

Mechanism of curcumin analog MHMD-induced cell death in A549 lung cancer cells

G.-Z. ZHOU, F.-K. CAO, J.-M. CHANG¹, G.-C. SUN², X.-B. CHEN³

College of Bioengineering, Henan University of Technology, Zhengzhou, China

¹The 3rd Affiliated Hospital of Henan Xinxiang Medical College, Xinxiang, China

²College of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou, China

³The Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China

Abstract. – **OBJECTIVE:** To investigate the anticancer properties of a chemosynthetic curcumin analog, (1E,6E)-4-((furan-2-yl)methylene)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (C₂₆H₂₂O₇, abbreviated MHMD) in A549 cells.

MATERIALS AND METHODS: Inverted microscope was used to observe the alteration on cytomorphology. MTT assay was used to detect cell viability. Acridine-orange staining was used to measure autophagy, and AnnexinV/PI staining and Hoechst/PI staining to measure apoptosis and necrosis.

RESULTS: MTT assays showed that at 12 h, 24 h, 48 h, MHMD reduced cell viability with an IC₅₀ of 27.46 μM, 18.86 μM, and 11.23 μM, respectively. Typical characteristics were observed in concert with cell death, including treated-cells getting brighter, rounder, and becoming non-adherent gradually. Additionally, acridine-orange staining suggested that autophagy didn't involve in MHMD-induced cell death. However, apoptosis and necrosis played important roles in MHMD-induced cell death by Hoechst33342/PI staining. It showed apoptosis was the main cause at low concentrations (≤ 4 μM), while with the concentrations rising, necrosis was the leading role. AnnexinV/PI staining again indicated the occurrence of apoptosis at 4 μM. Furthermore, the caspases inhibitor z-VAD-fmk could prevent MHMD-induced cell death, which showed much higher cell viability than those only treated with MHMD (4 μM). Moreover, MTT assay also demonstrated that MHMD did possess a greater anti-proliferative ability than curcumin.

CONCLUSIONS: The curcumin analog MHMD is able to induce A549 cell death in a time and dose-dependent manner via apoptosis and necrosis. And MHMD could be a more effective drug than curcumin.

Key Words:

Anticancer, Apoptosis, Necrosis, Curcumin analog.

Introduction

Curcumin, also termed diferuloylmethane, a yellow-colored polyphenol extracted from the rhizome of *Curcuma longa*, was a dietary constituent with the demonstrated capability of anti-carcinogenic through diverse mechanisms¹. Studies for curcumin have made it a charming candidate for cancer therapies recently. Curcumin is closely related to programmed cell death. Apoptosis is a major mode of cell death induced by curcumin, which includes the changes in morphological and biochemical in the cellular consisting of DNA fragmentation, as well as caspases activity and subsequent PARP cleavage; initiating mitochondrial pathways and releasing of cytochrome c^{1,2}. Anti-apoptotic proteins including Bcl-2, Bcl-xL, XIAP and survivin, PI3K-AKT activation, as well as the transcription factor NF-κB are inhibited under the effect of curcumin¹. Curcumin had been shown to induce autophagy by inhibiting the Akt/mTOR/p70S6K pathway and activating ERK1/2 Signaling Pathway³. Nevertheless, low bioavailability of this prominent anticancer drug restricts its appliance in clinical trials^{4,5}. Nowadays, a growing number of curcumin analogs appeared, hoping to be a replacement of curcumin with a better bioavailability and lower toxicity. For instances, curcumin analog EF24 had been demonstrated to disrupt the microtubule cytoskeleton and inhibit HIF-1⁴. GO-Y030 was shown to induce apoptosis by suppressing NF-κB signaling in thyroid, pancreatic cancers and cholangiocarcinoma cells⁵; and studies on CDF, suggested it's the inhibitor of colon cancer stem-like cells⁶. While another analog DM-1, had been proved to occupy an effect in breast cancer treatment when combined with paclitaxel^{7,8}. However, the precise pathway of these analogs inducing cell death still remains unknown.

