Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysins

Xiang-Wen Tan, Hong Xia, Jin-Hua Xu, Jian-Guo Cao

Xiang-Wen Tan, Department of Laboratory Animal Science, University of South China, Hengyang 421001, Hunan Province, China
Hong Xia, Institute of Cancer Research, University of South China, Hengyang 421001, Hunan Province, China
Jin-Hua Xu, Center of Biochemistry and Molecular Biology Laboratory, University of South China, Hengyang 421001, Hunan Province, China
Jian-Guo Cao, Laboratory of Medicine Engineering, Medical College, Hunan Normal University, Changsha 410006, Hunan Province, China

Abstract
AIM: To investigate the effect of 5-allyl-7-gen-difluoromethylenechrysins (ADFMCChR) on apoptosis of human liver carcinoma HepG2 cell line and the molecular mechanisms involved.

METHODS: HepG2 cells and L-02 cells were cultured in vitro and the inhibitory effect of ADFMCChR on their proliferation was measured by MTT assay. The apoptosis of HepG2 cells was determined by flow cytometry (FCM) using propidium iodide (PI) fluorescence staining. DNA ladder bands were observed by DNA agarose gel electrophoresis. The influence of ADFMCChR on the proxisome proliferator-activated receptor γ (PPARγ), NF-κB, Bcl-2 and Bax protein expression of HepG2 cells were analyzed by Western blotting.

RESULTS: MTT assay showed that ADFMCChR significantly inhibited proliferation of HepG2 cells in a dose-dependent manner, with little effect on growth of L-02 cells, and when IC50 was measured as 8.45 μmol/L and 191.55 μmol/L respectively, the potency of ADFMCChR to HepG2 cells, was found to be similar to 5-fluorouracil (5-FU). IC50 was 9.27 μmol/L. The selective index of ADFMCChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27). FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMCChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMCChR than when treated with 30.0 μmol/L ChR (16.0%) (P < 0.05) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%). DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMCChR for 48 h and 72 h resulted in typical DNA ladders which could be reversed by 10.00 μmol/L GW9662, a blocker of PPARγ. Western blotting analysis revealed that after 24 h of treatment with 3.0, 10.0, 30.0 μmol/L ADFMCChR, PPARγ and Bax protein expression in HepG2 cells increased but Bcl-2 and NF-κB expression decreased; however, pre-incubation with 10.0 μmol/L GW9662 could efficiently antagonize and weaken the regulatory effect of 3.0, 30.0 μmol/L ADFMCChR on PPARγ and NF-κB protein expression in HepG2 cells.

CONCLUSION: ADFMCChR induces apoptosis of HepG2 cell lines by activating PPARγ, inhibiting protein expression of Bcl-2 and NF-κB, and increasing Bax expression.

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Key words: Liver neoplasm; Chrysins; 5-allyl-7-gen-difluoromethylenechrysins; Apoptosis; Proxisome proliferator-activated receptor γ

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INTRODUCTION
Human liver carcinoma is the fifth most common cancer in the world and is responsible for > 600,000 deaths...
annually\textsuperscript{[3]}. The majority of patients with hepatocellular carcinoma die within 1 year after the diagnosis. At present, the treatment of hepatocellular carcinoma mainly includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough and they have numerous side effects. Therefore, searching for highly efficient antitumor drugs remains a hot research area.

 Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) is a member of the nuclear hormone receptor superfamily; a ligand-dependent transcription factor that plays an important role in lipid and glucose metabolism\textsuperscript{[28]}. In recent years, over-expression of PPAR$\gamma$ has been found in a variety of tumor cells and PPAR$\gamma$ agonists can induce apoptosis\textsuperscript{[4,5,6]}. It has been reported that chrysin (ChR) and its derivatives activate PPAR$\gamma$ to inhibit COX-2 and iNOS activity through various pathways distinguished from thiocarbamates\textsuperscript{[8]}.

