The Novel Curcumin Derivative 1g Induces Mitochondrial and ER-Stress-Dependent Apoptosis in Colon Cancer Cells by Induction of ROS Production

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Research

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Abstract

Background: A novel curcumin (Cur) derivative 1g can inhibit the proliferation of colon cancer *in vitro* and *in vivo*. The purpose of this study was to explore the role of 1g in inducing apoptosis of colon cancer cells, especially mitochondrial apoptosis and endoplasmic reticulum (ER)-stress caused by reactive oxygen species (ROS).

Methods: Bioinformatics was used to analyze differentially expressed mRNAs. Gene expression was measured by using qRT-PCR and protein expression was measured by using western blotting. Cell apoptosis, cycle, mitochondrial membrane potential and ROS were analyzed by flow cytometry. Experiments on transplanted tumors in animals.

Results: The mechanism of this effect was a change in mitochondrial membrane potential caused by 1g that increased its pro-apoptotic activity. In addition, 1g produced ROS, induced G1 checkpoint blockade, and enhanced ER-stress in colon cancer cells. [On the contrary](#), pretreatment with the ROS scavenging agent N-acetyl-L-cysteine (NAC) inhibited the mitochondrial dysfunction caused by 1g and reversed ER-stress, cell cycle stagnation, and apoptosis. Additionally, pretreatment with the p-PERK inhibitor GSK2606414 significantly reduced ER-stress and reversed the apoptosis induced by colon cancer cells.

Conclusions: This study not only found that 1g inherits the safety of Cur and has a more inhibitory effect on colon cancer cells than Cur, but also revealed that excessive production of ROS is one of the mechanisms of anti-tumor action.

Background

According to cancer statistics recently published by the American Cancer Society, the incidence of human colorectal cancer (CRC) is 10.2%, with a mortality rate of 9.2%, a rise from 4th to 2nd place[1, 2]. Currently available treatments for CRC include surgical intervention, radiotherapy, and chemotherapeutic drugs. However, the mechanisms by which the treatment operates remains largely unknown[3]. Therefore, new treatment strategies are required to improve survival in CRC patients.

Cur (Fig. 1a), a bioactive ingredient of *Curcuma longa* (turmeric) is a well-known cancer-preventative agent with no significant side effects at a therapeutic dose. Over the past decade, curcuma's anti-cancer properties have been well documented in a variety of cancer types, including lung, breast, pancreatic and prostate cancer[4–6]. In addition, a lot of studies have showed that Cur has anti-proliferative and anti-carcinogenic properties in varieties of cell lines and animal models, partly because it blocks the cell cycle, induces apoptosis, and inhibits the proliferation and metastasis of tumor cells[7–9]. A variety of studies have demonstrated the chemoprophylactic and antitumor effects of Cur in CRC *in vivo*[10–14]. Despite its safety and extensively-documented pharmacological effects, the utilization of Cur is challenging due to its extremely low water solubility, instability, and poor oral bioavailability[15, 16]. A variety of strategies have been studied to overcome the limitations of Cur, including chemical modification.

ROS is a highly active oxygen radical that is mainly derived from NADPH oxidase in the mitochondria[17]. It has been reported that ROS and tumor biology are inherently intertwined[18]. Over recent years, this laboratory has been working to chemically modify the Cur to create novel molecules that are more chemically stable and more pharmacologically powerful than Cur. Previously, a range of pyrimidine-substituted curcumins was synthesized and evaluated as a treatment against a variety of cancer cells[19]. One compound:(E,E)-2-(4-(4,6-bis(4-methoxystyryl)pyrimidin-2-yloxy)butyl)-1,1,3,3-tetramethylisothiuronium hydrobromide (1 g, Fig. 1a) exhibited excellent chemical stability combined with anti-cancer properties. Here, RNA-sequencing was conducted to identify differentially expressed mRNAs in a colon cancer cell line (HCT116) treated with the Cur analog 1 g and found that those mRNAs identified were closely related to cell apoptosis. Subsequently, the anticancer mechanism and capability of action of 1 g in colon cancer cells were explored. The results indicate the anticancer mechanism of action of 1 g was possibly via the induction of intracellular ROS generation, leading to a change in mitochondrial membrane potential, cell cycle arrest, activation of ER-stress, ultimately leading to cell apoptosis. This study not only found that 1 g inhibited the activity of colon cancer cells more than Cur, but also revealed that overproduction of ROS is an important key for the treatment of CRC.