In this study, we set out to elucidate the anticancer effect of a novel chemically synthetic curcumin analog, (1E, 6E)-4-((furan-2-yl)methylene)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (abbreviated MHMD) (Figure 1), whose chemical structures had been confirmed by NMR spectroscopy. We demonstrated that MHMD could induce A549 lung cancer cells death through apoptosis and necrosis. A549 cells showed a nuclear fragment in a caspase-dependent fashion under 4 μM , and apoptosis was the principle. While with higher concentrations, more cells were stained with Propidium Iodide (PI) and caspases inhibitor didn't seem to lead cells to survive much as low concentrations did, suggesting necrosis was taking in charge. And AnnexinV/PI staining was another proof of apoptosis induced by MHMD. Moreover, the bioavailability of MHMD is about 10 folds higher than curcumin. Still, profound investigation of MHMD on its anticancer therapy potential is warranted.

Materials and Methods

Chemicals and Cell Culture

RPMI Medium 1640 was purchased from GIBCO, Carlsbad, CA, USA. Newborn Calf Serum was purchased from DingGuo Biotechnology Co.Ltd., China. Trypsin was purchased from

Sango Biotech Co., Ltd. (Shanghai, China) and MTT was purchased from Genview, Genview, IL, USA. Acridine orange, Hoechst33342 and Propidium Iodide (PI) were purchased from Sigma Aldrich, St Louis, MO, USA. A549 cells were maintained in 1640 medium supplemented with 10% Newborn calf serum, 1% garamycin at 37°C in 5% CO₂ incubator. Cells were cultured in 96-well plates for cell viability assay, in 24-well plates for acridine orange staining or Hoechst/PI dual-staining analysis.

Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 cells were seeded (2×10^4 cells/well) in 96-well plates (Corning, USA) and incubated for 24 h. Then cells were treated with the different concentrations of MHMD at the designed time points. Caspases inhibitor z-VAD-fmk (20 μM) was added 30 min earlier before cells were treated with drugs. Then, 20 μl MTT (5 mg/ml) was added to each well four hours ahead of time when drugs stopped working. Thereafter, 100 μl dymethyl sulfoxide (DMSO) per well was used to dissolve MTT-formazan. Optical density was measured colorimetrically at a wavelength of 490 nm with a microplate Spectrophotometer (Bio-Tek Epoch, China).

