ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/263845638

Benzylidenetetralones, cyclic chalcone analogues, induce cell cycle arrest and apoptosis in HCT116 colorectal cancer cells

ARTICLE *in* TUMOR BIOLOGY · JULY 2014

Impact Factor: 3.61 · DOI: 10.1007/s13277-014-2289-y

CITATIONS
3

READS
88

10 AUTHORS, INCLUDING:

David Drutovic
Institute Animal Physiology and Genetics A…
5 PUBLICATIONS **9** CITATIONS
SEE PROFILE

Martina Pilatova
Pavol Jozef Šafárik University in Košice
32 PUBLICATIONS **273** CITATIONS
SEE PROFILE

Pal Perjesi
University of Pécs
72 PUBLICATIONS **549** CITATIONS
SEE PROFILE

Available from: David Drutovic
Retrieved on: 13 January 2016

Tumor Biol. (2014) 35:9967–9975
DOI 10.1007/s13277-014-2289-y

RESEARCH ARTICLE

Benzylidenetetralones, cyclic chalcone analogues, induce cell cycle arrest and apoptosis in HCT116 colorectal cancer cells

David Drutovic · Martina Chripkova · Martina Pilatova · Peter Kruzliak · Pal Perjesi · Marek Sarissky · Monica Lupi · Giovanna Damia · Massimo Broggini · Jan Mojzis

Received: 8 April 2014 / Accepted: 26 June 2014 / Published online: 10 July 2014
© International Society of Oncology and BioMarkers (ISOBM) 2014

Abstract Colorectal cancer is the third most common cancer in the world, with 1.2 million new cancer cases annually. Chalcones are secondary metabolite precursors of flavonoids that exhibit diverse biological activities, including antioxidant and antitumor activities. The aim of this study was to investigate the antiproliferative effect of new synthetic chalcone derivatives on HCT116 cells. (*E*)-2-(2′,4′-dimethoxybenzylidene)-1-tetralone (Q705) was found to be the most active (IC_{50}=3.44 ±0.25 μM). Based on these results, this compound was chosen for further analysis of its biochemical and molecular mechanisms. Our results showed that Q705 inhibited the growth and clonogenicity of HCT116 cells. The results of a flow cytometric analyses suggested that this compound caused a significant cell cycle arrest in G2/M phase and increased the proportion of cells in the subG0/G1 phase, marker of apoptosis. Q705-induced apoptosis was confirmed by TdT-mediated dUTP nick end labelling (TUNEL) assay. Treatment of HCT116 cells with this chalcone significantly increased the caspase-3,-7 activity and resulted in cleavage of poly-ADP-ribose polymerase (PARP). Changes in the nuclear morphology such as chromatin condensation were also observed. These effects were associated with a decreased expression of bcl-x_L and increased overall ratio of bax/bcl-x_L mRNA levels. Immunofluorescence and qRT-PCR analysis revealed that Q705 induced H2AX histone modifications characteristic of DNA damage, disruption of microtubule organization and downregulation of tubulins. In summary, these results suggest that the cyclic chalcone analogue Q705 has potential as a new compound for colorectal cancer therapy.

Keywords Benzylidenetetralones · Cyclic chalcone analogues · Cell cycle arrest · Apoptosis · Colorectal cancer cells

D. Drutovic · M. Chripkova · M. Pilatova · M. Sarissky · J. Mojzis
Department of Pharmacology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, Slovak Republic

P. Kruzliak (✉)
International Clinical Research Center, St. Anne's University Hospital, Pekarska 53, 65691 Brno, Czech Republic
e-mail: peter.kruzliak@savba.sk

P. Perjesi
Institute of Pharmaceutical Chemistry, Faculty of Medicine, University of Pécs, Pécs, Hungary

M. Lupi
Laboratory of Cancer Pharmacology, Department of Oncology, IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

G. Damia · M. Broggini
Laboratory of Molecular Pharmacology, Department of Oncology, IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

Abbreviations

ADP	adenosine diphosphate
cDNA	complementary DNA
Ct	threshold cycle
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
FITC	fluorescein isothiocyanate
mRNA	messenger RNA
PCR	polymerase chain reaction
qRT-PCT	quantitative reverse transcription PCR
RNA	ribonucleic acid
TdT	terminal deoxynucleotidyl transferase

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females with approximately

1.2 million new cancer cases worldwide and causes more than 600,000 deaths annually [1]. Although early diagnosis and rigorous screenings have reduced its incidence in recent years, chances of survival for patients with late stage colorectal carcinoma are low despite current treatments such as surgical excision, chemotherapy, and radiotherapy [2, 3]. Moreover, these therapeutic options are highly toxic and have harmful side effects [4]. Therefore, novel therapeutic agents for treatment of colorectal cancer are needed. Currently, the search for new drugs has refocused on natural products and the identification of anticancer constituents from plants is essential for advancing the treatment of colorectal cancer [3].

Natural products have been used as novel compounds in the treatment of many diseases, including cancer, because of their ability to inhibit cancer cell growth and induce apoptosis [5]. A number of clinically important cancer chemotherapeutic agents have been obtained from natural sources, by structural modification of natural compounds, or by the synthesis of new compounds, designed following a natural compound as a model and higher plants continue to be promising sources of new antitumor drugs [6, 7]. One major advantage of naturally occurring drugs is that many phytochemicals have shown promising anticancer results with little or no toxicity to normal cells. In addition, most of these phytochemicals are constituents of the human diet or are taken as dietary supplements [8].

