Synthesis and biological analysis of a new curcumin analogue for enhanced anti-tumor activity in HepG 2 cells

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Abstract. The aim of the present study was to investigate the apoptosis of human hepatocellular carcinoma cell line HepG 2 induced by a new curcumin analogue, GL63. HepG 2 cells were treated with increasing doses of GL63 and curcumin for 48 h. The proliferation of cells was detected with MTT. The apoptosis were examined by flow cytometry. The caspase-3 activity was detected by Western blotting. ER calcium stores were assessed by the fluorescent calcium indicator fura-2/AM. The protein expression of ER stress pathway, GRP78, XBP-1, ATF-4 and CHOP were examined with Western blotting. Growth inhibitory effect was observed for treatment with GL63 in a dose-dependent manner and with more potential than curcumin. GL63 at 20 μM induced significant apoptosis in HepG 2 cells. Furthermore, GL63 induced the ER stress response, up-regulation of CHOP, XBP-1, ATF-4 and GRP78 expression in a dose-dependent, while curcumin had no effect on ER stress. These results suggest that GL63 has more potent anti-tumor activity than curcumin, which is associated with activation of ER stress and induction of apoptosis in HepG 2 cells.

Introduction

Hepatocellular carcinoma is one of the most common cancers worldwide, and it has become the first among malignant tumors in China (1). The outcomes of current available chemotherapy drugs are poor, development of innovative new therapeutics for the management of liver cancer is especially urgent and this development is the long-term objective of this current project.

An increasing amount of attention has been paid to the use of complementary and alternative medicine as a part of the treatment for various cancers and the complications associated with current therapies (2). Curcumin, is a well-known food additive and constituent of traditional medicine, which has long been used to treat various inflammatory diseases in China and India (3). It has a surprisingly wide range of beneficial properties, including anti-inflammatory, antioxidant, chemopreventive and chemotherapeutic activity and has been used to treat different cancer types including liver cancer in clinical trials (4). However, the exact molecular mechanisms involved in the apoptotic effect of curcumin remain to be identified. A number of studies suggest that multiple mechanisms may be involved in different cancer cell lines (5-7). Recently, it has been reported that curcumin exerts its pro-apoptotic effect by inducing endoplasmic reticulum stress (ER stress) in human leukemia HL-60 cells (8).

Although curcumin is remarkably non-toxic and has promising anti-cancer activities, preclinical and clinical studies indicate that its poor bioavailability and pharmacokinetic profiles due to its instability under physiological conditions have limited its application in anti-cancer therapies (9-11). Great effort has been put into the chemical modification of curcumin to identify potential analogues with better bioavailability and anti-tumor activities (7,12-16). We also have designed and synthesized a new curcumin analogue, GL63. HepG 2 cells were treated with increasing doses of GL63 and curcumin for 48 h. The proliferation of cells was detected with MTT. The apoptosis were examined by flow cytometry. The caspase-3 activity was detected by Western blotting. ER calcium stores were assessed by the fluorescent calcium indicator fura-2/AM. The protein expression of ER stress pathway, GRP78, XBP-1, ATF-4 and CHOP were examined with Western blotting. Growth inhibitory effect was observed for treatment with GL63 in a dose-dependent manner and with more potential than curcumin. GL63 at 20 μM induced significant apoptosis in HepG 2 cells. Furthermore, GL63 induced the ER stress response, up-regulation of CHOP, XBP-1, ATF-4 and GRP78 expression in a dose-dependent, while curcumin had no effect on ER stress. These results suggest that GL63 has more potent anti-tumor activity than curcumin, which is associated with activation of ER stress and induction of apoptosis in HepG 2 cells.

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Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; Cur, curcumin

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(1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one (GL63) and compared the effect of GL63 and curcumin in preclinical model systems using liver cancer cell lines (HepG 2) at several doses on cell growth, apoptosis and protein expression on ER stress pathway. We demonstrated that GL63, as a new curcumin analogue, is more active than curcumin in the inhibition of HepG 2 cells proliferation and induction of apoptosis. GL63 appears to be more potent than curcumin in anti-tumor activity and has more stability in pharmacokinetic profiles. Therefore, the synthetic derivative of curcumin GL63 has potential as a new therapeutic agent for hepatoma.

Materials and methods

Synthesis of GL63. The general procedure of synthesis of GL63 was as follows. An amount of 7.5 mmol acetone was added to a solution of 15 mmol arylaldehyde in MeOH (10 ml). The solution was stirred at room temperature for 20 min, followed by dropwise addition of NaOCH 3/CH 3OH (1.5 ml, 7.5 mmol). The mixture was stirred at room temperature and monitored with TLC. When the reaction was finished, the residue was poured into saturated NH 4Cl solution and filtered. The precipitate was washed with water and cold ethanol, and dried in vacuum. The solid was purified by chromatography over silica gel using CH 2Cl 2/CH 3OH as the eluent to yield compounds.

Treatment of HepG 2 with GL63 and curcumin. Hepatocellular carcinoma cell line HepG 2 obtained from American Type culture Collection was grow in DMEM medium, containing 10% fetal bovine serum 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO 2. GL63 and curcumin were dissolved in DMSO. The cells were treated with various concentrations (5, 10 and 20 μM) of GL63 and curcumin for 48 h. Control cultures were treated with DMSO and were processed similarly.

MTT assay. HepG 2 cells and rat primary culture hepatocytes were grow in a 96-well plate for 24 h and then treated with different doses (5, 10 and 20 μM) of GL63 and curcumin for 48 h, followed by the MTT treatment (5 mg/ml) to the cells in each well for 4 h at 37˚C as described earlier. MTT was aspirated and 100 μl of DMSO was added to each well and absorbance at 570 nm was read in a plate reader. Each treatment was done in triplicate. Mean of three values was determined and the results are expressed as percent of control.

Apoptosis rate analysis. HepG 2 cells were grow for 24 h in a 6-mm plate and then treated with varying doses (5, 10, 20 μM) of GL63 and curcumin for 48 h. Cells were washed by PBS 3 times, then digested by 0.25% trypsin-EDTA. After centrifugation, cells were resuspended in 0.5 ml PBS. Cells were then stained with Annexin V and propidium iodide (PI) in the presence of 100 mg/ml RNAse and 0.1% Triton X-100 for 30 min at 37˚C. Flow cytometric (Becton-Dickinson, Mountain, CA, USA) analysis was performed using a fluorescence-activated cell sorter.

Assay of endoplasmic reticulum calcium pools. HepG 2 cells were grow on 22x40-mm coverslips and treated with vehicle, curcumin and GL63 for 12 h. The cells were loaded with 4 μM fura-2 AM and 0.3% plurinic F-127 in HBSS at 37˚C for 120 min and incubated in HBSS for an additional 30 min, then mounted on the stage of an Axioskop 2 plus upright fluorescence microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a x40 objective. After washing with HBSS without Ca 2+ and Mg 2+ three times, cells were stimulated with thapsigargin (TG, 100 nm; San Diego, CA, USA). Fluorescence images before and after addition of TG were collected at 15-sec intervals through a cooled CCD camera (TILL Photonics LLC, Martinsreid, Germany) which is attached to an image intensifier, an epifluorescent light source, a 515-nm dichroic beam splitter, and a 535-nm emission filter. The 340:380 ratios of individual cells in these images were analyzed using TILLvisION version 3.1 imaging software.

Western blotting. Caspase-3, GRP78, CHOP, XBP-1, ATF-4, Lamin B and Actin expression were examined by Western
blot assay based on previous reports (8). Briefly, Cells were lysed, supernatants were collected, and proteins were resolved on Tris-HCl polyacrylamide gels at 120 V. The proteins were transferred to a polyvinylidene difluoride (PVDF) blotting membrane, and the membranes were probed with rabbit polyclonal antibody (Santa Cruz, CA, USA). Details of the secondary antibodies used and their detection by chemiluminescence have been previously described (8). Equal loading was verified by determining protein concentration by Bio-Rad protein assay (Bio-Rad) and using the same amount of protein from protein lysates for electrophoretic analysis. ß-actin was used as standard, and the ratio between the analyzed protein and ß-actin from quantitative densitometric analysis was used for comparison of control and experimental samples.

Statistical evaluation. All assay were performed at least three times and levels of the parameters measured were expressed as mean ± standard deviation (SD). Statistical comparisons between cells treatment with GL63 and curcumin versus control were based on the t-test.

Results

GL63 inhibits cancer cell viability more potently than curcumin. We determined the effect of GL63 and curcumin on cell proliferation in HepG 2 cells. GL63 significantly suppressed proliferation of HepG 2 cells dose-dependently in a 48-h treatment (Fig. 2A). In contrast, curcumin, even in 20 μM, showed 86% cell viability after 24 h. To test the toxicity of curcumin analogues, rat primary culture hepatocytes were treated with GL63 and curcumin with 20 μM, neither GL63 nor curcumin affected the proliferation (Fig. 2B).

GL63 is more potent than curcumin in inducing apoptosis. At a sufficiently high dose, curcumin induces apoptosis of many cancer cells including liver cancer cells. We assessed the effect of GL63 and curcumin on the induction of apoptosis in HepG 2 cells by flow cytometry. The results (Fig. 3A and B) showed that GL63 dose-dependently increases cells apoptotic rate after 48 h treatment, GL63 at 20 μM significantly induce apoptosis (30%) compared with curcumin (13%). Caspase-3 is a key effector molecule in the apoptosis pathway involved in
amplifying the signal from initiator caspases, such as caspase-8 and -12. Increased activation of caspase-3 was observed within 24 h in HepG2 cells treated with 20 μM GL63, however, curcumin did not induce caspase-3 activation (Fig. 3C). These data suggest that GL63 is a potent inducer of apoptosis even at low dose where no such effect is observed with curcumin.

Depletion of ER calcium stores by GL63. Depletion of luminal ER calcium stores is believed to reflect ER stress, which can promote induction of ER stress. To investigate the possible mechanisms of curcumin and GL63 induced ER stress and activation of the unfold protein response (UPR), we assessed the effect of GL63 and curcumin on ER calcium stores in HepG2 cells with the fluorescent calcium indicator fura-2/AM. After treatment with different concentration of curcumin and GL63, for 12 h, cells were loaded with fura-2/AM and switched to calcium-free medium. ER calcium release induced by 100 nM thapsigargin was recorded by fluorescence microscopy. Treatment of HepG2 cells with GL63 decreased the ER calcium content in a dose-dependent manner. Cells treated with 20 μM GL63 remarkably reduced the response to TG, which suggested that ER calcium stores were depleted (Fig. 4B). However curcumin even at 20 μM had similar effects on ER calcium stores (Fig. 4A).

Activation of the ER stress pathway by GL63. Recently, a novel pathway of cell apoptosis via ER stress has been reported. Inductions of the downstream transcription factors, GRP78, XBP-1, ATF-4 and CHOP, are markers for activation...
of the UPR. In this study, we compared the effect of GL63 and curcumin on the UPR activation in HepG 2 cells. The results indicated that even though GL63 and curcumin inhibited cell growth, only GL63 induced the ER stress response in HepG 2 cells and the earliest response was observed at 6 h of treatment. As shown in Fig. 5, GL63 significantly induced the expression of GRP78, CHOP, ATF-4, and XBP-1 in a dose-dependent manner in HepG 2 cells. However, curcumin had no effect even at 20 μM concentration. These results suggest that B19-induced UPR activation may represent a major cellular mechanism of its anti-cancer activity.

Discussion
Because curcumin is poorly absorbed through the gut, more potent and soluble curcumin analogues have been developed (9). In our previous study (17-20), a series of monocarbonyl analogues of curcumin was designed by the deletion of the highly reactive ß-diketone moiety in the structure of curcumin which is considered to be responsible for the in vitro instability and the in vivo pharmacokinetic disadvantages. In this study, we also showed a new curcumin analogue GL63 significantly inhibit hepatocellular carcinoma cells proliferation and induce cell
apoptosis, but curcumin at the same doses did not have a therapeutic effect.

Synthetic chemical analogues to molecularly targeted chemotherapeutic drugs are a common way to develop new drugs. These events include the acquisition of self-sufficient growth signals, insensitivity to signals that usually inhibit proliferation, use of a survival pathway to evade apoptosis, initiation of angiogenesis to ensure sufficient oxygen and nutrient significantly higher levels of apoptosis. There are several curcumin analogues that have already been reported, such as dimethoxycurcumin and EF-24 (21-23). We compared the inhibitory efficacy of GL63 and curcumin in HepG2 cells. We demonstrated that GL63 are more potent than curcumin in the inhibition of cell viability in HepG2 cells studied. Curcumin has been found to be safe in clinical trials and dose-limiting toxicity was not observed in many studies. We also observed that neither GL63 nor curcumin induced apoptosis in normal liver cells, which indicates that GL63 has no significant toxicity, similar to curcumin. Further, it will be necessary to evaluate the possible toxicity of GL63 comparing to curcumin in an animal model to verify the toxicity in vivo.

In this study it was also found that GL63 induced apoptosis in HepG2 cells. GL63 is also more potent than curcumin in the induction of caspase-3 and -9, evidence of apoptosis. Two different pathways, the death receptor pathway and the mitochondrial pathway play major roles in regulating apoptosis in cancer cells. The caspase family includes critical mediators of apoptosis. The death receptor pathway and the mitochondrial pathway initially start by activation of caspase-8 and -9, respectively. Both the activated caspase-8 and -9 activate caspase-3 and finally induce apoptosis. In the present study, it was found that caspase-3 was activated in HepG2 cells treated with GL63, while curcumin had no significant effect. This suggests that GL63 might induce apoptosis via a pathway that is mediated by the caspase signal pathway.

A recent study found that intracellular organelles, including the ER, promote cell apoptosis signals. The UPR is an intracellular signaling pathway, which regulates the accumulation of unfolded or misfolded proteins in the ER and plays an important role in regulating cell growth, differentiation and apoptosis (24-26). Therefore, the possibility that GL63 induces apoptosis via ER stress was examined. CHOP is a typical ER stress-regulated protein involved in ER stress-induced apoptosis. Our results on CHOP induction by GL63 suggest that GL63 may trigger ER stress. Indeed, GL63 also increased the levels of XBP-1, ATF-4, GRP78, all of which are additional proteins increased during ER stress. However, curcumin at the same concentration had no effect on GRP78, CHOP, ATF-4 and XBP-1, suggesting that curcumin-inhibited hepatocellular carcinoma growth does not involve ER stress. Although some studies showed curcumin-induced ER stress in human leukemia HL-60 cells, murine melanoma B16-F10 cells, and lung cancer cells A549 (8,27,28), the concentration of curcumin was higher than in our studies.

The ER is a principal site of the synthesis for protein, sterols, cholesterol, and other lipids. Maintenance of ER calcium homeostasis is essential for many cellular functions. Perturbation of ER calcium homeostasis is expected to induce ER stress. Thapsigargin depletes the ER calcium stores and activates the UPR in many mammalian cells (29). We assessed the effect of GL63 on the mobilization of Ca\(^{2+}\). Treatment with GL63 resulted in a rapid increase in cytosolic calcium levels as early as 12 h after GL63 in HepG2 cells.

In conclusion, our studies show that the new monocarbonyl analogue of curcumin, GL63 treatment, of NSCLC cancer cells results in growth inhibition in vitro. Our studies in combination with the observation that GL63 did not affect proliferation of normal liver cells strongly suggest that GL63 has potential for use as a therapeutic or chemo-preventative agent for liver cancer. GL63 activation of ER stress may contribute to the anti-cancer activity. The enhanced potency and better pharmacokinetic profiles renders it a strong candidate for therapeutic applications for hepatocellular carcinoma, possibly also other cancers.

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