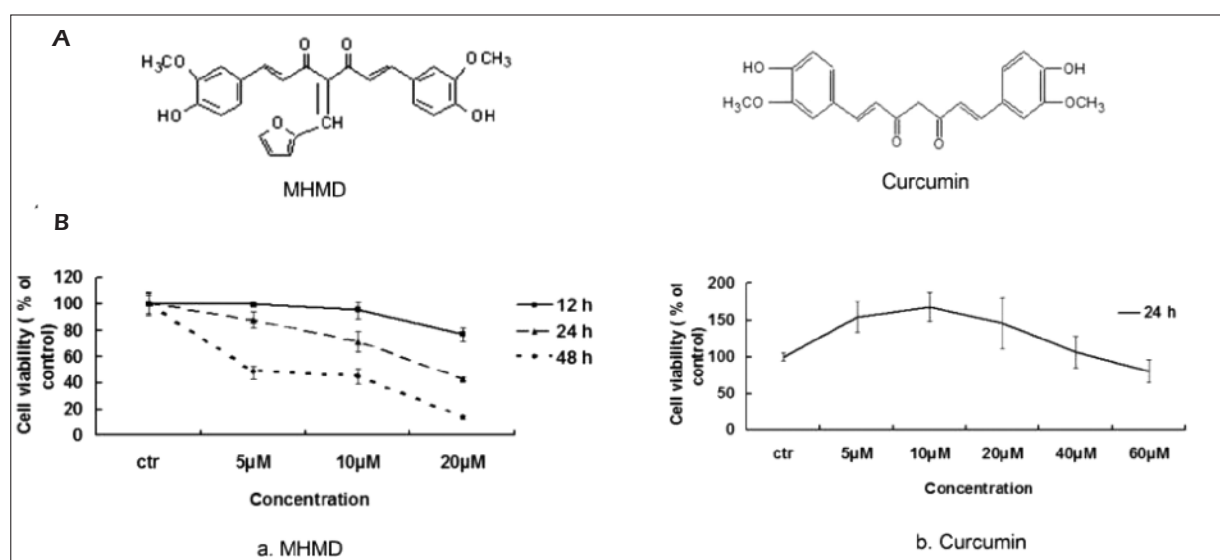


Figure 1. **A**, The chemical structures of curcumin and its analog MHMD. MHMD has a molecular formula $\text{C}_{26}\text{H}_{22}\text{O}_7$ with a molecular weight of 446.14 g/mol, which was dissolved in DMSO (0.1 M) as a stock solution. **B**, Effects of MHMD on cell viability. A549 cells were treated with MHMD at the indicated concentrations (0-20 μM) for 12, 24 or 48 h (**a**), and curcumin at the indicated concentrations (0-60 μM) for 24 h (**b**), and cell viability was performed by MTT assay. Each point represents as the mean \pm SD of at least triplicate experiments (* $p < 0.05$, ** $p < 0.01$, $n \geq 5$).

Acridine orange, Hoechst 33342/PI Staining and AnnexinV/PI Staining

Cells were treated with different concentrations of MHMD for indicated times, and stained with acridine orange (5 µg/ml) for 40 s, washed with PBS solution twice. Thereafter, the acidic vacuoles were detected under a fluorescence microscope (Nikon TE2000-U, Shizvoka, Japan). For Hoechst 33342/PI dual-staining, treated A549 cells were firstly stained with Hoechst 33342 (10 µg/ml) for 10 min and then stained with PI (10 µg/ml) for another 10 min. After washed with PBS twice, the cell nucleus was observed under the fluorescence microscope. AnnexinV/PI staining was operated using Annexin V- FITC Apoptosis Detection Kit, Beyotime institute of biotechnology, China.

Data Analysis

Each experiment was conducted at least three times. The data shown were expressed as the mean±SD and the *p* value was used the Student's *t*-test (**p* < 0.05, ***p* < 0.01, *n* ≥ 5 or *n* = 5).

Results

MHMD Reduced A549 Cells Viability

To assess the effect of MHMD, A549 cells were treated with MHMD at the indicated concentrations (5, 10 and 20 µM) for 12, 24 or 48 h, and cell viability was performed by MTT assay. The results showed that MHMD induced A549 cell death in a time and dose-dependent manner with an IC₅₀ of 27.46 µM, 18.86 µM and 11.23 µM, respectively (Figure 1B.a). And as we can see, curcumin in 60 µM had the same anti-proliferative ability as MHMD only in 4 µM (Figure 1B).

Alteration of A549 Cells in Cytomorphology

Cell morphology alteration was examined with an inverted microscope. A549 cells were treated with different concentrations (5, 10 and 20 µM), and pictures were taken at indicated times ranging from 0 h to 24 h. MHMD made A549 cells smaller in size compared to control group, and also the treated cells became rounded and granular. As time passed, cells were refractile and detached from the cell sheet (Figure 2).

Detection of MHMD-Induced Cell Death

There are three main cell death pathways: apoptosis, necrosis and autophagy. MHMD could

induce the A549 cell death, so next work is to identify which pathway is involved. Generally, autophagosomes fuse with lysosomes forming autophagolysosomes which are acidic vacuoles (AVO) that can bind acridine-orange in the process of cells autophagy. In that case AVOs could be investigated by the appearance of red fluorescence under a fluorescence microscope. However, our research showed that AVOs could not be detected in MHMD-treated A549 cells by acridine orange staining analysis (data not shown), which suggested that MHMD may not induce A549 cell autophagy.