Methods

Antibodies and reagents

The following antibodies were used: GADPH (#2118), anti-CHOP (#2895), anti-GRP78 (#3177), anti-cleaved caspase 9 (#7237), anti-cleaved caspase 3 (#9661), anti-cleaved PARP (#5625), anti-Bax (#5023), anti-Bcl-2 (#3498), anti-Cytochrome c (#4272), anti-PERK (#5683), anti-p44/42 MAPK (Erk1/2) (#9102), anti-phospho-p44/42 MAPK (Erk1/2) (#9101), anti-cyclin D1 (#2978), anti-phospho-Rb (#8516), anti-IRE1 α (#3294), and anti-p21 (#2947), anti-mouse IgG-HRP (#7076), anti-rabbit IgG-HRP (#7074) were purchased from Cell Signaling (Beverly, MA, USA), and anti-Phospho-EIF2 alpha (AF3087) and anti-Phospho-PERK (Thr982) (DF7576) were purchased from Affinity Biosciences Ltd., anti-ATF4 (0835-1-AP) obtained from Proteintech (Wuhan Sanying) and anti-p53 (ab26) aquired from Abcam (Cambridge, UK).

Other reagents used in the present study were: 1 g (> 98% purity, HPLC) prepared in our laboratory. Cur and DMSO were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8), N-acetylcysteine (NAC; ROS scavenger), and GSK2606414 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Antibody diluent and Restore™ PLUS Western Blot Stripping Buffer were purchased from ThermoFisher Scientific (Shanghai, China).

Cell Culture

The human colon cancer cell lines HCT116 and HT29 were obtained from the American Type Culture Collection (ATCC), SW480 and LOVO were obtained from the Cell Resource Center, Shanghai Institute of Life Sciences, Chinese Academy of Sciences. HCT116 and HT29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; BI, New York, NY, USA), and SW480 in Roswell Park Memorial Institute (RPMI)-1640 medium (BI). LOVO cells were maintained in DMEM/F-12 (Dulbecco's Modified Eagle

Medium/Nutrient Mixture F-12). The medium was supplemented with 10% fetal bovine serum (FBS; Biological Industries, Israel) and 1% penicillin G/streptomycin sulfate. Cells were maintained at 37 °C in a humidified incubator within 5% CO₂.

Transcriptome gene sequencing

Untreated HCT116 cells and those treated with 1 g for 24 h (n = 3) underwent transcriptomic analysis by Gooal Gene Technologies Ltd (Wuhan, China). Briefly, total RNA was processed by mRNA enrichment and rRNA removal. The RNA was fragmented with interrupt buffer, and random N6 primers were reversed transcribed, from which double-stranded cDNA was synthesized. The resultant double-stranded DNA ends were flattened and phosphorylated at the 5'-ends. A “sticky” end was formed at the 3' end from which an 'A' protruded to which a bubblelike junction with a 'T' was connected. The ligands were amplified by PCR using specific primers. The PCR product was thermally denatured into a single strand, which was cycled with a bridge primer to obtain a single-stranded circular DNA library which was finally machine sequenced. The process is detailed in a flowchart in **Additional file 1**.

Cell proliferation assay

Cell proliferation was detected using the CCK-8 kit. Briefly, 1 g-treated cells were plated at 5×10^3 cells per well in 96-well culture plates and cultured for 12 h, 24 h, and 48 h. A 10 µl aliquot of CCK-8 reagent was added to each well then incubated at 37 °C for 3 hours. The absorbance at 450 nm of each well was recorded using a microplate reader (Tecan Austria GmbH 5082, Austria) (n = 5).

Flow cytometry analysis

Cell apoptosis analysis. Cells were seeded in 6-well plates (5×10^5 cells/well). After incubation with specified drug treatments, the cells were suspended in Annexin V binding buffer then incubated with Annexin V–FITC and PI in accordance with the manufacturer's instructions (BD Biosciences, Bedford, MA), before flow cytometry analysis (n = 3).