Among currently identified anticancer agents, chalcones represent an important class of natural small molecules useful for cancer chemotherapy [9]. Chalcones are a group of polyphenols with widespread distribution in fruits, vegetables, tea, and spices such as hops or licorice [10, 11], and serve as secondary metabolite precursors for the synthesis of flavonoids and isoflavonoids [12]. Chemically, they are open-chain flavonoids in which two aromatic rings are joined by a three-carbon a,b-unsaturated carbonyl system (1,3-diphenyl-2-propen-1-ones) [13]. A good safety profile, abundance in plants, possibility of oral administration, and easy synthesis are the major factors contributing to the increasing interest in exploring therapeutic potential of chalcones [14, 15]. Chalcones and their derivatives have already been reported to exhibit several biological activities, including antitumor, antiangiogenic, antiinflammatory, antioxidant, immunomodulatory, antibacterial, antimalarial, antileishmanial, and trypanocidal activity [11, 12, 16–18]. Chalcones can induce apoptosis and cell cycle arrest in various cancer cells [19, 20], inhibit tumor promotion, and metastasis [21]. They are also effective in vivo as antiproliferative, antitumor promoting and chemopreventing agents [15].

In this study, antiproliferative effects of new synthetic chalcone derivatives on various cancer cell lines were investigated.

Materials and methods

Tested compounds

Chalcones 4-Methoxychalcone (Q126), 4-hydroxychalcone (Q797), 4-methylchalcone (Cs171);

Benzylideneindanones (*E*)-2-(4′-methylbenzylidene)-1-indanone (Q397), (*E*)-2-(4′-dimethylaminobenzylidene)-1-indanone (Q560), (*E*)-2-(2′,4′-dimethoxybenzylidene)-1-indanone (Q707), (*E*)-2-(4′-hydroxybenzylidene)-1-indanone (Q764);

Benzylidenetetralones (*E*)-2-(4′-hydroxybenzylidene)-1-tetralone (Q701), (*E*)-2-(2′,4′-dimethoxybenzylidene)-1-tetralone (Q705), (*E*)-2-(4′-methylbenzylidene)-1-tetralone (TE2), (*E*)-2-(4′-methoxybenzylidene)-1-tetralone (TE3).

Compounds were synthesized and purified as described before [22–24]. The structure and purity of the synthesized compounds were verified by spectroscopic (IR, ^{1}H NMR) and chromatographic (TLC, GC) methods [22].

Cell culture

Cancer cell lines HCT116 (human colorectal carcinoma), HeLa (human cervical adenocarcinoma), HepG2 (human hepatocellular carcinoma), and Jurkat (human leukemic T cell lymphoma) were cultured in RPMI 1640 medium (PAA Laboratories, Pasching, Austria) and A549 (human alveolar adenocarcinoma) and NIH/3 T3 (fibroblasts) was maintained in growth medium consisting of high glucose Dulbecco's modified Eagle medium (Invitrogen, USA). Both media were supplemented with a 10 % fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) (all from Invitrogen, USA). All cancer cell lines were cultured in an atmosphere containing 5 % CO_2 in humidified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95 % before each experiment.

MTT assay

The effect of synthetic chalcone derivatives on the viability and proliferation of cancer cell lines was determined using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5×10^3 cells/well in 96-well polystyrene microplates (SARSTEDT, Nümbrecht, Germany) in the culture medium containing the tested compounds at final concentrations of 1–100 μM. After 72 h, cells in each well were incubated with 10 μL of MTT (5 mg/mL, Sigma-Aldrich Chemie, Steinheim, Germany) at 37 °C. After an additional 4 h, during which insoluble formazan was produced, 100 μL of a 10 % sodium dodecyl

sulphate (SDS) was added in each well and another 12 h were allowed for the formazan to dissolve. The absorbance was measured at 540 nm using the automated uQuant™ Universal Microplate Spectrophotometer (BioTek). Three independent experiments had been conducted for each test. Results from the MTT assay had been used to derive a half maximal inhibitory concentration (IC_{50}) of each drug.

Colony formation assay

To determine colony formation, cells were seeded in 6-well plates (5×10^3 cells/well) and allowed to adhere for 24 h before treatment. A culture medium containing variable concentrations of the tested compound was added to cells and incubated for 10 days to allow colony formation. Colonies were then fixed in 4 % formaldehyde at room temperature for 30 min and stained with 0.01 % crystal violet. The crystal violet stain was then extracted with 10 % acetic acid for 60 min and read at 540 nm. Cell survival at each drug concentration was expressed as a percentage of survival of controls.

Flow cytometry analysis

1. Cell cycle analysis

   Cellular deoxyribonucleic acid (DNA) content was determined by flow cytometric analysis of propidium iodide (PI)-labeled cells. HCT116 cells (1×10^6) after treatment with tested compound (5 μM) for 24, 48, and 72 h were trypsinized, washed twice in phosphate-buffered saline (PBS) and fixed overnight in 70 % ethanol at −20 °C. After washing twice with PBS, fixed cells were treated with 1 mg/mL of RNase A for 1 h and stained with 10 μg/mL PI in the dark at room temperature. Cell cycle distribution and subdiploid population were analyzed on a FACS Vantage SE flow cytometer using CellQuest software (Becton Dickinson, USA). Ten thousand cells were required per analysis. Results were analyzed using WinMDI software. Percentages of cells corresponding to G0/G1, S, and G2/M phases of the cell cycle were calculated. A sub-G0/G1 fraction of cells was identified as an apoptotic population.
2. Two-parameter flow cytometry analysis: DNA content and FITC-conjugated dUTP

   DNA fragmentation was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling technique (TUNEL), which uses TdT to catalyze the binding of fluorescein isothiocyanate (FITC)-conjugated dUTP to DNA strand breaks. This technique detects DNA fragmentation induced during apoptosis. At the end of each time point, cells were washed with PBS and harvested by trypsinization. After centrifugation, the cells were resuspended in saline GM, fixed with 70 % cold ethanol on ice for 30 min and then stored at 4 °C. About 1×10^6 fixed cells were washed in PBS and permeabilized for 2 min on ice in 0.1 % Triton X-100, 0.1 % sodium citrate. The cells were washed, resuspended in 50 μL of TUNEL reaction mixture (Roche, USA) containing dUTP-FITC and TdT, and incubated for 90 min at 37 °C in the dark. After that, the samples were washed and resuspended in 1 μg/mL PI plus 25 μL of 1 mg/mL RNAse in water and incubated overnight at 4 °C. Samples were analyzed by FACS.