Then, we identified whether MHMD-induced A549 cells death via apoptosis or necrosis. The Hoechst/PI dual-staining assay was used. Hoechst is a reactive dye that combines with DNA specifically, which is capable of entering into normal cytomembrance and staining nucleolus in blue. Contrast to Hoechst, PI is a nucleic acid dye that only has access to impaired cytomembrance and stains nucleolus in red. In our results, MHMD-treated cells were seriously shrunk than control cells. A severe nuclear fragmentation was examined under 4 µM (Figure 3A i, j) while it couldn't be found on cells pretreated with the caspases inhibitor z-VAD-fmk at 48 h (Figure 3(A) k). Simultaneously, those cells preprocessed with z-VAD-fmk showed much higher cell viability than that only treated with 4 µM MHMD at the same time (Figure 3B). However, there were no significant differences in

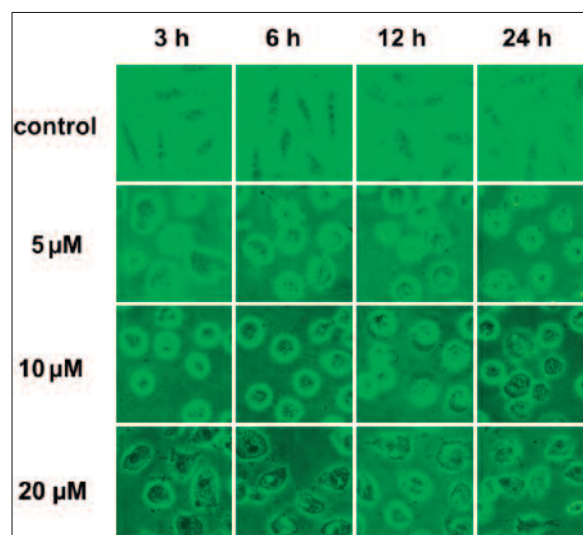


Figure 2. Morphology alteration of MHMD treated cells. Cells were incubated with MHMD (0-20 µM) for indicated times. Images of morphology were taken under inverted microscope (magnification: 400×).