Cell cycle analysis. Cells were treated with 1 g or Cur for 18 h, collected, fixed and permeated overnight in 75% ethanol overnight at -20 °C. All samples were washed twice with PBS, and then incubated with PI and RNase at room temperature for 30 min. The results were analyzed using Modifit software (n = 3).

ROS measurement. ROS levels were determined with a dichloro-dihydrofluorescein diacetate (DCF-DA) probe. After treatment, the cells were collected and incubated with 20 µM DCF-DA for 30 min. After washing twice in PBS, the fluorescence intensity of DCF was measured by flow cytometry. The results were analyzed using Flowjo v10 software (n = 3).

Mitochondrial membrane potential ($\Delta\Psi_m$) evaluation. Following treatment with 1 g or Cur for 24 h, the cells were incubated with JC-1 fluorescent probe (Elabscience, Wuhan, China) in a 5% CO₂ humidified incubator at 37 °C for 20 min. The cells were then washed twice with a staining buffer and analyzed using a TMFC500 flow cytometer (Beckman Coulter, CA, USA) (n = 3).

Western blot analysis

Cells and tissues were collected in RIPA buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton-X100), and supplemented with PMSF protease inhibitor and phosphatase inhibitor. The cells and tissues were then sonicated (8 bursts of 12 s, 4 °C, 100 W, using a Labsonic sonicator, Hielscher, Teltow, Germany), and the protein concentration of the samples determined using a BCA protein assay. The same quantity of protein (35 µg) was added to an appropriate volume of 5x sample buffer (Beyotime Biotechnology, Shanghai, China), and then heated until boiling. The samples were then separated on a 10-12.5% SDS-polyacrylamide gel (PAGE) after which they were transferred to PVDF membranes using a Bio-Rad electro-transfer system (Bio-Rad Laboratories, Munich, Germany). Each membrane was then hybridized with a primary antibody overnight on a shaking table at 4°C, washed three times with TBST buffer (3 × 10 min), then incubated with the corresponding secondary antibody at room temperature for 1 h. Finally, Immobilon Western chemiluminescent HRP matrix (Millipore, Burlington, MA, USA) was added to develop the membrane. The positive bands were imprinted using the Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and the density of digital images was measured using ImageJ software (NIH, Bethesda, MD, ver.1.52a) and expressed as a ratio of the GAPDH loading control.

Quantitative real-time PCR analysis

qRT-PCR was used to measure the expression of GRP78 and CHOP. Total RNA was extracted using Trizol reagent (Invitrogen; Life Technologies Corporation, Grand Island, NY, USA) in accordance with the manufacturer's instructions. A 1 µg quantity of RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara, Tokyo, Japan). The cDNA was diluted (2 µl) and selectively amplified by real-time PCR using SYBR Green I (Takara) with specific primers. The sequences of human CHOP, GRP78, and GAPDH were obtained from previously published articles[20]. The samples were amplified using a Roche LightCycler 480II (Switzerland). The relative amount of each mRNA was calculated by a comparative method ($2^{-\Delta\Delta C_t}$) against a GAPDH endogenous control.

Colon cancer xenografts in nude mice

All animal husbandry and experimental procedures were approved by the Animal Research Ethics Committee of the Affiliated Hospital of Qingdao University in Shandong Province. Four six-week-old female Balb/c-nu/nu mice were from the Beijing Viton Lihua Experimental Animal Technology Co. Ltd. At an ambient temperature of 20–25 °C, all mice were maintained in specific pathogen-free ventilation chambers 45–50% relative humidity, and a 12 h light-dark cycle. All mice adapt to the environment for 7 days before the experiment, and get sterilized food and water free of charge. Suspensions of HCT116 cells were injected subcutaneously into each mouse (at a cell density of 5×10^6 in 150 µl PBS). When the tumor volume had reached approximately 150mm³, the mice were randomly divided into control and treatment groups (n = 6). The groups were: control group with vehicle; 1 g group (40 mg/kg); 1 g group (20 mg/kg), and Cur group (40 mg/kg). Each treatment group received an intraperitoneal injection once per day. The mice were monitored for 14 days during treatment. Bodyweight and tumor volume were

measured every 2 days. The tumor volume was calculated using the formula: $([\text{width}]^2 \times [\text{length}]/2)$. Moreover, weight was recorded throughout the experiment.