 Chrysin (5,7-dihydroxy flavone, ChR) is a kind of flavonoid with pharmacological activities and is widely distributed in the plant kingdom. It has been demonstrated that ChR can markedly inhibit the growth of human thyroid cancer cells\textsuperscript{[7]}, and has an effect on the inhibition of proliferation and induction of apoptosis in human myeloid leukemia cells as well\textsuperscript{[8,9]}. Comte et al\textsuperscript{[8]} reported that, through alkylation, the hydrophobicity of ChR is increased, its KD value decreased, and its binding affinity towards P-glucoprotein (P-gp) enhanced. We confirmed that a series of B-ring trifluoromethylated derivatives of ChR markedly inhibited the growth of HT-29 and SGC-7901 cell lines\textsuperscript{[10]}) and that 5,7-dihydroxy-8-nitrochrysin (NOChR) had an inhibitory effect on subcutaneously transplanted primary Lewis lung carcinoma in mouse and its spontaneous metastasis in a dose-dependent manner\textsuperscript{[12]}. Our previous study showed that the suppressive effect of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) on proliferation of the CoCl2 cell line was stronger than that of ChR\textsuperscript{[13]}. However, whether ADFMChR has antitumor effects on human liver carcinoma is unknown.

 In this study, we aimed to investigate whether ADFMChR induces apoptosis of HepG2 cell line by activation of PPAR$\gamma$ and whether NF-κB, Bcl-2 and Bax are involved in this mechanism, thereby providing a new opportunity for research with regard to the pharmaceutical prevention and cure of human liver cancer.

\section*{MATERIALS AND METHODS}

\subsection*{Cell lines and cell culture}

HepG2 cells and L-02 cells were purchased from the China Center for Type Culture Collection (CCTCC) and were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc) at 37°C in a 5% CO$_2$ incubator.

\subsection*{Medicines and chemical reagents}

ADFMChR was synthesized in the Medical College, Hunan Normal University as previously described\textsuperscript{[14]}; with a molecular weight of 344 ku, characteristic yellow crystals and purity of 99.0%, its molecular formula is C$_{16}$H$_{16}$O$_{8}$F$_{5}$ ADFMChR was dissolved in dimethyl sulfoxide (DMSO), diluted with phosphate buffer solution (PBS), and finally prepared as 2 mmol/L storage solution after filtration sterilization. RPMI-1640, ChR, MIT and DMSO were purchased from Sigma Company; 5-fluouracil (5-FU) was from Jinghua Pharmaceutical Corporation Ltd, Nantong, Ladder Apoptotic DNA Ladder Detection Kit was purchased from Bodataike Corporation Ltd, Nantong.

\subsection*{Flow cytometry (FCM) with propidium iodide (PI) staining}

HepG2 cells were treated with serum-free medium for 24 h, followed by treatment with media containing 3.0, 10.0, 30.0 μmol/L ADFMChR, 30.0 μmol/L ChR and 30.0 μmol/L 5-FU for 48 h, respectively. Cells were collected and prepared as a single cell suspension by mechanical blowing with PBS, washed with cold PBS twice, fixed with 700 mL/L alcohol at 4°C for 24 h, stained with PI and cell apoptosis was detected using FCM (American BD Company, FACS420).

\subsection*{DNA agarose gel electrophoresis}

As previously described\textsuperscript{[16]}, cells were cultured with 10.0 μmol/L ADFMChR and 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662, a PPAR$\gamma$ antagonist, for 0, 24, 48 and 72 h, respectively. Cells were washed twice with PBS and DNA was extracted with an Apoptotic DNA Ladder Detection Kit according to the manufacturer’s instructions. The extracted DNA was kept at 4°C overnight. Then 8.5 μL of DNA sample was mixed with 1.5 μL of 6 × Buffer solution, electrophoresed on 20.0 g/L agarose gel containing ethidium bromide at 40 V, and observed through DBT-08 gel image analysis system.