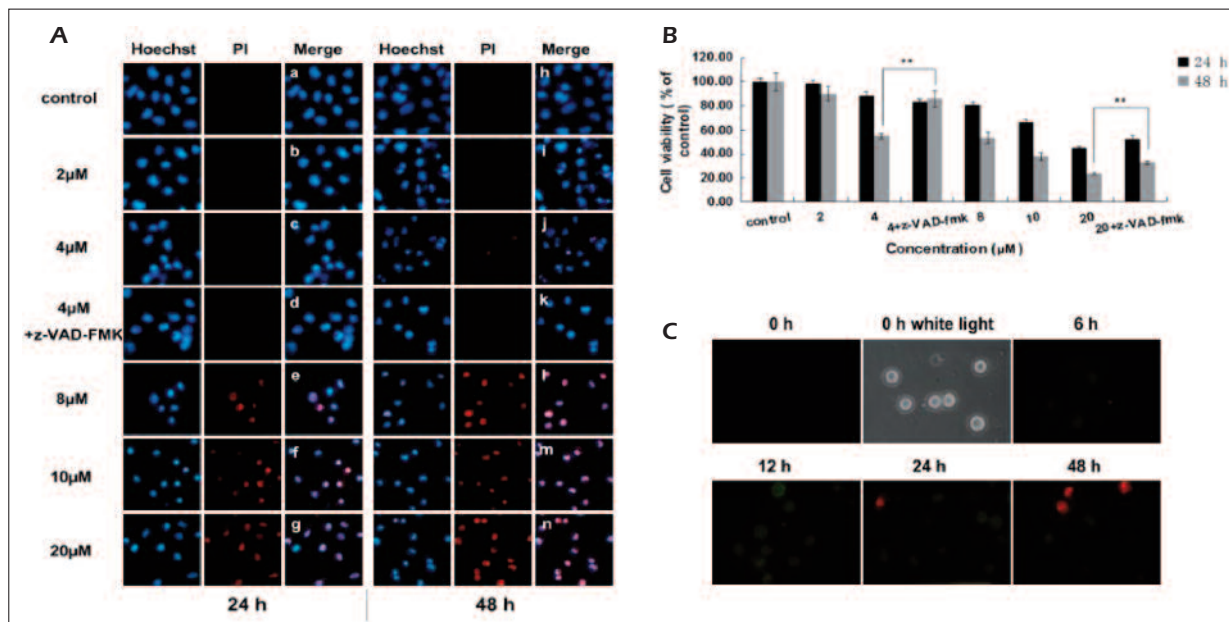


Figure 3. **A**, Detection of apoptosis and necrosis in MHMD treated A549 cells. Cells were treated with MHMD (0-20 μM) in the presence or absence of z-VAD-fmk for indicated times. **A**, Treated cells were stained with Hoechst and PI. Images were taken by fluorescence microscope. **B**, MTT assay was used to detect cell viability. Data were presented as mean \pm SD of at least triplicate experiments ($*p < 0.05$, $**p < 0.01$, $n^* = 5$). **C**, Cells were treated with MHMD (4 μM) for indicated times and then stained with AnnexinV and PI. Images were taken by fluorescence microscope.

the presence or absence of z-VAD-fmk at 24 h (Figure 3A c, d, 3B). With increasing of MHMD concentrations, more cells were stained with PI (Figure 3A e-g, 3A l-n). In accordance with MTT, cell viability with z-VAD-fmk was still higher when concentrations went up to 20 μM at 48 h (Figure 3B). It could be easily found that z-VAD-fmk didn't work as significantly as low concentrations did (Figure 3B).

To further confirm the activation of apoptosis induced by MHMD, AnnexinV/PI staining was performed. A marker for apoptotic cells is the AnnexinV conjugated with the fluorochrome FITC for it has a strong binding affinity to phosphatidylserine (PS), which redistributes from the inner to the outer membrane. Cells treated with MHMD (4 μM) showed AnnexinV positively. And PI positive cells represented cells undergoing the late stage of apoptosis or necrosis (Figure 3C). This data was much in agreement with Hoechst/PI staining that MHMD did induce cell apoptosis.

Discussion

In this study, curcumin analog MHMD was firstly certificated to be a potential anticancer agent, which exerts its activity through apoptosis

and necrosis pathways. MHMD could effectively inhibit cell proliferation. Evidences had been gained that MHMD induced apoptosis in A549 cancer cells, while necrosis was gradually taking in charge as the concentration of MHMD increased. Nuclear fragmentation disappeared at 4 μM in the presence of z-VAD-fmk when time up to 48 h, suggesting that caspases are essential for MHMD-induced cell death. In addition, much higher cell viability of cells pretreated with z-VAD-fmk at 4 μM (Figure 3B) suggested that caspases did take part in the process of MHMD-induced cell death. What's more, no difference by MTT and Hoechst/PI analysis of treated A549 cells (MHMD at 4 μM) in the presence or absence of z-VAD-fmk at 24 h implied caspases didn't work yet at that time. The phenomena that more cells were stained with PI as concentrations went up and the little higher cell viability with cells pretreated with z-VAD-fmk at 20 μM certificated that necrosis was the major mode of cell death at higher concentrations, though apoptosis was took part at the same time. However, the no increased AVOs in A549 cells, promoting that autophagy didn't join in. Whereas, other methods to detect autophagy should be done, but it could be hardly viewed AVOs based on cellular morphology either.