HE staining

Transplanted tumor tissue biopsies were embedded in paraffin, sectioned, then processed in xylene for 20 min, xylene for 20 min, ethanol for 5 min, anhydrous ethanol for 5 min, and 75% alcohol for 5 min prior to washing in water. Sections were stained with hematoxylin stain for 3–5 min, rinsed with tap water, differentiated, rinsed with tap water, immersed in basic bluing reagent prior to rinsing in running water. Sections were then dehydrated through an 85%-95% gradient of alcohol for 5 min respectively, then stained in eosin solution for 5 min. The sections were placed in anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, anhydrous ethanol for 5 min, dimethyl for 5 min, xylene for 5 min for clearing then sealed in neutral rubber. Finally, the sections were examined by light microscopy and images acquired and analyzed.

Statistical analysis

GraphPad InStat 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. The results were expressed as means of arbitrary values $\pm$ SD. Differences were analyzed using a one-way analysis of variance followed by a Bonferroni's post-hoc test using GraphPad InStat 8.0 software. *P*-values < 0.05 were considered significant.

Results

Bioinformatics analysis of differentially expressed mRNAs and microarray data of 1 g-treated colon cancer cells

There are 181 differentially expressed mRNAs were confirmed in the HCT116 cells after exposure to 1 g (10 μ M) for 24 h. Of these, 80 were up-regulated and 101 were down-regulated compared with the control group. From the results, a volcano plot was created (Fig. 1b) and a heat map of the mRNA expression levels (Fig. 1c). To determine the key factors that inhibit colon cancer cells by 1 g, gene ontology (GO) and pathway analyses were performed. From the GO enrichment bubble map (Fig. 1d), it is apparent that 1 g inhibits HCT116 cells principally by regulation of metabolic processes and adhesion, biological regulation, cellular processes, and catalytic activity. Enrichment was conducted from information in the KEGG database to identify pathways that mediate significant change to the function of the differentially expressed genes, identifying 20 significant pathways associated with those genes may play a key role in the processing of HCT116 cells by 1 g (Fig. 1e). Interestingly, of these, the apoptotic pathway was the most significant. Therefore, we further explored the effects and mechanisms of 1 g on apoptosis in HCT116 cells.

The Cur derivative, 1 g, inhibits colon cancer cell proliferation

Using a CCK-8 assay, the effect of 1 g on the proliferation of HCT116, HT29, SW480, and LOVO colon cancer cell lines treated with either DMSO, increasing concentrations of 1 g for 12, 24, or 48 h, was investigated. The results indicated that 1 g significantly inhibited the proliferation of four different colon cancer cells proliferation in a time- and dose-dependent manner (Fig. 2a, b, c, d). However, The activity of normal CHO fibroblasts was not affected by 1 g (10 μ M), as reported previously[19]. This suggests that 1 g has the safety and stability of Cur but is more selective against colon cancer cells.

1 g induces apoptosis and cell cycle arrest in colon cancer cells

To confirm that the apoptosis caused by 1 g was consistent with transcriptomic sequencing, HCT116 cells were further treated with 1 g and Cur for 24 h then analyzed by Annexin V-FITC/PI double staining flow cytometry. The number of apoptotic colon cancer cells was positively correlated with the concentration of 1 g, and the number of apoptotic colon cancer cells was significantly greater when treated with 10 μ M 1 g than with 10 μ M Cur (Fig. 3a,b). In addition, Apoptosis-related proteins proteins cleaved-PARP, p-ERK, cleaved-caspase 9, and cleaved-caspase 3 was detected by Western blotting. HCT116 cells were treated with 1 g (10 μ M) during cell culture (0–24 h), followed by DMSO, 1 g or Cur for 24 h. The results demonstrate that the protein expression levels increased after treatment with 1 g in a time- and dose-dependent manner (Fig. 3c,d). Cyclin is closely involved in the checkpoint control mechanism and the signal to undergo apoptosis[21]. PI staining then flow cytometry was also used to ascertain whether 1 g induced HCT116 cell cycle arrest. HCT116 cells undergoing exponential growth were treated with 1 g and Cur for 18 hours then analyzed by flow cytometry. Interestingly, after treatment with 1 g (10 μ M), the number of cells in G1 phase increased significantly, while the number in S phase decreased significantly and no significant change in number was observed in G2/M phase (Fig. 3e,f). These observations are different from those previously reported for Cur treatment of colon cancer cells, which were observed to undergo cycle arrest in the G2/M phase[8]. Subsequently, the colon cancer cells were treated with 1 g (10 μ M) for up to 24 h. The expression levels of p-Rb and Cyclin D1 were greatest at 4 h and then decreased, while the expression levels of p21 were highest at 8 h (Fig. 3g). In addition, the expression levels of P-Rb and Cyclin D1 were measured in HCT116 cells treated with increasing concentrations of 1 g or Cur at 24 h. The expression levels of p21 and p53 were measured at 8 h. P-Rb and Cyclin D1 expression decreased with increasing 1 g concentration, while the expression levels of p21 and p53 increased. This is consistent with the regulation of the cell cycle by these proteins (Fig. 3h).