\subsection*{Western blotting analysis}

As previously described\textsuperscript{[17]}, cells were treated with 3.0, 10.0, 30.0 μmol/L ADFMChR and 30.0 μmol/L ChR for 24 h, respectively. Cells were collected, washed three times with PBS, lysed in cell lysis buffer containing 0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L
EDTA (pH 8.0), 1 μg/mL Aprotinin, 100 μg/mL PMSF, and then centrifuged at 13000 × g for 10 min at 4°C. The extracted protein sample (25 μg total protein/lane) was added in the same volume of sample buffer and subjected to denaturation at 100°C for 10 min, then electrophoresed on 100 g/L or 60 g/L SDS-PAGE, at 100 mA for 3 h, and finally transferred onto PVDF membrane. The PVDF membrane was treated with TBST containing 50 g/L skimed milk at room temperature for 2 h, followed by incubation with the primary antibodies PPARγ, NF-κB, Bcl-2 and Bax (1:500 dilution), respectively, at 37°C for 2 h or at 4°C overnight. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST. Fluorescence was visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The results were analyzed with Image analyzer and the product of area and optical density was expressed as integral absorbance (IA).

Statistical analysis
Experimental data in each group were presented as mean ± SD. Analysis of variance was performed with SPSS software for windows 15.0 by using one way ANOVA and pairwise comparison with Student’s t test. P < 0.05 was considered statistically significant.

RESULTS

Determination of proliferation of HepG2 and L-02 cell lines by MTT assay
MTT assay showed that ADFMChR markedly inhibited proliferation of HepG2 cells in a dose-dependent manner (Figure 1), with little effect on growth of L-02 cells, and when IC50 were measured as 8.45 μmol/L and 191.55 μmol/L, respectively, the potency of ADFMChR to HepG2 cells was found to be similar to 5-fluorouracil (5-FU, IC50 was 9.27 μmol/L). The selective index of ADFMChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27).

Analysis of the effect of ADFMChR on apoptosis of HepG2 cell lines by FCM with PI staining
FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMChR than when treated with 30.0 μmol/L ChR (16.0%) (P < 0.05) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%) (Figure 2).

Detection of ADFMChR-induced apoptosis of HepG2 cells by agarose gel electrophoresis
DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMChR for 48 h and 72 h resulted in typical DNA ladders, which could be eliminated or attenuated by treating with 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662 for 48 h and 72 h (Figure 3).

Analysis of the effect of ADFMChR on PPARγ, NF-κB, Bax and Bcl-2 protein expression of HepG2 cell line
Western blotting analysis showed that the relative densities of PPARγ, NF-κB, Bcl-2 and Bax protein bands of HepG2 cells treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h were 109.3%, 126.4%, 147.7% and 92.9%, 89.0%, 72.4% and 94.1%, 85.5%, 77.3% and 106.8%, 116.3%, 125.7% of the HepG2 cells not treated with ADFMChR, respectively (P < 0.05) (Figure 4). This indicates that ADFMChR can increase the PPARγ and Bax protein expression and decrease NF-κB and Bcl-2 protein expression.

Effect of GW9662 on regulation of PPARγ and NF-κB protein expression by ADFMChR
Western blotting analysis demonstrated that when HepG2 cells were pre-incubated with 10.0 μmol/L GW9662, a blocker of PPARγ, for 30 min, the effects of 3.0, 30.0 μmol/L ADFMChR on PPARγ protein expression and NF-κB protein expression were antagonized or weakened (Figure 5), suggesting that the effects of ADFMChR on up-regulation of PPARγ protein expression and down-regulation of NF-κB protein expression were associated with the activation of PPARγ.

DISCUSSION
Tumorigenesis and tumor progression are strongly associated with abnormal apoptosis. A number of antitumor drugs exert their therapeutic effects by inducing or promoting apoptosis. Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. There is evidence to support the concept that luteolin, apigenin and chrysin have great potential to be developed into novel cancer preventative agents18.
Our previous research showed that ADFMChR potently inhibited the proliferation of ovarian cancer CoC1 cells in a dose-dependent manner\(^1\), and could induce apoptosis of SMMC-7721 cells \(\textit{in vitro}\), with its mechanism possibly associated with G1 phase cell cycle arrest\(^2\). Li \textit{et al}\(^3\) and Xu \textit{et al}\(^4\) found that the ability of ADFMChR to induce induction of apoptosis in CoC1 cells may be mediated by activation of PPAR\(\gamma\), sequentially accompanied by reducing NF-\(\kappa\)B and Bcl-2 levels and increasing Bax expression. Our experiment was to investigate the apoptosis of human liver carcinoma HepG2 cell line induced by ADFMChR and to provide experimental evidence for its application as an antitumor drug.