We have demonstrated the two mechanisms of curcumin analog MHMD-induced cell death. Curcumin owns hydroxyl groups and methoxy groups which had been reported to be essential for its antioxidant, anti-inflammatory and anti-proliferative activity. MHMD possesses the same two groups with curcumin. In addition, as with most curcumin analogs that are confirmed to induce cell death at a low drug level, MHMD could be another more potent compound than curcumin, which acted with higher concentrations in many cell lines^{4,6,9,10}. Our data demonstrated that MHMD in 4 μM and curcumin in 60 μM have the same ability to inhibit cell proliferation in 24 h. MHMD is at least 10 folds more effective than curcumin. Curcumin, together with its analogs, is closely related to apoptosis. Our results gave evidence for MHMD-induced visible apoptosis at the concentration under 4 μM ; though its regulative pathways are unknown, MHMD is already seemed to be a promising anticancer drug. Nevertheless, higher concentrations in which necrosis is the major mode of cell death are probably not delightful. It's said that necrosis has proinflammatory and tumor promoting potential¹¹. In that case, necrotic cell death may do more damage than good in the end.

Conclusions

We revealed that MHMD induced cell death through apoptosis and necrosis, rather than autophagy, and MHMD could be as a novel potential anticancer potential agent.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- 1) RAVINDRAN J, PRASAD S, AGGARWAL BB. Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J* 2009; 11: 495-510.
- 2) VIOLA G, BORTOLOZZI R, HAMEL E, MORO S, BRUN P, CASTAGLIUOLO I, FERLIN MG, BASSO G. MG-2477, a new tubulin inhibitor, induces autophagy through inhibition of the Akt/mTOR pathway and delayed apoptosis in A549 cells. *Biochem Pharmacol* 2012; 83: 16-26.
- 3) SHINOJIMA N, YOKOYAMA T, KONDO S, KONDO Y. Roles of the Akt/mTOR/p70S6K and ERK1/2 signaling pathways in curcumin-induced autophagy. *Autophagy* 2007; 3: 635-637.
- 4) THOMAS SL, ZHONG D, ZHOU W, MALIK S, LIOTTA D, SNYDER JP, HAMEL E, GIANNAKAKOU P. EF24, a novel curcumin analog, disrupts the microtubule cytoskeleton and inhibits HIF-1. *Cell Cycle* 2008; 7: 2409-2417.
- 5) SATO A, KUDO C, YAMAKOSHI H, UEHARA Y, OHORI H, ISHIOKA C, IWABUCHI Y, SHIBATA H. Curcumin analog GO-Y030 is a novel inhibitor of IKK β that suppresses NF- κ B signaling and induces apoptosis. *Cancer Sci* 2011; 102: 1045-1051.
- 6) KANWAR SS, YU Y, NAUTIYAL J, PATEL BB, PADHYE S, SARKAR FH, MAJUMDAR AP. Difluorinated-curcumin (CDF): a novel curcumin analog is a potent inhibitor of colon cancer stem-like cells. *Pharm Res* 2011; 28: 827-838.
- 7) FAIAO-FLORES F, SUAREZ JA, PARDI PC, MARIA DA. DM-1, sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate: a curcumin analog with a synergic effect in combination with paclitaxel in breast cancer treatment. *Tumour Biol* 2012; 33: 775-785.
- 8) SHIBATA H, YAMAKOSHI H, SATO A, OHORI H, KAKUDO Y, KUDO C, TAKAHASHI Y, WATANABE M, TAKANO H, ISHIOKA C, NODA T, IWABUCHI Y. Newly synthesized curcumin analog has improved potential to prevent colorectal carcinogenesis *in vivo*. *Cancer Sci* 2009; 100: 956-960.
- 9) MUKHOPADHYAY A, BANERJEE S, STAFFORD LJ, XIA C, LIU M, AGGARWAL BB. Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. *Oncogene* 2002; 21: 8852-8861.
- 10) O'SULLIVAN-COYNE G, O'SULLIVAN GC, O'DONOVAN TR, PIWOCKA K, MCKENNA SL. Curcumin induces apoptosis-independent death in oesophageal cancer cells. *Br J Cancer* 2009; 101: 1585-1595.
- 11) HANAHAHAN D, WEINBERG RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-674.