Assessment of caspase-3,-7 activity

Cells cultured in 6-well plates for 24 h were treated with the tested compound and then washed with PBS and lysed for 30 min on ice. Then, the lysates were centrifuged at 12,000*g* for 10 min at 4 °C. The protein concentrations in the supernatants were determined using the BioRad assay kit (BioRad, USA). The enzymatic activities of caspase-3 and -7 were assayed with 20-mM Ac-DEVD-AMC fluorogenic substrate (Sigma-Aldrich) in an assay buffer. The fluorescence was measured at 460 nm using a fluorescence spectrofluorometer, with excitation at 370 nm.

Western blot analysis

Cells were treated with the tested compound (5 μM) for 24, 48, and 72 h. Protein extracts were obtained using a lysis buffer containing 10-mM Tris–HCl pH 7.4, 150-mM NaCl, 0.1 % Nonidet NP-40, 5-mM EDTA, and 50-mM NaF in the presence of aprotinin, leupeptin, and PMSF as protease inhibitors, for 30 min on ice. After the insoluble materials were removed by centrifugation at 12,000*g* for 10 min at 4 °C, total protein concentrations were quantified using the BioRad assay kit (BioRad, USA). Thirty micrograms of total cellular proteins were separated on 8 % SDS polyacrylamide gels and electrotransferred onto nitrocellulose membranes (PROTRAN, Schleicher e Schuell). Membranes were blocked in 5 % skim milk in Tris-buffered saline (TBS) containing 0.1 % Tween-20 for 1 h at room temperature to minimize non-specific binding and incubated with the primary antibodies overnight at 4 °C. Immunoblotting was carried out with PARP Antibody (Cell Signaling Technology, Inc.) and Actin Antibody (C-11, Santa Cruz Biotechnology, Inc.). After incubation with primary antibodies, membranes were washed 1×5 min with TBS-Tween followed by an incubation of 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibodies (Goat anti-rabbit IgG-HRP, Donkey anti-goat IgG-HRP, all from Santa Cruz Biotechnology, Inc.). After washing 4×10 min with TBS-Tween expression was detected by chemiluminescence emission using ECL Western Blotting Detection Reagent (Amersham) and then the blots were exposed to x-ray films.

RNA isolation and quantitative real time PCR

Total RNA was extracted from HCT116 cells using the TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's instruction. The total RNA quality was verified on an agarose gel. One microgram of total RNA was converted to complementary DNA (cDNA) by the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas GmbH) according to the manufacturer's instruction and used for quantitative real time polymerase chain reaction (PCR).

α-tubulin, α_1-tubulin, β_5-tubulin, bcl-x_L and bax gene expression were quantified by quantitative real time PCR analysis, performed in Light Cycler (Roche, USA) using iQTM SYBR Green Supermix (Bio-Rad Laboratories, USA). The PCR program was initiated by 5 min at 95 °C before 40 thermal cycles, each of 30 s at 95 °C and 45 s at 60 °C. Data was analyzed according to the comparative threshold cycle (Ct) method and was normalized by β-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

DAPI staining

At the indicated time after treatment, cells grown on cover slips were fixed with 2 % paraformaldehyde for 30 min at 4 °C followed by permeabilization with 0.3 % Triton X-100 and incubation with a blocking solution containing 0.1 % FBS in PBS. After incubation, the cells were washed briefly with 0.1 % FBS in PBS and incubated with 8 % FBS in PBS for 30 min. Cells were then washed with PBS and incubated at room temperature with SlowFade® Gold antifade reagent with 4′,6-diaminidino-2-phenyl-indole, dihydrochloride (DAPI) (Invitrogen, USA) for nuclear visualization and observed under fluorescence microscope (Leica DMI6000 B, Leica Microsystems, Inc., Bannockburn, IL, USA).

Immunofluorescence analysis

HCT116 cells were grown on glass coverslips. Cells were then fixed in 4 % paraformaldehyde for 30 min at room temperature. After permeabilization with Triton X-100 0.5 % in PBS for 15 min, cells were blocked in 2 % bovine serum albumin (BSA), 0.2 % Triton X-100 in PBS (blocking buffer) for 1 h and then incubated with the anti-phospho-histone H2A.X primary antibody (1:1000, clone JBW301, EMD Millipore Corporation, Billerica, MA, USA). After 1 h, a secondary antibody (1:500, Alexa Fluor, 594, Molecular Probes) was added. For tubulin, visualization cells were incubated with an anti alpha-tubulin primary antibody (1:100, clone YOL1/34, Oxford Biotechnology Ltd.) overnight and then a secondary antibody (1:100, Anti-Rat IgG-FITC antibody, Sigma-Aldrich) was added. DAPI (final concentration 30 ng/mL in PBS, Sigma-Aldrich) was added for 10 min to stain nuclei and cells were mounted with the mounting medium Fluor Save (Calbiochem, Darmstadt, Germany). Immunofluorescence images were acquired on a confocal microscope.

Statistical analysis

Statistical data are expressed as mean±standard deviation (SD). Student's *t* test and analysis of variance were employed to determine statistical significance. Values of $P<0.05$ were considered to be statistically significant.