Apoptosis usually results in typical morphological and biochemical characteristics, including condensed chromatin in cells, appearance of apoptotic bodies, presence of hypodiploid peak in FCM analysis and DNA ladder bands on agarose electrophoresis\(^5\). In

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**Figure 2** Induction of apoptosis of HepG2 cells by ADFMChR. A: Treated with 0.2% DMSO; B: Treated with 30.0 \(\mu\)mol/L 5-FU; C: Treated with 30.0 \(\mu\)mol/L ChR; D: Treated with 3.0 \(\mu\)mol/L ADFMChR; E: Treated with 10.0 \(\mu\)mol/L ADFMChR; F: Treated with 30.0 \(\mu\)mol/L ADFMChR; G: Quantification of induction of apoptosis analysis of HepG2 cells. \(a\) \(\text{P} < 0.05\) vs treatment with DMSO (mean ± SD, \(n = 3\)).

**Figure 3** DNA ladder assay showing ADFMChR-induced apoptosis of HepG2 cells. Lane 1: DNA marker; lane 2: Control; lane 3: 10.0 \(\mu\)mol/L ADFMChR (24 h); lane 4: 10.0 \(\mu\)mol/L ADFMChR + GW9662 (24 h); lane 5: 10.0 \(\mu\)mol/L ADFMChR (48 h); lane 6: 10.0 \(\mu\)mol/L ADFMChR + GW9662 (48 h); lane 7: 10.0 \(\mu\)mol/L ADFMChR (72 h); lane 8: 10.0 \(\mu\)mol/L ADFMChR + GW9662 (72 h).

**Figure 4** Western blotting analysis showing regulation of PPAR\(\gamma\) (A), NF-\(\kappa\)B (B), Bcl-2 (C) and Bax (D) protein expression in HepG2 cells by ADFMChR. (mean ± SD, \(n = 3\)).

**Figure 5** PPAR\(\gamma\) antagonist GW9662 blocked the effects of ADFMChR on PPAR\(\gamma\) and NF-\(\kappa\)B protein expression in HepG2 cells. A: PPAR\(\gamma\); B: NF-\(\kappa\)B. HepG2 cells were pretreated with 10.0 \(\mu\)mol/L GW9662 for 30 min, then exposed to 3.0, 30.0 \(\mu\)mol/L ADFMChR for 24 h, respectively (mean ± SD, \(n = 3\)).
this study, treatment of HepG2 cells with ADFMChR resulted in formation of DNA ladder bands and the appearance of marked hypodiploid peak. Thus, this experiment suggested that ADFMChR can induce apoptosis of human liver carcinoma HepG2 cell line in vitro.

PPARγ is a kind of ligand-activated nuclear transcription factor belonging to a nuclear receptor superfamily and has been implicated in metabolic diseases and is associated with cell proliferation, differentiation and apoptosis[24]. NF-κB inhibits apoptosis, promotes cell survival and reduces the expression of Bcl-2[25]. Chen et al[26] confirmed that PPARγ ligands may markedly inhibit NF-κB expression and reduce Bcl-2 expression leading to inhibited cell growth and induction of apoptosis of colonic cancer HT-29 cell line by activation of PPARγ. Liang et al[27] have recently shown that ChR is activated in different ways with thiazolidinones, and PPARγ inhibits activation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. 8-bromo-7-methoxychrysin (BrMChR) or 5,7-dihydroxy-8-nitrochrysin (NOCChR) induce apoptosis of SGC-2-7901 cell line by activating PPARγ[28,29]. In order to find out whether ADFMChR decreases NF-κB and Bcl-2 protein expression to induce apoptosis of HepG2 cells by activation of PPARγ, we pre-incubated HepG2 cells with GW9662, a selective antagonist of PPARγ, and observed the effect of ADFMChR on apoptosis and PPARγ and NF-κB protein expression of HepG2 cells. Our results showed that preincubation with GW9662 could effectively antagonize ADFMChR-induced apoptosis of HepG2 cells and down-regulation of NF-κB protein expression, suggesting that apoptosis of HepG2 cells induced by ADFMChR is dependent on activation of PPARγ.

