Antitumor and apoptotic effects of 5-methoxypsoralen in U87MG human glioma cells and its effect on cell cycle, autophagy and PI3K/Akt signaling pathway

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Abstract

Introduction: The main purpose of the current study was to investigate the antitumor effects of 5-methoxypsoralen in U87MG human glioma cells along with studying its effects on cell cycle progression, autophagy and the PI3K/Akt signaling pathway.

Material and methods: The cytotoxic effects of the drug were demonstrated by the MTS cell viability assay while its effects on cellular morphology associated with cell apoptosis were evaluated by phase contrast and fluorescence microscopic techniques. Effects of 5-methoxypsoralen on the cell cycle were studied by flow cytometry while effects on PI3K/Akt proteins were evaluated by western blot assay.

Results: The results indicate that 5-methoxypsoralen led to time-dependent as well as dose-dependent inhibitory effects on U-87MG human glioma cells. 5-Methoxypsoralen led to a substantial decrease of cell count along with distorted cell morphology. The molecule also led to DNA ladder formation which increased with increasing doses of 5-methoxypsoralen. 5-methoxypsoralen also led to dose-dependent G2/M phase cell cycle arrest. 5-Methoxypsoralen-treated cells also exhibited altered cell ultrastructure with the appearance of autophagic vacuoles and the number of these vacuoles increased with increasing drug dose.

Conclusions: In brief, the results indicate that 5-methoxypsoralen exerted potent anticancer and apoptotic effects in U-87MG human glioma cells along with inducing cell cycle arrest, autophagy and m-TOR/PI3K/Akt signaling pathway inhibition.

Key words: 5-methoxypsoralen, coumarins, apoptosis, glioma, flow cytometry.

Introduction

Tumors, also called neoplasms, are a heterogeneous disease characterized by uncontrolled cell division [1]. They are either solid or filled with fluids [2]. A tumor does not necessarily mean cancer; in fact tumor can exist in three forms: benign tumor (not cancerous), pre-malignant tumor (pre-cancerous) and malignant tumor (cancerous) [2]. Malignant gliomas constitute the most common kind of primary brain cancers, with glioblastoma being the most common type of glioma [3]. Annually more than 5 persons per 100,000 individuals are diagnosed with malignant glio-
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Ma [3]. Glioma patients have a median survival of 14–16 months, while gliomas which relapse have a poor prognosis, decreasing the median survival to 3–6 months only [4, 5]. Glioma treatment initially involves surgical resection followed by radiotherapy and simultaneous use of temozolomide, which is a chemotherapeutic agent [6]. Temozolomide is a potent alkylating agent that destroys cancer cells by making O6-methylguanine in DNA, which later miss-matches with thymine, ultimately resulting in cell death [6].

Coumarins are a vast class of natural products found in a large variety of plant species in different parts including fruits, roots, stem and leaves. In addition, some essential oils such as cinnamon bark, lavender oil and cassia leaf oil are a good source of coumarins [7–9]. 5-Methoxypsoralen (5-MOP), also called bergapten, belongs to the furanocoumarin class of natural products and is commonly isolated from Psoralea corylifolia. Previous studies have reported that furanocoumarins exhibit both in vitro and in vivo antitumor and apoptotic effects in a range of cancer cells [10–12]. 5-Methoxypsoralen has been reported to exert a cytotoxic effect in a human hepatocellular carcinoma (HCC) cell line [13]. Moreover, furanocoumarins such as angelicin and 4,6,4′-trimethylangelicin (TMA) exhibit antiproliferative activity in human keratinocytes through cell cycle arrest [14]. Moreover, a related furanocoumarin, psoralidin, was reported to induce autophagy in lung cancer cells [15]. Consistent with this, the present study was designed to evaluate the antitumor and apoptotic effects of 5-methoxypsoralen in U87MG human glioma cells along with its effects on the cell cycle, autophagy and the m-TOR/P13K/Akt signaling pathway.

Material and methods

Chemicals and other reagents

5-Methoxypsoralen (≥ 95% by HPLC), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and dimethyl sulfoxide were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Acridine orange and propidium iodide were procured from Wuhan Boster Biological Technology Ltd. (Wuhan, China). Dulbecco’s modified Eagle’s medium and RPMI-1640 medium were obtained from Gibco Life Technologies (Grand Island, NY, USA). Heat-inactivated fetal calf serum, penicillin, and streptomycin were obtained from Thomas Scientific, High Hill Road, Swedesboro, U.S.A.

Cell line and cell culture medium

The U87MG human glioma cancer cell line was procured from the Cancer Research Institute of Beijing, China and maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified incubator.

MTS assay for cell viability

The cell death induced by 5-methoxypsoralen was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, which is a CellTiter 96 Aqueous One Solution Cell Proliferation assay. The wells of the 96-well plate were seeded with 1 × 10^4 U87MG human glioma cells per well, incubated for 12 h and then subjected to treatment with increasing doses of 5-methoxypsoralen (0, 2.5, 5, 10, 20, 50 and 75 μM) for two different durations (48 and 72 h). After incubation, MTS solution was added to the cells according to the instructions provided by the manufacturer and absorbance was measured at 490 nm using an ELISA plate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Morphological evaluation using inverted phase contrast microscopic technique

U87MG human glioma cells were seeded in 24-well plates at a density of 2 × 10^4 cells per well. The cells were treated with varying doses of the drug (0, 5, 20, 50 μM), Dimethyl sulfoxide (DMSO 1.5%) acted as the vehicle control. The cells were incubated for 48 h and the cells were visualized under an inverted phase contrast microscope at 200× magnification (Nikon, Tokyo, Japan).

Fluorescence microscopic study of apoptosis

The apoptosis induced by 5-methoxypsoralen in U87MG human glioblastoma cells was evaluated by fluorescence microscopy using the double staining dye acridine orange/propidium iodide. The U87MG cells were seeded in 6-well plates at a density of 1 × 10^5 cells/well. The cells were treated with varying doses of 5-methoxypsoralen drug (0, 5, 20, 50 μM) for 48 h. Subsequently, the treated and untreated cells were incubated with acridine orange and propidium iodide (20 μg/ml each) for 1 h. The cell morphology was finally examined under a fluorescence microscope (Nikon, Tokyo, Japan) at 400× magnification.

DNA fragmentation analysis

In brief, U87MG human glioblastoma cells were seeded in a 60-mm cell culture plate, incubated for 48 h and then treated with 0, 5, 20, 50 μM of 5-methoxypsoralen for 48 h. Subsequently the U87MG cells were harvested and washed twice with PBS before the pellets were lysed with a DNA lysis buffer for 50 min. The sample was centrifuged
at 12,000 rpm and the supernatant was prepared in an equal volume of 2.5% sodium-dodecyl sulfate, then incubated with 10 mg/ml RNase A for 4 h. After the addition of 10 M ammonium acetate, the DNA was precipitated with cold ethanol and collected by centrifugation at 12,000 rpm for 30 min. Finally, the DNA was dissolved in gel loading buffer, separated by electrophoresis in 3.5% agarose gel, stained with ethidium bromide and examined under UV.

Cell cycle analysis

For this assay, U87MG human glioblastoma cells were seeded in 60-mm plates and treated with 0, 5, 20, 50 μM of 5-methoxypsoralen for 48 h. After drug treatment, the cells were subjected to trypsinization and then washed twice with PBS. The cells were then fixed with 70% cold ethanol overnight and treated with 20 μg/ml RNase A, and then stained with 5 μg/ml of PI. Finally, the DNA content and cell cycle distribution were analyzed with a flow cytometry FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Transmission electron microscopy study for the evaluation of autophagy

A transmission electron microscope (TEM) was used to demonstrate that 5-methoxypsoralen induces autophagy in U87MG human glioblastoma cells. In brief, the cells were seeded in a flask. The cells were then treated with varying doses (0, 5, 20, 50 μM) of 5-methoxypsoralen for 48 h. The cells were harvested and washed with PBS twice before adding 2.5% glutaraldehyde for microtome sectioning using an ultramicrotome (JEOL Co; Japan). TEM analysis was performed using a TEM (JEOL Co; Japan). Autophagy valuation was carried out by examining the appearance of vacuoles and their prevalence in 5-methoxypsoralen-treated cells.

Western blot assay

The fact that 5-methoxypsoralen targets some of the key apoptotic protein signaling pathways was demonstrated using western blot assay. In brief, U87MG human glioblastoma cells were seeded in a 16 cm plate for 24 h. Subsequently, the medium was removed and replaced with fresh medium. The cells were then treated with increasing doses (0, 5, 20 and 50 μM) of 5-methoxypsoralen followed by 48 h incubation. After medium removal, the cells were washed with PBS solution twice before detaching cells and then lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) and protease inhibitor for 30 min. Following centrifugation, the protein content was estimated by the BCA method. The protein lysates (20 μg/lane) were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Millipore, MA, USA). Each membrane was blocked with 5% skim milk, and then incubated with the designated primary antibodies overnight at 4°C.

Statistical analysis

The results are expressed as mean ± SD of three independent experiments. Differences between the control and treatment groups were examined using Student’s t-test with SPSS 17.0 software. A p-value < 0.05 was considered to be statistically significant.

Results

5-Methoxypsoralen exhibits potent anticancer effects in U87MG human glioblastoma cells

5-Methoxypsoralen (5-MOP), also called bergapten, belongs to the furanocoumarin class of natural products (Figure 1). The cytotoxic effects of 5-methoxypsoralen against U87MG human glioblastoma cells were evaluated by MTS cell viability assay. The findings revealed that 5-methoxypsoralen effectively inhibits the growth of U87MG cells. The results are graphically depicted in Figure 2 and show that the drug induced both time-de-
Pendent and concentration-dependent cytotoxic effects. It was observed that a 75 μM dose of the drug led to 92.1% and 99.3% cell growth inhibition at 48 h and 72 h respectively. The potency of the drug was also assessed by calculating the IC₅₀ value, which was found to be 22.7 μM after 48 h incubation time and 12.9 μM after 72 h incubation.

Morphological changes induced by 5-methoxypsoralen in U87MG human glioblastoma cells

The fact that 5-methoxypsoralen induced apoptotic-related morphological changes in U87MG human glioblastoma cells was demonstrated using inverted phase contrast microscopy. Treatment of cells with 5, 20 and 50 μM of 5-methoxypsoralen led to a substantial decrease of cell count along with distorted cell morphology. As compared to the untreated control cells, 5-methoxypsoralen-treated cells at concentrations of 5, 20 and 50 μM resulted in drastic changes in cell morphology including rounding and withering of cells with unsystematic cell layers (Figures 3 A–D).

Apoptotic morphological changes induced by 5-methoxypsoralen in U87MG human glioblastoma cells

To further substantiate that 5-methoxypsoralen does indeed induce apoptotic morphological changes in U87MG human glioblastoma cell, fluorescence microscopy with acridine orange/propidium iodide double staining was used. The findings of this assay are depicted in Figure 4.
which indicates that untreated control cells exhibited normal green fluorescence (green arrows in Figure 4) while 5-methoxypsoralen-treated cells showed orange/red fluorescence (yellow arrows in Figure 4) indicative of the fact that higher doses of the drug led to significant levels of apoptosis. A dose-dependent relationship was observed between the 5-methoxypsoralen concentration to which the cells were subjected and the percentage of apoptotic cells.

5-Methoxypsoralen led to DNA damage in U87MG human glioblastoma cells

DNA damage which was induced by 5-methoxypsoralen in U87MG human glioblastoma cells was examined using gel electrophoresis, which involves the formation of a DNA ladder. The findings of this assay are depicted in Figure 5 and indicate that DNA ladder formation was observed and this effect increased with increasing doses of 5-methoxypsoralen. In the case of the untreated control group, no such DNA laddering was seen, indicating no DNA damage at all. The DNA fragmentation is believed to be a hallmark of the apoptotic process which initiates within the cell, further confirming that the drug led to cell death through inducing apoptosis.

5-Methoxypsoralen induces G2/M cell cycle arrest in U87MG human glioblastoma cells

In this experiment, the effects of 5-methoxypsoralen on the cell cycle progression was examined using flow cytometry. It was observed that 5-methoxypsoralen has a tendency to disrupt the normal cell cycle in these cells. The findings are shown in Figure 6, and reveal that the untreated control cells showed only 14.8% of cells in the G2/M phase of the cell cycle. However, on treating cells with 5, 20 and 50 μM doses of 5-methoxypsoralen, the percentage of G2/M phase cells in-
creased to 19.2%, 47.3% and 68.3% respectively. Thus it can be concluded from this experiment that 5-methoxypsoralen induces apoptosis by inducing G2/M phase cell cycle arrest.

5-Methoxypsoralen led to autophagic cell death in U87MG human glioblastoma cells

Transmission electron microscopy was used to study the effect of 5-methoxypsoralen on autophagic cell death in U87MG human glioblastoma cells. The findings are shown in Figure 7 and indicate that as compared to the untreated control group (Figure 7 A), which showed normal ultrastructural cellular morphology with no signs of vacuoles, 5-methoxypsoralen-treated cells exhibited altered cell ultrastructure with the appearance of autophagic vacuoles. The number of these autophagic vacuoles also increased with increasing doses of the drug (Figures 7 B–D).

5-Methoxypsoralen led to downregulation of m-TOR/PI3K/AKT signaling cascade

The m-TOR/PI3K/Akt signaling pathway is considered to be one of the decisive signaling pathways imitated in cancer cells. To examine whether 5-methoxypsoralen could curb the protein expression of the m-TOR/PI3K/Akt signaling cascade, we carried out western blotting analysis. The results
Figure 7. 5-Methoxypsoralen induces autophagic cell death in U87MG human glioblastoma cells. The cells were treated with 0, 5, 20 and 50 μM doses of 5-methoxypsoralen for 48 h and then analyzed using a transmission electron microscope at 8000× resolution. Arrows indicate autophagic vacuoles in Figure 8 and indicate interesting observations. In comparison to the untreated control cells, 5-methoxypsoralen administered cells exhibited concentration-dependent inhibition of m-TOR and pm-TOR proteins. It also caused inhibition of PI3K/Akt protein expression. Therefore, it may be suggested that 5-methoxypsoralen incited anticancer action via modulation of the m-TOR/PI3K/Akt signaling pathway.

Discussion

5-Methoxypsoralen (bergapten) is a natural product and belongs to the coumarin class of compounds. These compounds show anticancer property against a wide range of cancer cells [8]. Interest in the field was increased by the reports of Thornes, who has reported the immunomodulatory activity and utility in malignant melanoma of coumarin [16]. It has been reported that coumarins led to the significant inhibition of bladder cancer cells, muco-epidermoid carcinoma [17], mammary cancer cell [18] and human melanoma cell lines [19], showing their promise in clinical treatments. Adhesion and motility of neoplastic cells were affected by some of the naturally occurring coumarins. This aspect has been well elucidated in the highly invasive murine melanoma cell line B16–F10. In the maintenance of proliferation and survival signals complex interactions are involved, e.g. between epidermal growth factor (EGF), ER-α and polypeptide growth factors such as IGF-I, transforming growth factor (TGF)-α and TGF-β [20]. The activation of the transduction pathways by psoralens in the target cells was not known until a few years ago, but the growing interest in this field led to the identification of the main signals by which they exert their an-
Antitumor and apoptotic effects of 5-methoxypsoralen in U87MG human glioma cells and its effect on cell cycle, autophagy and PI3K/Akt signaling pathway

Figure 8. 5-Methoxypsoralen led to dose-dependent inhibition of m-TOR/PI3K/Akt signaling pathway proteins. The effect of the drug on expression of these proteins was evaluated by western blot assay.

5-methoxypsoralen dose [µM]

- PI3K
- p-PI3K
- AKT
- p-AKT
- mTOR
- p-mTOR
- Actin

The authors declare no conflict of interest.

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