Results

Antiproliferative effect of synthetic chalcone derivatives

The antiproliferative effect of synthetic chalcone derivatives on cancer cells was evaluated by MTT assay using different concentrations of tested compounds. As shown in Table 1, synthetic chalcones suppressed cell proliferation with IC_{50} values ranging from 3.4 to >100 μM. Among these derivatives, compound (*E*)-2-(2′,4′-dimethoxybenzylidene)-1-tetralone (Q705), was the most effective. In NIH/3 T3 cells, significant effect was observed after incubation with Q705 at concentrations 50–100 μM. Therefore, further experiments studying the antiproliferative effect of this chalcone derivative were performed. Because HCT116 cells were more sensitive to compound Q705 than other cell lines tested, HCT116 cells were used in the subsequent experiments. To evaluate the effect of compound Q705 on HCT116 colony-forming ability, a colony formation assay was performed. As shown in Fig. 1, compound Q705 again inhibits the proliferation of HCT116 cells in a concentration-dependent manner.

Effect of compound Q705 on cell cycle and apoptosis

To examine whether Q705 treatment could affect cell cycle progression, HCT116 cells were treated with compound Q705 at 5 μM—a dose close to its IC_{50}. As shown in Table 2, Q705 treatment caused a marked increase in the number of cells arrested at G2/M phase in HCT116 cells (41.87 vs 27.02 % in non-treated cells) and a significant accumulation of cells in sub-G0/G1 from 0.93 % in control cells to 22.90, 42.33, and 48.68 % in treated cells after 24-, 48-, and 72-h incubation, respectively. These results indicate that Q705 treatment induced apoptosis.

We used the TUNEL assay, which measures the amount of fragmented DNA during apoptosis, to confirm the proapoptotic activity of compound Q705 in HCT116 cells. A significant increase of dUTP-positive cells was observed when cells were treated at concentrations of 5 μM, reaching

Table 1 IC_{50} values of synthetic chalcone derivatives on cancer cell lines

IC_{50}±SD (μmol l^{-1})					
Compound	A549	HCT116	HeLa	HepG2	Jurkat
Q126	>100	66.84±2.83	>100	63.18±8.99	>100
Q797	>100	32.64±1.21	71.40±1.54	73.06±1.70	33.78±2.14
Cs171	77.13±5.21	33.02±0.56	>100	>100	33.45±1.98
Q397	>100	>100	>100	94.52±7.76	>100
Q560	>100	>100	>100	>100	>100
Q707	>100	>100	>100	>100	>100
Q764	>100	98.91±1.03	>100	95.78±3.96	>100
Q701	>100	97.99±1.68	>100	>100	58.27±3.11≠
Q705	8.75±1.28	3.44±0.25	23.90±2.18	41.31±9.49	58.38±4.22
TE2	>100	>100	>100	>100	>100
TE3	>100	73.73±2.11	>100	>100	47.10±2.25

Cancer cells were cultured for 24 h prior to synthetic chalcones treatment. Cells were incubated with synthetic chalcones for 72 h. Cell viability was determined by MTT colorimetric assay. Results are presented as mean±SD of three independent experiments.

the highest amount of apoptotic cells after 48 h of treatment (Fig. 2a). The TUNEL assay showed that Q705 induced apoptosis in HCT116 cells.

The nuclear morphological changes of HCT116 cells under apoptosis were studied using fluorescence nuclear staining. In control groups, cells appeared to be round and manifested homogeneous nuclei (Fig. 2b). In cells treated with compound Q705 for 24 h, the cells displayed condensed and fragmented nuclei. These observations denote the proapoptotic potential of compound Q705 in HCT116 colon cancer cells.

To elucidate whether caspase activation was involved in Q705-induced apoptosis, we examined the activity of caspase-3 and -7 in the HCT116 cells in the same experimental setting. As shown in Fig. 2c, a significant increase of caspase-3,-7 activity was observed, reaching a maximum after 24 h (2.67-fold increase in activity).

The poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP) cleavage, which is a known marker of apoptosis and a downstream target of activated caspase-3 [25], was analyzed by Western blotting and again the cleaved form of PARP (89 kDa) was significantly increased in HCT116 cells treated with 5 μM of compound Q705 compared to controls at all treatment times from 24 to 72 h (Fig. 2d).

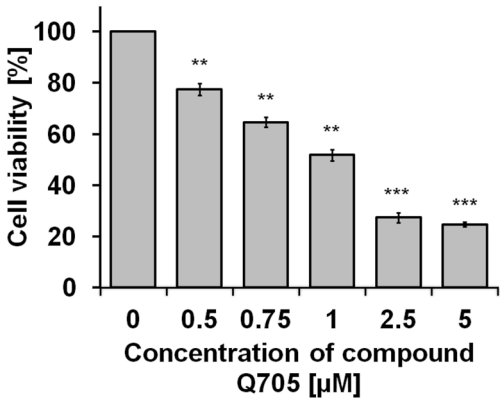

Fig. 1 Effect of compound Q705 on colony formation of HCT116 cells. Results are presented as mean±SD of three independent experiments. $^{**}P<0.01$ vs. untreated cells (control) $^{***}P<0.001$ vs. untreated cells (control)

Effect of chalcone Q705 on the expression of apoptosis genes and tubulins

The effect of Q705 treatment on the expression of antiapoptotic and proapoptotic members of Bcl-2 family was examined. A significant decrease of the antiapoptotic bcl-x_L gene expression was observed (Table 3). The overall ratio of bax/bcl-x_L messenger ribonucleic acid (mRNA) levels was significantly increased in HCT116 cells following the treatment with Q705.

