see Figure 1B). TMPyP4 caused the increase of LC3BⅡ protein level in Hep-2 and MCF-7 cells, while the decrease of P62 protein level suggested that TMPyP4 may induce autophagy in 2 types of cells. Further, Hep-2 cells stably expressing the mcherry-LC3B fusion gene were used to detect the effect of TMPyP4 on autophagy. The results showed that compared with the DMSO negative control group, the red fluorescent spots in the TMPyP4 treated group were significantly brighter and significantly increased in number (see Figure 1C), indicating that TMPyP4 caused an increase in the number of autophagosomes and autophagolysosomes in the cells. . The above results indicate that TMPyP4 may induce autophagy in tumor cells.

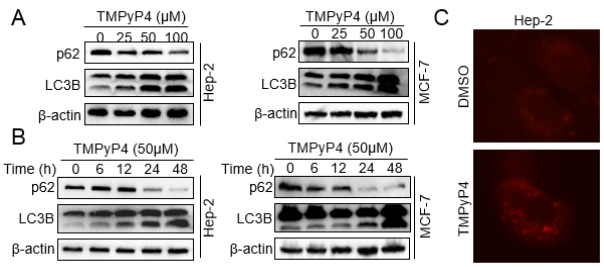

Fig 1. Effect of TMPyP4 on Autophagy in cancer cells

Hep-2 and MCF-7 cells were treated with the indicated doses of TMPyP4 for 48h (A) or 50μM TMPyP4 for different times (B), and then subjected to detect the protein level of LC3B and p62 by WB. β-actin was used as loading control. (C) Effect of TMPyP4 on autophagosomes and autophagolysosomes.

3.2 TMPyP4 induces Autophagic Flux of Tumor Cells

Autophagy is a highly dynamic process of directed multi-step successively, also called autophagy flow. The changes in the autophagy protein level and the number of autophagosomes caused by TMPyP4 may be caused by the increase of autophagy levels, or may be caused by the inhibition of the degradation of autophagosomes. Therefore, further changes in autophagic flow need to be detected. First, the effects of TMPyP4 on the autophagy flow of Hep-2 and MCF-7 cells were detected by the combined treatment of autophagy inhibitors TMPyP4, CQ and TMPyP4 targeting different levels of autophagy regulation. The results are shown in Figure 2A. In the two types of cells, compared with the TMPyP4 alone treatment group, the combined treatment of LC3BⅡ and p62 protein levels was significantly reduced; similarly, in Figure 2B, compared with the TMPyP4 and CQ alone treatment groups, The levels of LC3BⅡ and p62 protein in the combined treatment were significantly increased. The results of LY294002 inhibiting TMPyP4-induced increase in LC3BⅡ level and CQ promoting TMPyP4-induced increase in LC3BⅡ level indicate that TMPyP4 induces autophagic flow in Hep-2 cells.

In addition, siRNA was used to interfere with the autophagy genes ATG5 and ATG7 to detect the effect of TMPyP4 on autophagy flow. Both ATG5 and ATG7 are important autophagy genes and play an important role in regulating the formation of autophagosomes. Therefore, silencing ATG5 and ATG7 leads to the blockage of autophagic flow. The results showed that the use of specific siRNAs to interfere with the expression of ATG5 and ATG7, respectively, can inhibit TMPyP4-induced increase in LC3BII levels (see Figure 2C and D). The reverse shows that TMPyP4 induces autophagic flow in Hep-2 cells.

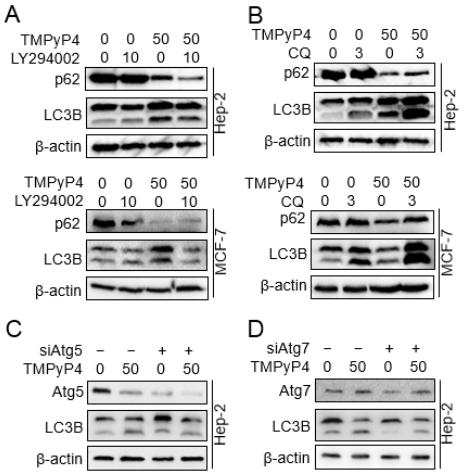

Fig 2. Effect of TMPyP4 on autophagy flux in cancer cells

(A) and (B) Effect of LY294002 or CQ on TMPyP4 induced autophagy. After pretreated with LY294002 (10mM) or CQ (3μM) for 1h, Hep-2 and MCF-7 cells were treated with 50μM TMPyP4 for 24h and subjected to detect the protein level of LC3B and p62 by WB. (C) and (D) Effect of knockdown ATG5 or ATG7 on TMPyP4 induced autophagy by WB. β-actin was used as loading control.