1 g induces changes in mitochondrial membrane potential in colon cancer cells

It is well-known that many cytotoxic stimuli, including chemotherapeutic drugs, induce apoptosis via endogenous pathways in which mitochondria are involved[22, 23]. Apoptosis-related gene expression was assessed to test the hypothesis that 1 g operates similarly, finding that Bax and Cyto C expression levels increased and anti-apoptotic Bcl-2 expression levels decreased after the treatment of HCT116 cells

with 1 g, in a time and dose-dependent manner (Fig. 4a,b). As the ratio of Bax/Bcl-2 expression increased, mitochondrial membrane dysfunction and permeability increased[24]. The mitochondrial membrane potential of HCT116 cells treated with 1 g or Cur for 24 h was found to have undergone a clear change (Fig. 4c).

1 g induces colon cancer apoptotic death by evoking ROS generation and transduction of the ER stress-related cell death pathway

As is well known, in eukaryotic cells, mitochondria are the principal source of ROS. The destruction of mitochondrial function is generally related to an enhancement of the production of mitochondrial ROS[25]. Excessive production of ROS can lead to DNA damage[26], endoplasmic reticulum stress[27], and apoptosis[28]. To test the hypothesis that 1 g was able to stimulate colon cancer cells to produce ROS, production was measured in 1 g-treated cells, which demonstrated increased ROS production in a time-dependent manner (0-120 min) (Fig. 5a). N-acetyl-L-cysteine (NAC) is an antioxidant that can eliminate all ROS subclasses[29]. The ROS produced by HCT116 cells was measured after treatment with different concentrations of 1 g and pretreatment with 10 mM NAC for 1 h. ROS increased with increasing concentration of 1 g, while ROS production was significantly reduced in the NAC pretreatment group (Fig. 5b). To verify that the ROS produced in colon cancer cells led to cell apoptosis and cell cycle stagnation, ultimately leading to decreased cell activity, the cells were pretreated with NAC for 1 h, followed by treatment with 1 g (10 μ M) for 24 h. The expression of mitochondrial proteins (Fig. 5c) and cycle proteins (Fig. 5d) was detected by Western blotting. The activity of HCT116 and HT29 cells was measured using a CCK-8 assay (Fig. 5e). In order to further explore whether 1 g (10 μ M)-stimulated ROS production of colon cancer cells can cause their endogenous apoptosis by ER-stress, the gene expression of CHOP and GRP78 was quantified by RT-PCR in HCT116 cells at various times (0 to 8 h) (Fig. 6a), and CHOP and GRP78 gene expression in 1 g and Cur-treated HCT116 cells for 8 h (Fig. 6b). The results showed that the mRNA transcription of GRP78 and CHOP was time-dependent and increased with the increasing 1 g concentration. The time dependency of ER-stress related protein expression was then measured by Western blotting: PERK, p-PERK, and p-EIF2 α (0-120 min); ATF4 and CHOP (0–12 h); and IRE1 α and BIP (0–24 h). Maximum protein expression of p-PERK occurred at 1 h, P-EIF2 α at 2 h, ATF4 and CHOP after 6 h, and IRE1 α and BIP at 24 h (Fig. 6c). The effect on ER-stress related protein expression of different concentrations of 1 g and Cur was investigated at the specific exposure times above. The protein expression of p-EIF2 α , p-PERK, CHOP, ATF4 were increased with increasing 1 g concentration, while the expression of IRE1 α and BIP was highest at 1 g (10 μ M) (Fig. 6d). After 1 h of pretreatment with NAC, HCT116 cells were treated with 1 g (10 μ M) after which they were analyzed for protein expression. It showed that the expression of CHOP, ATF4 and p-PERK was almost entirely blocked by NAC pre-treatment and the expression of BIP significantly reduced (Fig. 6e). To determine if ER-stress was the cause of 1 g-induced apoptosis and death of the colon cancer cells, the molecule p-PERK inhibitor GSK2606414 was used[30]. Compared with the untreated group, p-PERK was significantly reduced after 1 h pretreatment with GSK2606414 (2 μ M). Similarly, the expression of proteins downstream of CHOP decreased significantly (Fig. 6f). These results indicated that ER stress signal activation at least partially mediated 1 g-induced apoptosis of HCT116 cells.