To better understand the mechanisms of chalcone Q705-induced G2/M arrest, we conducted quantitative real time PCR analysis for selected genes. After 48-h incubation of

Table 2 Effect of compound Q705 on cell cycle distribution (percentage) in HCT116 cells

	Sub-G0/G1	G0/G1	S	G2/M
Control	0.93±0.15	53.86±3.42	18.61±5.37	±
24 h	22.90±2.82**	19.01±4.79*	16.75±3.37	±
48 h	42.33±1.94**	20.25±2.45**	17.81±5.30	±
72 h	48.68±5.47**	17.67±0.74**	18.01±0.41	

Cells were treated with 5 μM of Q705 for 24, 48, and 72 h, stained with PI and analyzed for cell cycle phase using flow cytometry. Results are presented as mean±SD of three independent experiments.

$^{*}P<0.05$ vs. untreated cells (control)

$^{**}P<0.01$ vs. untreated cells (control)

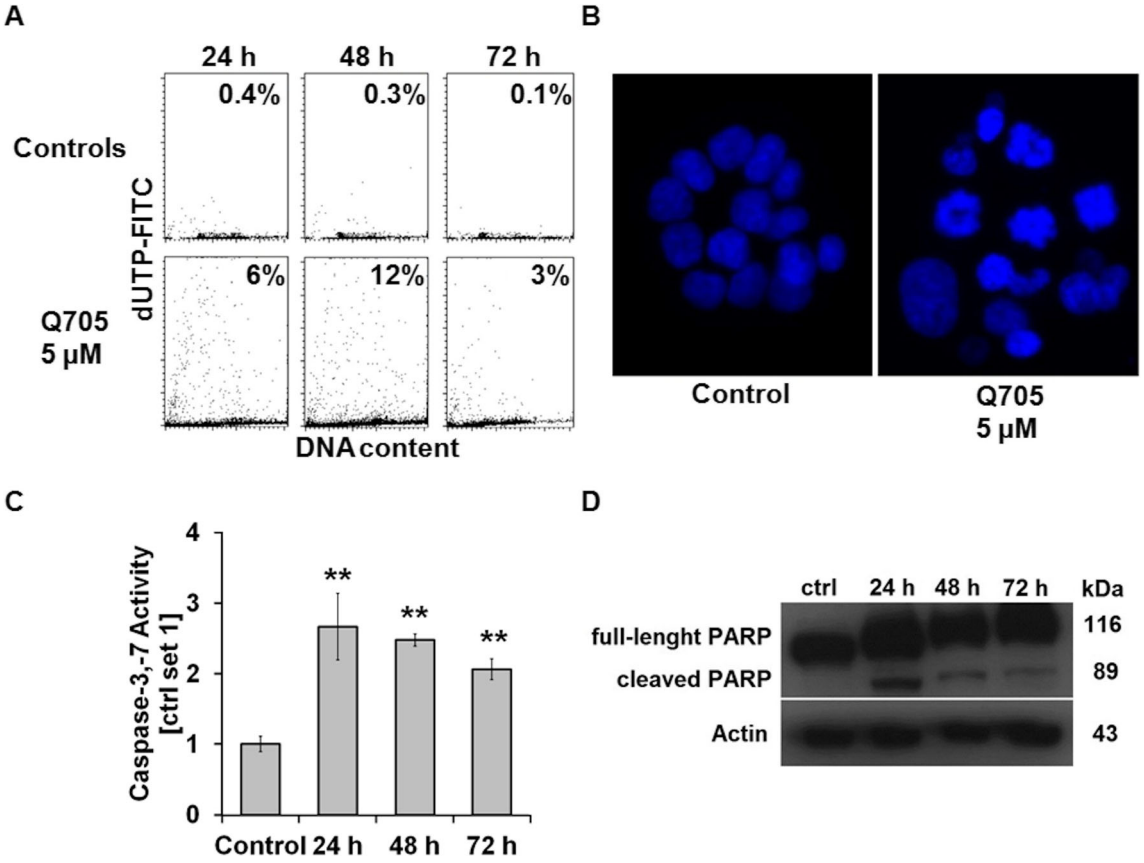

Fig. 2 Apoptotic effect of compound Q705 on HCT116 cells. **a** Biparametric staining with dUTP-FITC and PI (DNA content) of HCT116 cells following treatment with 5 μM of compound Q705 for 24, 48, and 72 h. Cells with DNA fragmentation induced during apoptosis are dUTP-positive (detected above the *straight line*) and their percentage is reported on each plot. **b** Morphological changes of HCT116 cells treated with 5 μM of compound Q705 for 24 h. Cells were cultured in chamber slides and stained with DAPI to show typical apoptotic morphology. **c** Caspase-3,-7 activity in HCT116 cells after incubation with compound Q705 at a concentration of 5 μM for the time intervals 24, 48, and 72 h. Data represents mean±SD, compared to control, $^{**}P<0.01$ vs. untreated cells (control). **d** HCT116 cells were treated for 24, 48, and 72 h with the compound Q705 (5 μM). Expression of cleaved PARP, the index for apoptosis, was determined by using Western blotting. Actin served as a loading control

cancer cells with Q705 (5 μmol/L), we found a downregulation of the expression of α-tubulin, α_1-tubulin, and β_5-tubulin in HCT116 cells (Table 3) suggesting the inhibitory effect of this chalcone on microtubules.

γH2AX nuclear foci formation and immunofluorescence analysis of α-tubulin

To gain insights into the mechanisms regulating DNA damage, histone modifications were analyzed. Formation of γH2AX nuclear foci, a characteristic of double strand breaks, was detected by immunofluorescence with γH2AX-specific antibodies in HCT116 cells treated with 5 μM of compound Q705 for 16 h. An increase in a number of cells with γH2AX nuclear foci was observed in treated cells, compared with controls (Fig. 3a). These results indicate that compound Q705 can induce H2AX histone modifications characteristic of DNA damage, preceding the activation of apoptotic cell death.