3.3 Tmpyp4 Induces Autophagy in Tumor Cells by Activating AMPK/Mtor

The foregoing test results confirmed that TMPyP4 can induce autophagy in tumor cells, but the mechanism by which TMPyP4 induces autophagy is a further study. By examining the effects of TMPyP4 treatment on the autophagy regulatory genes AMPKa and mTOR, it was found that TMPyP4 treatment resulted in a significant decrease in AMPKa levels in Hep-2 and MCF-7 cells,

while the level of P-AMPKa T172 was significantly increased; TMPyP4 also caused Both mTOR and P-mTOR S2448 levels were significantly down-regulated (see Figure 3A). These results indicate that TMPyP4 treatment leads to AMPK activation and mTOR complex inactivation, suggesting that TMPyP4 may induce tumor cell autophagy through the AMPK/mTOR signaling pathway. To verify this idea, siRNA silencing AMPK expression was used to detect the effect on TMPyP4 induced autophagy. The results are shown in Figure 3B. In two types of cells, silencing AMPKa significantly inhibited the increase in TMPyP4-induced increase in LC3BⅡ levels, further proving TMPyP4 induces autophagy by activating the AMPK signaling pathway.

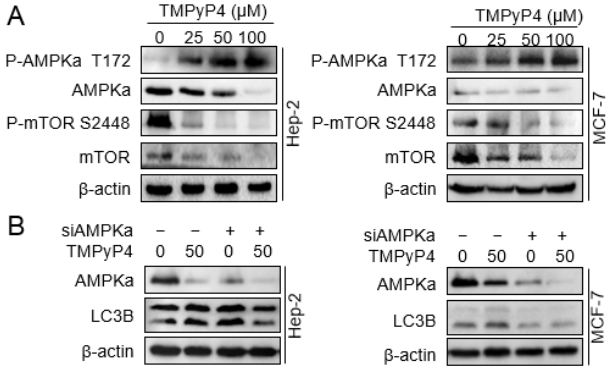

Fig.3. Effect of TMPyP4 on AMPK/mTOR signal pathway in tumor cells

(A) Effect of TMPyP4 on AMPK and mTOR activities in tumor cells. Hep-2 and MCF-7 cells were treated with different doses of TMPyP4 for 48h and subject to detect the AMPK and mTOR activities by WB. (B) Effect of knockdown AKPK on TMPyP4 induced autophagy by WB. β-actin was used as loading control.

3.4 **Inhibition of autophagy can enhance the apoptosis of TMPyP4 on tumor cells**

Studies have reported that autophagy has a "double-edged sword" effect to promote the survival or death of tumor cells. What effect does autophagy induced by TMPyP4 have on its anti-tumor efficacy? To investigate this issue, the autophagy inhibitors LY294002 or CQ and TMPyP4 were used to treat Hep-2 and MCF-7 cells in order to examine the effect on TMPyP4-induced apoptosis. The results are shown in Figure 4. Compared with the TMPyP4 alone treatment group, the combined treatment of the two autophagy inhibitors and TMPyP4 both resulted in a decrease in the proprotein level of procaspase-8 and procaspase-9, and an increase in the cleavage products of caspase-3 and RARP, indicating inhibition Autophagy can enhance TMPyP4-induced apoptosis of tumor cells, suggesting that TMPyP4 induces protective autophagy in tumor cells and has an inhibitory effect on its anti-tumor activity.

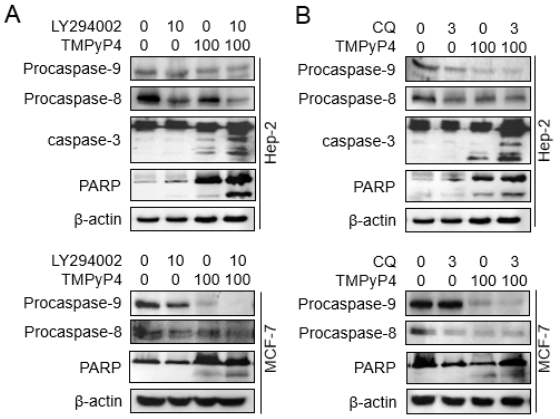

Fig 4. Effect of autophagy TMPyP4 anti-tumor activity

(A) and (B) Effect of LY294002 or CQ on TMPyP4 anti-tumor activity. After pretreated with LY294002 (10mM) or CQ (3μM) for 1h, Hep-2 and MCF-7 cells were treated with 50μM TMPyP4

for 24h and subjected to detect the protein level of procaspase-8, procaspase-9, caspase-3 and RARP by WB. β-actin was used as loading control.

4. Discussions

The porphyrin compound TMPyP4 has good anti-tumor activity, but its anti-cancer mechanism is not yet fully understood. The autophagy identification test in this paper proves that TMPyP4 can induce autophagy in tumor cells. Many small molecule drugs have been reported to induce autophagy in tumor cells. Kessel et al. [12] confirmed that the two porphyrin photosensitizers porphycene-CPO and mesophrion (MC) combined with PDT treatment can induce autophagy in mouse leukemia cells L1210, and that these two photosensitizers induced tumor cell autophagy in dose and time There are differences. Zhu et al. [13] found that sodium porphyrin-mediated PDT (DVDMS-PDT) can induce autophagy in colorectal cancer cell HCT116, confirming that porphyrin photosensitizer combined with PDT can induce autophagy. But do porphyrins alone induce tumor cell autophagy? Our research proves that TMPyP4 alone can induce tumor cell autophagy. Gyongyosi et al. [14] also found that the porphyrin compounds heme chloride or cobalt protoporphyrin IX (CoPPIX) alone can induce cardiomyocyte H9c2 autophagy. These studies confirmed that porphyrins can induce cell autophagy, which may be related to the dual activities of chemical damage and photodynamic effects of many porphyrins. TMPyP4 also has dual activities. TMPyP4 not only mediates PDT's anti-tumor effect, but also has good cytotoxicity. Our research confirmed that TMPyP4 itself can induce cell autophagy. It is speculated that TMPyP4-mediated PDT may also induce autophagy.

Cellular autophagy is an important cell physiological activity, and the process of autophagy is strictly controlled by signals. Protein kinase mTOR is a key negative regulator of autophagy [15]. Our results found that TMPyP4 treatment resulted in significant down-regulation of mTOR and P-mTOR S2448 levels, indicating that TMPyP4 can inhibit mTOR function. At the same time, it was also found that TMPyP4 treatment leads to the inhibition of the negative AMPK signal pathway upstream of mTOR. AMPK is an energy-sensing molecule in cells. When stress stimulation leads to cell energy production disorders, the AMP: ATP ratio increases, and AMPK is phosphorylated at Thr172 to activate. Activated AMPK indirectly negatively regulates mTORC1 and activates autophagy [16]. Autophagy mechanism test results prove that TMPyP4 can induce tumor cell autophagy by activating the AMPK/mTOR signaling pathway. However, it is unclear how TMPyP4 activates the AMPK/mTOR signaling pathway, and there is a lack of relevant research reports. It is speculated that there are two possible mechanisms. The treatment of the photosensitizer TMPyP4 may induce the cells to produce a large amount of ROS, damage the mitochondrial function, lead to ATP production disorders, and cause the activation of the AMPK signaling pathway to activate autophagy; TMPyP4-induced ROS accumulation may also cause mitochondrial membrane communication Permeability changes, causing calcium ions to be released into the cytoplasm, which in turn activates the CaMKK-AMPK signaling pathway to induce autophagy.

The results of autophagy test prove that TMPyP4 induces protective autophagy in tumor cells, that is, autophagy plays a role in protecting tumor cells against TMPyP4 cytotoxicity. Studies on the relationship between autophagy and tumors have shown that autophagy has a dual role in the development and treatment of tumors [5]. On the one hand, autophagy degrades non-essential components in the cell, promotes energy metabolism, helps the rapid growth of tumor cells and responds to adverse growth environments such as hypoxia. On the other hand, continued excessive autophagy can also lead to self-acting cell death. Therefore, autophagy has a dual effect of antagonism or assistance to the cytotoxic effects of small molecule drugs. The dual role of autophagy has been reflected in a large number of drug-induced tumor cell autophagy studies. The dual role of autophagy is related to both the molecular structure and activity of the drug and the dose of the drug. For tumor cells, treatment with cytotoxic small-molecule drugs is equivalent to applying unfavorable stimuli. Tumor cells maintain survival by promoting autophagy, thereby exhibiting certain drug antagonistic capabilities. Tumor cell autophagy induced by the

above-mentioned multiple photosensitizers mediated PDT all showed antagonism [13]. Our study found that the natural product triptolide is also induced by endoplasmic reticulum stress to activate CaMKK-AMPK signaling pathway to cause protective autophagy in prostate cancer cells [6]. These findings suggest that the combination of low toxicity autophagy inhibitors with anticancer drugs or PDT may enhance the therapeutic effect and reduce the phenomenon of drug antagonism.

The TMPyP4 may induce protective autophagy in tumor cells by activating the AMPK/mTOR signaling pathway, suggesting that in clinical applications, TMPyP4 can be combined with autophagy inhibitors such as CQ to enhance TMPyP4 or TMPyP4-mediated PDT The killing effect of tumor cells in order to obtain better therapeutic effect.

Funds

Funded by the National Natural Science Foundation of China (31300654) and the Natural Science Foundation of Shaanxi Province (2014JQ3098)

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