1 g significantly inhibits HCT116 cell growth in the mouse models

To verify the anti-tumor effect of 1 g *in vivo*, HCT116 cells were injected as an *in vivo* tumor xenograft experiments in mice. The results demonstrated that the volume of the resultant tumors in mice administered saline increased in a time-dependent manner, while both 1 g and Cur displayed antitumor effects (Fig. 7a). The size of the tumors in mice of 1 g groups was distinctly smaller than those in mice of Cur the control and groups (Fig. 7b). Furthermore, all mice survived 14 days of treatment and were approximately the same weight. The results indicate that 1 g displayed good safety at the specified dose (Fig. 7c). Western blotting and immunohistochemical analysis indicated that 1 g significantly enhanced the expression of cleaved-caspase 3 (Fig. 7d). From the results of hematoxylin-eosin (HE) staining. A large number of pathological mitotic figures were found in the tumor tissue treated with 1 g (Fig. 7e). These data are consistent with the results of 1 g in anti-colon cancer cell proliferation experiments *in vitro*. In addition, 1 g had stronger anti-tumor effects *in vivo* than Cur at the same dose.

Discussion

Cur is the biologically active ingredient in turmeric, known since ancient times for its medicinal properties[31]. Cur is thought to induce apoptosis in all types of cancer cells[32–34]. Over the past decade, cur has demonstrated remarkable biological properties, but its clinical use has been limited by its instability at physiological pH and low bioavailability[35, 36]. Multiple studies have shown that the reason for Cur's poor bioavailability is its early biological transformation and metabolism[37, 38]. In order to overcome this limitation, Cur was chemically modified to improve its stability and bioavailability, the modified compound termed 1 g. In previous study, we preliminarily investigated the inhibitory effect of 1 g on colon cancer proliferation, but the mechanism was unknown[19].

Here, we identified 181 differentially expressed mRNAs in HCT116 cells following exposure to 1 g (10 μ M) for 24 h, and additional bioinformatics analysis indicated that these differentially expressed mRNAs were closely related to cell apoptosis. As a result, we explored the effects and mechanisms of 1 g on apoptosis in HCT116 cells. The data indicate that 1 g reduces the activity of colon cancer cells in a time- and dose-dependent manner. Furthermore, transcriptional genome sequencing revealed that it mainly induces apoptosis and death of colon cancer cells. As mentioned above, the molecular mechanism by which Cur induces apoptosis has been confirmed to be principally through the mitochondria-dependent pathway[32, 38]. Flow cytometry analysis and Western blotting revealed that 1 g changed the mitochondrial membrane potential of HCT116 cells and induced apoptosis. Interestingly, the change in mitochondrial membrane potential induced by 1 g activated apoptotic signals, unlike the mechanism of Cur. In addition, Western blot analysis of the apoptotic proteins demonstrated that 1 g effectively induced apoptosis by releasing cytochrome c and activating downstream caspase-9 and caspase-3 cascade reactions.

In recent years, increasing attention has focused on therapeutic drugs that can regulate different stages of the cell cycle in cancer cells, including G0/G1, S, and G2/M[39]. Regulation by inhibition of the cell

cycle may be considered a beneficial event leading to the induction of apoptotic cell death during the treatment of colon cancer[40]. In the present study, in comparison with Cur[8], 1 g induced cell blockade of the G1 phase in HCT116 cells. The results indicate that 1 g caused apoptosis in colon cancer cells by blocking the cell cycle.

Cur also produced ROS, leading to apoptosis in cancer cells[41, 42]. As important multifaceted signaling molecules, ROS regulate multiple cellular pathways and play an important role in deciding cell fate[43]. There is growing evidence that excessive oxidative stress maybe an effective way in the elimination of cancer cells[44]. After excessive production of ROS, a number of pro-apoptotic signaling pathways, including ER-stress, is activated[45]. ER-stress is a conservative cellular defense mechanism that responds to the environment within the ER[46]. A number of anticancer drugs have previously been reported to induce apoptosis in cancer cells by ER-stress, such as sphenol[47], farnicol[48], or polyfiline D[49]. Similarly, 1 g also promotes ROS-mediated ER-stress. The response of the unfolded protein reaction causes ER stress over a long period and activates the mammalian apoptosis pathway[50]. During the commitment stage of ER-stress-induced apoptosis, activation of the downstream transcription factor CHOP caused by ATF-4 signaling can trigger pro-apoptotic signals, thereby triggering the specific cascade of ER-stress that results in apoptosis[51]. We used Western blotting identify the association between apoptosis and ER-stress in colon cancer cells induced by 1 g. We further investigated whether apoptosis induced by 1 g was related to ER stress. HCT116 cells were pretreated with PERK inhibitor. Inhibition of P-PERK by GSK2606414 significantly inhibited the expression of CHOP and p-PERK in 1 g-induced HCT116 cells. Importantly, however, the inhibition of ROS production by combined NAC treatment nearly completely reversed HCT116-induced apoptosis, including cell cycle blockade, mitochondrial apoptosis and ER-stress. These results further show that ROS production plays a vital key role in cell survival. The results suggest, as illustrated in Fig. 8, that 1 g can induce the production of ROS in colon cancer cells, resulting in cell cycle stagnation, change in mitochondrial membrane potential and ER-stress, thus leading to apoptosis in colon cancer cells. Indeed, besides cellular effects, we have demonstrated that 1 g has a strong inhibitory effect on tumor growth in an HCT116 transplant tumor mouse model. Moreover, 1 g displays an excellent profile in terms of safety.

Conclusions

In summary, The Cur derivative 1 g significantly reduces the activity of colon cancer cells and induces apoptosis of colon cancer cells *in vitro* and *in vivo* through the production of ROS. The properties of 1 g should be further studied in order to develop an effective anticancer drug to treat human colon cancer. The results also suggest that activation of ROS production may be a key point in the treatment of CRC. Nevertheless, the direct target of the 1 g molecule remains unknown and the specific origin of ROS is still unclear. Current data indicate a high probability that it is generated in mitochondria, but other aspects require additional study. Therefore, we believe that the over-production of ROS following the modification of natural anticancer drugs may provide a novel strategy for anticancer treatment.

Abbreviations

ATCC

American Type Culture Collection

CRC

colorectal cancer

Cur

Curcumin

DMSO

Dimethyl Sulfoxide

ER

endoplasmic reticulum

GO

gene ontology

HE

hematoxylin-eosin

KEGG

Kyoto Encyclopedia of Genes and Genomes

NAC

N-acetyl-l-cysteine

qRT-PCR

Quantitative real-time PCR

ROS

reactive oxygen species

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of The Affiliated Hospital of Qingdao University (AHQU20200605).

Consent for publication

Not applicable.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Competing interests

No potential conflicts of interest are disclosed.

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Authors' contributions

HW, JLS designed experiments and helped write the manuscript. YYX designed experiments and checked the whole article. ZGS designed experiments and funded the experiment.

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Figures

Figure 1

Differentially expressed genes profile and bioinformatics analysis. HCT116 cells were exposed to 1g (10 μM) for 24h, after which the transcriptome was sequenced. (a) The structures of Cur and 1g. (b) Volcano plot of differentially expressed mRNAs. (c) Heat map of differentially expressed mRNAs. (d) GO enrichment bubble map for differentially expressed mRNAs. (e) KEGG Pathway enrichment bubble map for differentially expressed mRNAs.

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Figure 2

1g inhibited the proliferation of colon cancer cells. (a) HCT116, (b) HT29, (c) SW480, and (d) LOVO cells were treated with 1g (2-32 μM) for 12h, 24h and 48h, The inhibition effect of 1g on cell proliferation was dose-dependent and time-dependent (n=5).

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1g induced colon cancer apoptosis and inhibited cell cycle arrest in G1 phase. (a) Flow cytometry was used to detect the induction of apoptosis of HCT116 cells after treatment with 1g or Cur for 24h. (b) The number of apoptotic cells was determined using GraphPad Prism software. (c) Levels of apoptotic proteins were evaluated in HCT116 cells treated with 1g (10μM) for varying durations. (d) 1g or Cur-treated HCT116 cells for 24h, followed by protein levels evaluated by Western blot analysis. (e) Flow

cytometry was used to determine the induction of colon cancer cell cycle arrest after treatment with 1g or Cur for 18h. (f) Ratio of HCT116 cells in each phase determined using GraphPad Prism. (g) Expression of relative protein levels of the cell cycle determined by Western blot analysis after treatment with 1g (10 μ M) for varying durations. (h) Protein levels evaluated by Western blot analysis of 1g or Cur-treated HCT116 cells for the time indicated. Data represent means $\pm$ SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Statistical significance indicated as *P<0.05, **P<0.01, ***P<0.001. ns indicates that the comparison was not statistically significant.

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1g induces change in mitochondrial membrane potential in colon cancer cells. (a) HCT116 cells were treated with 1g (10μM) for varying durations. (b) Protein levels evaluated by Western blot analysis of 1g or Cur-treated HCT116 cells for 24h. (c) Mitochondrial membrane potential was analyzed by flow cytometry in HCT116 cells treated with 1g or Cur for 24h (n=3).

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Induction of cytotoxicity of 1g to colon cancer cells depends on the production of intracellular ROS. (a) Flow cytometric analysis of intracellular ROS generation induced by 1g over increasing durations measured in HCT116 cells after staining with DCFH-DA (10μM). (b) HCT116 cells pre-incubated with NAC (10mM) for 1h prior to exposure to 1g (10μM) for 8h. (c, d) HCT116 cells pretreated with NAC (10mM) for 1h, then treated with 1g (10μM) for 24h. Apoptotic proteins and cyclins measured by Western blotting. (e)

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ER-stress is involved in 1g induced apoptosis of colon cancer cells. (a) GRP78 and CHOP mRNA levels of HCT116 cells were measured after treatment 1g (10 μ M) from 0 to 8h. (b) GRP78 and CHOP mRNA levels of HCT116 cells were measured after treatment with various concentrations of 1g ranged from 0-10 μ M for 8h. (c) ER-stress protein levels were measured by Western blot analysis of HCT116 cells treated with 1g (10 μ M) for various durations. (d) Protein levels were measured by Western blot analysis of HCT116 cells treated with 1g or Cur for the duration indicated. (e) Expression of proteins measured by Western blot analysis of HCT116 cells pre-incubated with 10mM NAC for 1 h prior to treatment with 1g (10 μ M) for the duration indicated. (f) Expression of PERK, p-PERK, or CHOP determined by Western blot analysis of HCT116 cells incubated with p-PERK inhibitor (GSK2606414), after stimulation with 1g (10 μ M) for 2 h or 6 h.

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Figure 7

1g inhibits tumor growth in a xenograft mouse model of human colon cancer. (a) 7 days after HCT116 cell implantation, the mice were treated with either vehicle (control), 1g (20 mg/kg), 1g (40 mg/kg), or Cur (40 mg/kg) for an additional 14 days. The size of the tumor was shown in each group (n=6 each group). (b) Representative images of tumors in each treatment group. (c) Weight of Balb/c mice (n=6). (d) Cleaved caspase 3 levels evaluated in 1g-treated and Cur-treated xenografts. Relative density

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Figure 8

The picture of the mechanism of inhibition of colon cancer growth by 1g. 1g leads to the production of mitochondrial ROS, which then leads to cell cycle stagnation and ER-stress, leading to apoptotic cell death.

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