The observation that Q705 downregulated the expression of tubulins prompted us to investigate whether this compound actually leads to the disruption of microtubule organization. Using confocal microscopy, we found that compound Q705 altered the organization of microtubules compared to control

Table 3 Effect of compound Q705 (5 μM) on specific genes expression in HCT116 cells

Gene	Normalized ratio to β-actin		
	24 h	48 h	72 h
Bcl-X_L	0.39	0.69	0.25
Bax	0.79	1.53	1.46
α-Tubulin	1.3	0.73	0.48
α_1-Tubulin	0.9	0.42	0.48
β_5 Tubulin	1.11	0.48	0.63

β-actin was used as a housekeeping gene to normalize each sample.

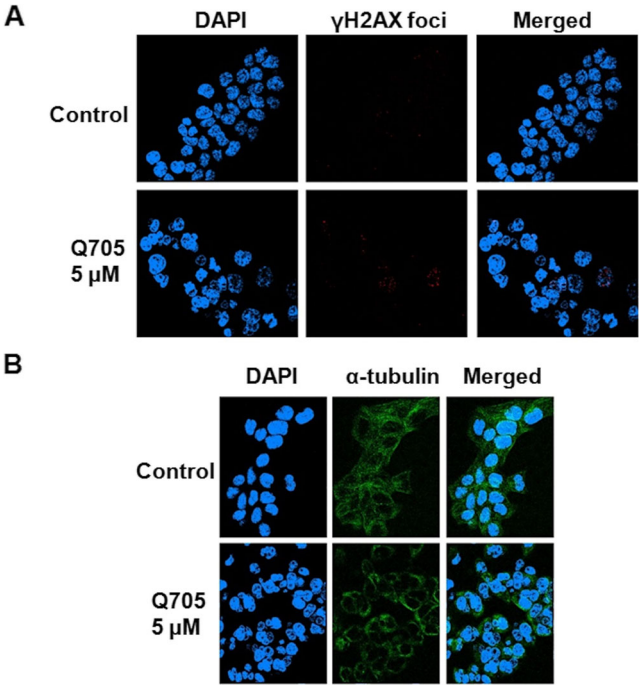

Fig. 3 Immunofluorescence analysis of HCT116 cells after incubation with compound Q705. **a** Compound Q705 induces H2AX phosphorylation. HCT116 cells were immunostained with anti-γH2AX antibody and counterstained with DAPI after treatment with 5 μM of compound Q705 for 16 h. **b**Effect of compound Q705 on the microtubule structure of HCT116 cells. Control cells and cells treated with compound Q705 at concentration of 5 μM for 24 h, were fixed and stained with α-tubulin antibody and counterstained with DAPI. α-tubulin are shown in *green*, DNA, stained with DAPI, is shown in *blue*

cells with well-organized microtubule network (Fig. 3b). This data suggests that compound Q705 can affect the microtubule organization in HCT116 cells and thus contribute to induction of cell cycle arrest.

Discussion

In the last decade, the focus of cancer control has been on the search for new antitumor drugs, which are safer and have higher patient acceptability. Various phytochemicals, which are generally a part of human diet or traditional herbal medicine, have been receiving attention in this regard. In this context, the naturally occurring chalcones and their synthetic derivatives are extensively studied in vitro and in vivo for their anticancer potential [26, 27].

In the present study, we aimed to analyze the effect of synthetic chalcone derivatives on the viability and proliferation of various cancer cell lines. First, we evaluated the antiproliferative effect of these compounds by MTT assay. Among them, Q705 was the most effective compound on HCT116 cells with IC_{50}=3.44 μM. In our in vitro studies, the treatment of HCT116 cells with this chalcone inhibited the growth of cells in a concentration-dependent manner. Consistent with this result, it also inhibited clonogenic cell growth of HCT116 cells and caused a significant decrease of cell viability at a concentration of 0.5 μM. Interestingly, this chalcone derivative did not exert any significant cytotoxic effect in HUVECs even at a concentration of 100 μM [28]. Further study of the mechanism underlying the antiproliferative activity of this derivative by flow cytometry indicates that growth inhibition may be due to cell cycle arrest (G2/M accumulation) as well as apoptotic cell death (increase in subG0/G1 fraction). We observed a similar effect on cell cycle progression and subG0/G1 accumulation with another synthetic chalcone derivative on Jurkat and Hela cells [28, 29].

Emerging evidence demonstrates that the anticancer activities of several chemotherapeutic agents are involved in the induction of apoptotic cell death [30, 31]. Apoptosis is characterized by several morphological appearances such as condensation of the nucleus associated to DNA fragmentation, cell membrane blebbing and caspase activation [27]. Cysteine aspartases (caspases), a protease family, are required for apoptosis induced by various stimuli. In the death receptor pathway of apoptosis, receptor ligation leads to the activation of caspase-8. In the mitochondrial pathway, on the other hand, various apoptosis-inducing triggers such as DNA damage cause activation of caspase-9. In these two different pathways, activated initiators caspases finally activate effector caspases, such as caspase-3 and -7. Caspase-3 is considered the main effector of caspases and has been identified as being activated in response to cytotoxic drugs [32]. Its activation is an important step in the execution of the apoptotic signal through proteolytic degradation of its substrate PARP, which occurs at the onset of apoptosis [25]. Our study indicates that treatment of HCT116 cells with compound Q705 resulted in significant increase in caspase-3,-7 activity, leading to cleavage of PARP and increase of cell apoptosis. The increased proportion of condensed and fragmented nuclei determined by fluorescence staining with DAPI and the detection of dUTP-positive cell by TUNEL assay reinforce the fact that treatment with Q705 induces cell apoptosis.

Apoptosis key regulators are the bcl2 family genes, including antiapoptotic members (bcl2, $bclx_L$) and proapoptotic members (such as bim, bax, bak) [27]. In this work, the antiapoptotic protein Bcl-x_L was studied. It belongs to the Bcl-2 family of proteins, whose over-expression prevents or delays apoptosis induced by a variety of stimuli [33]. We observed a decrease in bcl-x_L expression in HCT116 cells after the treatment with compound Q705, facilitating the apoptotic processes.

Altogether, the present study demonstrates that HCT116 cells exposed to compound Q705 exhibited morphological and biochemical changes characteristic of apoptosis, including a loss of cell viability, internucleosomal DNA fragmentation, chromatin condensation, sub-G0/G1 accumulation, activation of effector caspases, degradation of PARP and

dysregulation of bcl-x_L and bax. Our findings are consistent with study of Dimmock et al. who found stronger antiproliferative effects of benzylidenetetralones compared with benzylideneindanones [22]. They suggest that a difference between the location of the arylmethylene ring in benzylideneindanones and the benzylidenetetralones may contribute to variations in the cytotoxicities between the groups [22]. Furthermore, proapoptotic effects of various naturally occurring chalcones, such as flavokawain B [5], isoliquiritigenin [34], and butein [35] and their synthetic derivates [36, 37] in HCT116 cells and other colorectal cancer cell lines were documented.

In addition, we were able to demonstrate that the compound also induced a cell cycle inhibition: a G2/M arrest. Inhibition of the cell cycle is one of the therapeutic targets for the control of tumor growth [38, 39]. A large number of phytochemicals have been shown to inhibit cell cycle progression [12].

It has previously been reported that the cytotoxic activity of various compounds containing the chalcone skeleton is often associated with tubulin inhibition and the ability to interfere with microtubule formation [40, 41]. The chalcone analogues of combretastatin, a natural product with high affinity binding in the colchicine site of tubulin, have been synthesized and developed by Ducki et al. These chalcone analogues showed potent inhibition of tubulin assembly and possesses promising anticancer activity [42]. Therefore we focused on the effect of chalcone derivative Q705 on microtubules to elucidate the mechanism by which this compound arrests cells in G2/M phase.

Microtubules, composed of α/β-tubulin heterodimers, are major components of the cytoskeleton of eukaryotic cells and play an important role in several critical cellular functions [41]. Their most important role is in the formation of the mitotic spindle, and are considered essential in the mitotic division [43]. Interference with the assembly or disassembly of microtubules in dividing cells blocks the cell division machinery and leads to an increase in the number of cells in metaphase arrest, triggering signals that induce apoptotic cell death [44, 45]. Therefore, dynamic mitotic-spindle microtubules have become an important target for the design and development of new anticancer agents [43]. Here, RT-PCR analysis showed that Q705 treatment led to a decreased expression of tubulins in HCT116 cells and immunofluorescence analysis of α-tubulin showed disruption of the microtubule structure. On the other hand, cells treated with Q705 showed an accumulation of nuclear γH2AX foci. Phosphorylation of H2AX, a variant of histone H2A at Ser-139 (γH2AX) is considered to be a sensitive molecular marker of DNA damage, such as presence of DNA double-stranded breaks [46]. It can form nuclear foci surrounding the damage sites and its activation has an important role in the DNA damage response including stimulation of DNA repair and activation of cell cycle checkpoints. In addition, a recent finding suggests a function of H2AX in apoptosis [47–49]. These results show that Q705 treatment can induce cell cycle arrest at the G2/M phases via the interference with microtubule formation and induction of the double strand breake type of DNA damage in HCT116 cells.

In conclusion, we herein demonstrated that Q705-treatment is associated with inhibition of HCT116 colorectal cancer cells growth via induction of apoptosis and inhibition of cell cycle. Based on its proapoptotic effect, the chalcone derivative Q705 is a potential inhibitor of proliferation in colorectal cancer cells and might be a novel promising chemotherapeutic agent for colorectal cancer treatment.

Acknowledgments This work was supported by SEPO-II (ITMS code: 26220120039) and European Regional Development Fund—Project FNUSA-ICRC (No. CZ.1.05/1.1.00/02.0123). We would like to thank Elisa Caiola and Laura Carrassa for technical support and Tom Billingham for his careful proof-reading of the manuscript.

Conflict of interest None.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
2. Figueredo A, Coombes ME, Mukherjee S. Adjuvant therapy for completely resected stage II colon cancer. Cochrane Database Syst Rev. 2008:CD005390.
3. Wang CZ, Calway TD, Wen XD, Smith J, Yu C, Wang Y, et al. Hydrophobic flavonoids from Scutellaria baicalensis induce colorectal cancer cell apoptosis through a mitochondrial-mediated pathway. Int J Oncol. 2013;42:1018–26.
4. Velmurugan B, Gangar SC, Kaur M, Tyagi A, Deep G, Agarwal R. Silibinin exerts sustained growth suppressive effect against human colon carcinoma SW480 xenograft by targeting multiple signaling molecules. Pharm Res. 2010;27:2085–97.
5. Kuo YF, Su YZ, Tseng YH, Wang SY, Wang HM, Chueh PJ. Flavokawain B, a novel chalcone from *Alpinia pricei* Hayata with potent apoptotic activity: involvement of ROS and GADD153 upstream of mitochondria-dependent apoptosis in HCT116 cells. Free Radic Biol Med. 2010;49:214–26.
6. Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol. 2007;9:767–76.
7. Cassidy CE, Setzer WN. Cancer-relevant biochemical targets of cytotoxic *Lonchocarpus* flavonoids: a molecular docking analysis. J Mol Model. 2010;16:311–26.
8. Tang Y, Li X, Liu Z, Simoneau AR, Xie J, Zi X. Flavokawain B, a kava chalcone, induces apoptosis via up-regulation of death-receptor 5 and Bim expression in androgen receptor negative, hormonal refractory prostate cancer cell lines and reduces tumor growth. Int J Cancer. 2010;127:1758–68.
9. Romagnoli R, Baraldi PG, Carrion MD, Cruz-Lopez O, Cara CL, Balzarini J, et al. Hybrid alpha-bromoacryloylamido chalcones. Design, synthesis and biological evaluation. Bioorg Med Chem Lett. 2009;19:2022–8.
10. Szliszka E, Mazur B, Zydowicz G, Czuba ZP, Krol W. TRAIL-induced apoptosis and expression of death receptor TRAIL-R1 and TRAIL-R2 in bladder cancer cells. Folia Histochem Cytobiol. 2009;47:579–85.

11. Kim SY, Lee IS, Moon A. 2-Hydroxychalcone and xanthohumol inhibit invasion of triple negative breast cancer cells. Chem Biol Interact. 2013;203:565–72.
12. Rao YK, Fang SH, Tzeng YM. Synthesis and biological evaluation of 3′,4′,5′-trimethoxychalcone analogues as inhibitors of nitric oxide production and tumor cell proliferation. Bioorg Med Chem. 2009;17: 7909–14.
13. Ku BM, Ryu HW, Lee YK, Ryu J, Jeong JY, Choi J, et al. 4′-Acetoamido-4-hydroxychalcone, a chalcone derivative, inhibits glioma growth and invasion through regulation of the tropomyosin 1 gene. Biochem Biophys Res Commun. 2010;402: 525–30.
14. Orlikova B, Tasdemir D, Golais F, Dicato M, Diederich M. The aromatic ketone 4′-hydroxychalcone inhibits TNFalpha-induced NF-kappa B activation via proteasome inhibition. Biochem Pharmacol. 2011;82:620–31.
15. Sashidhara KV, Kumar A, Kumar M, Sarkar J, Sinha S. Synthesis and in vitro evaluation of novel coumarin-chalcone hybrids as potential anticancer agents. Bioorg Med Chem Lett. 2010;20:7205–11.
16. Mojzis J, Varinska L, Mojzisova G, Kostova I, Mirossay L. Antiangiogenic effects of flavonoids and chalcones. Pharmacol Res. 2008;57:259–65.
17. Yadav VR, Prasad S, Sung B, Aggarwal BB. The role of chalcones in suppression of NF-kappaB-mediated inflammation and cancer. Int Immunopharmacol. 2011;11:295–309.
18. Orlikova B, Tasdemir D, Golais F, Dicato M, Diederich M. Dietary chalcones with chemopreventive and chemotherapeutic potential. Genes Nutr. 2011;6:125–47.
19. Hsu YL, Kuo PL, Tzeng WS, Lin CC. Chalcone inhibits the proliferation of human breast cancer cell by blocking cell cycle progression and inducing apoptosis. Food Chem Toxicol. 2006;44:704–13.
20. Zi X, Simoneau AR. Flavokawain A, a novel chalcone from kava extract, induces apoptosis in bladder cancer cells by involvement of Bax protein-dependent and mitochondria-dependent apoptotic pathway and suppresses tumor growth in mice. Cancer Res. 2005;65: 3479–86.
21. Tabata K, Motani K, Takayanagi N, Nishimura R, Asami S, Kimura Y, et al. Xanthoangelol, a major chalcone constituent of Angelica keiskei, induces apoptosis in neuroblastoma and leukemia cells. Biol Pharm Bull. 2005;28:1404–7.
22. Dimmock JR, Kandepu NM, Nazarali AJ, Kowalchuk TP, Motaganahalli N, Quail JW, et al. Conformational and quantitative structure-activity relationship study of cytotoxic 2-arylidenebenzocycloalkanones. J Med Chem. 1999;42:1358–66.
23. Varinska L, van Wijhe M, Belleri M, Mitola S, Perjesi P, Presta M, et al. Anti-angiogenic activity of the flavonoid precursor 4-hydroxychalcone. Eur J Pharmacol. 2012;691:125–33.
24. Dimmock JR, Zello GA, Oloo EO, Quail JW, Kraatz HB, Perjesi P, et al. Correlations between cytotoxicity and topography of some 2-arylidenebenzocycloalkanones determined by x-ray crystallography. J Med Chem. 2002;45:3103–11.
25. Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, et al. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem. 1999;274:22932–40.
26. Kaur P, Kaur S, Kumar N, Singh B, Kumar S. Evaluation of antigenotoxic activity of isoliquiritin apioside from *Glycyrrhiza glabra* L. Toxicol In Vitro. 2009;23:680–6.
27. Champelovier P, Chauchet X, Hazane-Puch F, Vergnaud S, Garrel C, Laporte F, et al. Cellular and molecular mechanisms activating the cell death processes by chalcones: critical structural effects. Toxicol In Vitro. 2013;27:2305–15.
28. Pilatova M, Varinska L, Perjesi P, Sarissky M, Mirossay L, Solar P, et al. In vitro antiproliferative and antiangiogenic effects of synthetic chalcone analogues. Toxicol In Vitro. 2010;24:1347–55.
29. Rozmer Z, Berki T, Perjesi P. Different effects of two cyclic chalcone analogues on cell cycle of Jurkat T cells. Toxicol In Vitro. 2006;20: 1354–62.
30. Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407: 770–6.
31. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res. 1999;59:1391–9.
32. