

Cytotropic heterogeneous molecular lipids inhibit the growth of glioma cells by inducing apoptosis and autophagy

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Abstract: The cytotropic heterogeneous molecular lipid (CHML) is a mixture of lipids isolated from natural products. CHML is an effective therapy for various kinds of cancers; however, the effect of CHML on glioma cells was seldom reported. Here, we aim to explore the cytotoxicity of CHML on glioma cells, and analyze the possible mechanisms. U251 glioma cells were cultured with CHML at different concentration, and the growth inhibition was measured by CCK-8 assay. Induced apoptosis were detected by flow cytometry, and the induced autophagies were observed by a transmission electron microscope. The key molecules involved in apoptosis and autophagy were detected by quantitative PCR and western-blot. CHML might inhibit the growth of U251 cells and promote apoptosis by up-regulating the expressions of Caspase-8 and Caspase-3; CHML also induced autophagy of U251 cells by promoting the expressions of MAP LC-3 and Beclin-1. CHML can inhibit proliferation of U251 cells by promoting cell apoptosis and inducing autophagy.

Keywords: CHML, glioma, apoptosis, autophagy.

INTRODUCTION

Gliomas are the most common primary brain tumor and are among the most challenging tumors for neurosurgeons. So far, the therapeutic effect of glioma is still not satisfied, even combining surgery, radiotherapy and chemotherapy, with a median survival times being less than 15 months from time of diagnosis (Stupp *et al.*, 2005). Thus, there is increased interest in finding new agents for glioma treatment, because of the lack of extraordinarily effective treatment (Stewart, 2002, Babcock *et al.*, 2008, Packer and Vezina, 2008, Ullrich *et al.*, 2008).

CHML is the abbreviation of cytotropic heterogeneous molecular lipids; here, the cytotropic means that the lipids have an affinity for cytological cells, especially for the membrane structure of cancer cells. "Heterogeneous" indicates the similarity of biological properties as lipids of the membranes of cancer cells. Molecular lipids suggest the molecular size of the lipids.

All the components of CHML are isolated from natural products and prepared by lipid-activated methods. CHML mainly consists of unsaturated fatty acids and its phospholipid composition facilitates penetration through the cellular plasma membrane (Chen *et al.*, 2007). Thus, CHML has active orientation to penetrate into the membrane of cancerous cells and has relatively high idiosyncratic absorbing capacity by target cells. Results showed that CHML, as a biological molecular missile, can easily penetrate through the target cancerous cells to perform programmed cell death (PCD). It has been reported that CHML is an effective therapy for hepatocellular carcinoma (Chen *et al.*, 2007), breast

carcinoma (Zhan *et al.*, 2001), colorectal carcinoma, lung cancer (Zhan and Xu, 1999), *et al.* Our previous research showed that CHML could induce apoptosis on U87 glioma cell line (Zhou *et al.*, 2014) and later we confirmed the induced apoptosis of CHML on U251 glioma cell line. Moreover, we also found that CHML could induce autophagy in U251 cells. For the effect of CHML on glioma was seldom published in English literatures, here, we reported that CHML could inhibit the proliferation of glioma cells by not only enhancing cellular apoptosis and but also inducing autophagy.

MATERIALS AND METHODS

Cell line and reagents

The U251 human glioma cell line (purchased from the American Type Culture Collection, Shanghai, China) was cultured in RPMI-1640 (Gibco, Shanghai, China) containing 10% fetal bovine serum (Hyclone, Shanghai, China). The real-time quantitative PCR kit was Sso Fast Eva Green Supermix, from Fermentas (USA). The CCK-8 kit for cytotoxic effect study was bought from Sigma Co. (Shanghai, China). The Annexin V-PE/7-AAD kit for apoptosis detection was products of BD Biosciences (Shanghai, China). The antibodies against Caspase-8 and Caspase-3 were bought from Cell Signaling (Shanghai, China), and antibodies against MAP-LC3, Beclin-1 and β -actin were purchased from Santa Cruze (USA).

CCK-8 assay

The *in vitro* cytotoxic effect of CHML on U251 glioma cells was evaluated by CCK-8 assay. First, the U251 glioma cells were seeded into a 96-well culture plates with a cellular density of 1×10^4 cells/well. Followed by

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24 hours' incubation at 37, then cells were treated with CHML at different concentrations (0 μ g/ml, 50 μ g/ml, 100 μ g/ml, 150 μ g/ml and 200 μ g/ml). Seventy-two hours after treatment, the viability of U251 glioma cells was detected with CCK-8 kit based company's protocol. Moreover, we calculated the inhibition ratios by the formula: Inhibition ratio= (OD₄₄₀ of control group- OD₄₄₀ of experimental group)/ OD₄₄₀ of control group.