Apoptosis is a complex process involving several genes, such as Bcl-2, Bax, and great attention has been given to the Bcl-2 family. The Bcl-2 family can positively and negatively regulate apoptosis[30]. Bcl-2 and Bax are two members of the Bcl-2 family, and play different roles in programmed cell death[31]. When Bax is over-expressed in cells, apoptosis in response to death signals is accelerated, leading to its designation as a death agonist[32]. When Bcl-2 is over-expressed it heterodimerizes with Bax and death is repressed[33]. Therefore, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis[34]. The results in this study confirmed that Bcl-2 expression in non-treated HepG2 cells was higher than in those treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h; in contrast, Bax expression was lower. Thus, the ratio of Bcl-2 to Bax in HepG2 cells treated with ADFMChR was lower than that of non-treated HepG2 cells, which indicated that ADFMChR-induced HepG2 cells apoptosis was associated with down-regulation of Bcl-2 expression, up-regulation of Bax expression and reduction of the ratio of Bcl-2 to Bax.

In summary, ADFMChR possesses stronger antihepatic cancer effect in vitro than parent compound ChR, and was similar to 5-FU, and it exerts its apoptotic effect by activation of PPARγ, down-regulation of NF-κB and Bcl-2 protein expression, up-regulation of Bax protein expression, and reduction of the ratio of Bel-2 to Bax. ADFMChR might be a promising candidate for the development of antitumor drugs.

COMMENTS

Background
Human liver carcinoma is the fifth most common cancer in the world. Unfortunately, the disease is often diagnosed at a late stage. For these patients, medical treatments, including chemotherapy, chemoembolization, ablation, and proton beam therapy, are not adequate. Most patients show disease recurrence that rapidly progresses to the advanced stages with multiple intrahepatic metastases and their 5-year relative survival rate is only 7%. Clearly, there is an urgent need for new therapies for this disease.

Research frontiers
Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. Natural compounds have been extensively studied and have shown anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis. Flavonoids are a group of polyphenolic substances widely distributed in the plant kingdom and present in human diets. Previous reports have shown that flavonoids (such as chrysin, apigenin) could inhibit the proliferation and induce apoptosis in tumor cells. In this study, the authors demonstrate that 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) could induce apoptosis of human liver carcinoma HepG2 cells in vitro by activation of PPARγ.

Innovations and breakthroughs
Recent research has shown that chrysin and its derivatives possess a strong anticancer effect. This is the first study to report that ADFMChR, the derivative of chrysin, has a greater suppressive effect on proliferation of HepG2 cells than that of chrysin, and induces apoptosis of HepG2 cells. These data support the idea that ADFMChR has great potential to be developed into novel cancer preventative agents.

Applications
This finding may provide a molecular basis for the clinically observed cancer-preventive effects of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) and new clues for research about pharmaceutical prevention and cure of human liver carcinoma.

Terminology
ADFMChR, a Chrysin derivative, which was taken as the principle compound to design and synthesize, was prepared by alkylation, methylation, and difluoromethylation of chrysin, and was found to have stronger anticancer activity than parent compound chrysin.

Peer review
The authors demonstrate that the effects of ADFMChR on induction of apoptosis in HepG2 cells may be associated with activation of PPARγ, sequentially accompanied by inhibition of protein expression of NF-κB and Bcl-2 and reduced ratio of Bcl-2 to Bax. The results provide a new idea for cure of human liver carcinoma.

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