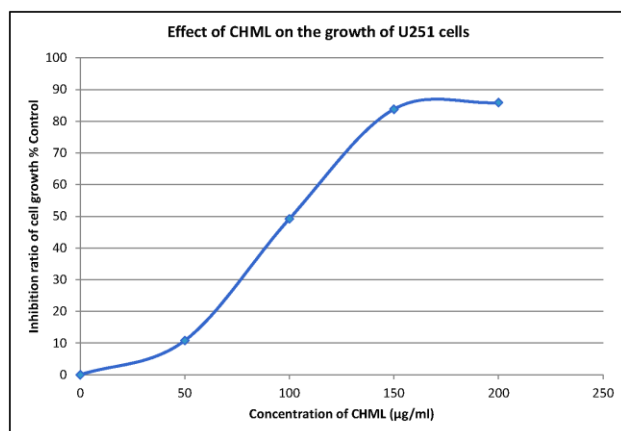


Fig. 1: the *in vitro* cytotoxic effect of CHML on U251 cells was detected by CCK-8 assay.

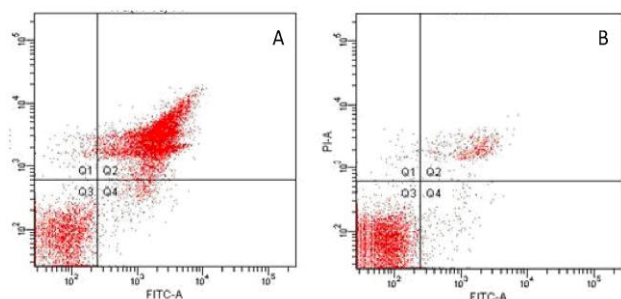


Fig. 2: Apoptosis of U251 cells. (A) U251 cells were treated with CHML (150 μ g/ml), the sum of apoptosis ration in Q2 and Q4 was 62 $\pm$ 4.16%; (B) in the control group, the sum of apoptosis ration in Q2 and Q4 was 5.7 $\pm$ 0.9%; which was significantly lower than that of experiment group, $p < 0.05$, $n = 5$ replicates/condition.

Flow cytometric analysis of apoptosis

The U251 glioma cells (1×10^6) in experiment group were treated with CHML (150 μ g/ml) or cells in control group were treated with medium only. Forty-eight hours later, treatments were terminated, and cells were rinsed in cold PBS. Then apoptosis in both groups were detected by flow cytometric analysis with Annexin V-PE/7-AAD kit. Briefly, 1×10^6 cells in either group were incubated with 5 μ l Annexin V-PE (as the early apoptotic marker) and 5 μ l 7-AAD (as the late apoptotic marker) in 100 μ l of $1 \times$ Annexin V binding buffer at room temperature for 15min. Then, 400 μ l of $1 \times$ binding buffer was added and the apoptotic cells were analyzed by a flow cytometry.

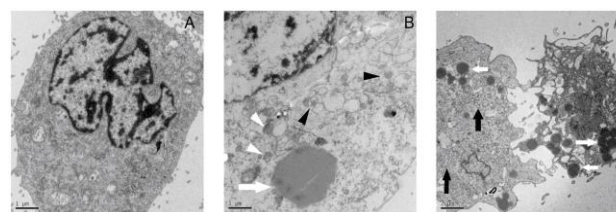


Fig. 3: the ultra-structural characteristics of U251 cells under a TEM. A, the ultra-structure of U251 cells in the control group seems to be intact (bar=1 μ m). B, the cytoplasm of U251 cells treated with CHML was swelling and lightly stained. A white arrow showed the liposome-like structure; the white arrowheads showed denatured mitochondria, and black arrowheads showed autophagosomes (bar=1 μ m). C, showed two U251 cell in experiment group. There were plenty of liposome-like structures in either cell (white arrows). The left cell was lightly stained with swelling RER (black arrows); the right one was darkly stained with a shrunken appearance, where the cell body began to disintegrate, (bar=2 μ m).

Transmission electron microscope (TEM)

U251 cells in both groups were treated at 4 in sodium cacodylate buffered (0.1M, pH 7.4) 5% glutaraldehyde fixative for 2 hours. Cells then were rinsed twice in PBS, postfixed with 1% PBS-buffered osmium tetroxide for half an hour, and dehydrated with a series of graded ethanol. During dehydration, cells were stained with a saturated uranyl acetate solution in 70% ethanol, and then were embedded for the ultra structural study. Ultrathin sections were prepared by a diamond knife and stained by lead citrate for 8min. Grids were examined under a JEOL-1230 TEM (Tokyo, Japan).

Real-time Quantitative PCR

U251 cells in either group were treated for 48 hours, then total RNA was extracted with Trizol and cDNA was generated. The forward and reverse oligo-nucleotide primers, specific to Caspase-8, Caspase-3, MAP LC3, Beclin-1 and GAPDH (as control) were mixed with cDNA. PCR cycles duration temperature was 10 min at 95 $^{\circ}$ C, then followed by 50 rounds of 10 s at 95 $^{\circ}$ C for and 30 s at 60 $^{\circ}$ C. The amplification specificity was analyzed by melting curves. Threshold cycle (Ct), which correlated inversely with the candidate mRNA levels, was calculated using the second derivative maximum algorithm. The mRNA levels of each target molecule were normalized to the level of GAPDH mRNA.

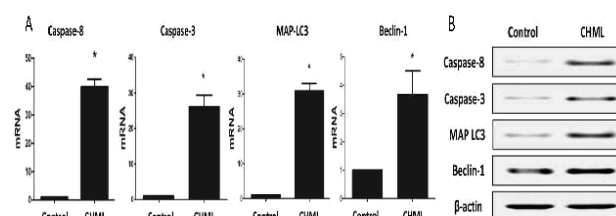


Fig. 4: CHML modulates apoptosis and autophagy related molecules. A, The transcriptions of Caspase-8, Caspase-3,

MAP LC3, and Beclin-1 in U251 cells were detected by Real-time quantitative PCR. * $p < 0.05$ compared with control group, $n = 3$ replicates per condition. B, The translations of Caspase-8, Caspase-3, MAP LC3, and Beclin-1 in U251 cells were detected by western blot. β -actin was kept as the control. The results showed obvious up-regulations of these molecules in U251 cells treated by CHML.

Western blot analysis

Cells were washed with cold PBS and lysed in lysis buffer containing 10mM Tris-HCl (pH 7.4), 150mM NaCl, 1% Triton X100, 1% sodium deoxycholate, 0.1% SDS, 5mM EDTA, 1mM PMSF, 0.28ku/L aprotinin, 50mg/L leupeptin, 1mM benzamidine, and 7mg/L pepstatin A. Protein concentration was determined using a BCA kit (Pierce). Total cellular lysates (30mg per sample) was subject to electrophoresis on 10% SDS PAGE gel using a constant current, and subsequently transferred onto nitrocellulose membranes; Blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with a primary antibodies specific for Caspase-8, Caspase-3, MAP-LC3, Beclin-1, and β -actin in blocking solution for three hours. Membranes were washed and followed by incubation with horseradish peroxidase conjugated second antibody in TBST containing 3% non-fat dry milk for another 1 hour. Immunological activity was detected with enhanced chemo luminescent autoradiography according to the manufacturer's instructions. The signal intensity of primary antibody binding was quantitatively analyzed with Sigma Scan Pro 5 and was normalized to a loading control β -actin. Statistical analysis was carried out by paired t-test.

STATISTICAL ANALYSIS

All data are presented as the means $\pm$ standard deviation. Significant difference was evaluated by t-test between two samples. A value of $p < 0.05$ was considered as statistical significance.

RESULTS

Inhibition of cell growth by CHML

To test the effect of CHML on U251 glioma cells, CCK-8 assay were performed. The *in vitro* U251 cells viability was examined 3 days after treatment with CHML. Compared with control group, CHML could inhibit the growth of U251 cells by a dose-dependent manner. In addition, when the CHML concentration was 150 μ g/ml, the inhibition reached the plateau stage. Therefore, in the following experiments, we kept the concentration of CHML being 150 μ g/ml in experiment group (fig. 1).

Induced apoptosis by CHML

To explore the mechanism by which CHML inhibits U251

cells growth; the apoptosis of U251 cells in either group were analyzed using Annexin V-PE (early apoptotic marker) and 7-AAD (late apoptotic marker) double staining by flow cytometry. As shown in fig. 2, CHML (150 μ g/ml) treatment resulted in 62 $\pm$ 4.16% of U251 cells apoptosis, whereas there was only 5.7 $\pm$ 0.9% apoptotic U251 cells occurring in control group.

Induced autophagy by CHML

The inhibition ratio of CHML on U251 cells was higher than the ratio of induced apoptosis, there may be some other mechanisms leading to the growth inhibition from CHML. To answer this question, we detected the ultra-structure changes of U251 cell before and after treatment with CHM. As shown in fig. 3, under a TEM, the ultra-structure of U251 cells in control group was intact. The morphological structures of organelles, such as rough endoplasmic reticulum (RER) and mitochondrion were normal. As well, there were no obvious pyknosis and chromatin condensation (fig. 3A). However, the ultra-structures of U251 cells treated with CHML were different. In cytoplasm, there were plenty of liposome-like structures, which factually were the aggregates of particulate CHML, with diameters being 0.4 μ m-2 μ m (generally being 0.8 μ m). Some cells were swelling, where the electronic density of cytoplasm decreased, with expanded RER, denatured mitochondria, and even lots of autophagosomes (fig. 3B, C). Some cells were shrunken, where cytoplasm, containing abundant liposome-like structures, began to disintegrate, with chromatin pyknosis and deeply stained nuclei (fig. 3C).

Molecular mechanisms

In order to make clear the molecular mechanisms for enhanced apoptosis and induced autophagy, we detected the transcriptions of some key-molecules for either apoptosis or autophagy by Real-time quantitative PCR, and the translations of those molecules by western blot. The results showed that both Caspase-8 and Caspase-3 had increase transcriptional and translational expressions; in addition, the transcriptions and translations of both MAP-LC3 and Beclin-1 were enhanced significantly (fig. 4).

DISCUSSION

Cytotropic heterogeneous molecular lipid (CHML), a mixture of lipids isolated from natural products, was developed as an anticancer drug by Glory F & D Co. Ltd, (USA). Our CCK-8 assay results showed that CHML could inhibit the growth of U251 cells by a dose-dependent manner (fig. 1). Then, what are the mechanisms leading to the growth inhibition? According to the biochemical characteristics of CHML and previous research, we first believed that PCD probably is the underlying reason. PCD, referring to apoptosis and autophagy, is proposed to be cell death in any

pathological format mediated by an intracellular program. Sometimes, programmed necrosis is also regarded as a type of PCD. Then we detected apoptosis in both control group and experiment group using Annexin V-PE and 7-AAD double staining by flow cytometry. Our results confirmed a significantly enhanced apoptosis in U251 cells treated with CHML (fig. 2).

Apoptosis is also considered as type I PCD. There are two pathways leading to apoptosis, i.e. the extrinsic-death receptor pathway and intrinsic- mitochondrial pathway (Eum and Lee, 2011). The activation of extrinsic pathway will finally activate pro-caspase-8, which proceeds to trigger pro-caspase-3, the penultimate enzyme for execution of the apoptotic process (Kerr *et al.*, 1972). The intrinsic pathway will lead to activation of Caspase-9 (Wen *et al.*, 2012), which then activates Caspase-3, subsequently activating the downstream Caspase cascade finally generating apoptosis (Muzio *et al.*, 1996). Being cysteine proteases, Caspases family members have an important role in the apoptotic process, among which, Caspase-3 is the key enzyme and a major effector. In our study, we detected the expressions of both Caspase-8 and Caspase-3, and our results showed there were significantly enhanced expressions of both molecules in U251 after CHML treatments. Therefore, CHML could promote U251 glioma cells' apoptosis by triggering both the extrinsic and intrinsic pathway.

When we found that the inhibition ratio of CHML on U251 cells was higher than the ratio of induced apoptosis, we suspected autophagy may involve in the growth inhibition from CHML. Autophagy, or type II PCD, is a process of protein degradation and turnover of the destroyed cell organelles, which were wrapped in double or multimembrane autophagic vesicles and delivered to their own lysosomal system (Gozuacik and Kimchi, 2004). In general, autophagy plays a crucial pro-survival role in homeostasis when there is deprivation of nutrients and/or growth factors. (He and Klionsky, 2009). However, it was reported that autophagic cells may commit suicide by undergoing cell death when under excessive stress (Eisenberg-Lerner *et al.*, 2009, Bialik *et al.*, 2010).

Beclin-1/PI3K-III complex is involved in the formation of autophagosomes and initiation of autophagy (Blommaert *et al.*, 1997). Thus the expression level of Beclin-1 may indicate the activity of autophagy. Nowadays, MAP-LC3 is regarded as a specific marker of autophagosomes (Kabeya *et al.*, 2000, Mizushima *et al.*, 2001, Munafo and Colombo, 2001, Kanzawa *et al.*, 2004). However, the ultra structure study by TEM is the most convincing and standard method to detect autophagy (Gozuacik and Kimchi, 2004). Here, we examined the ultra structure of U251 cells, together with the detection of the expressions of both Beclin-1 and MAP LC3 by Real-time PCR and western blot. Our results showed that the autophagic

activity in U251 cells increased significantly after CHML treatments. Therefore, CHML could also inhibit the growth of U251 glioma cells by the induction of autophagy.

However, are there intricate interrelationships or crosstalk between the apoptosis and autophagy in glioma cells after treatment of CHML? Can apoptosis and autophagy exert synergetic effects, or conversely? These questions remain further study in the future.

CONCLUSION

CHML can inhibit proliferation of U251 cells by promoting cell apoptosis and inducing autophagy.

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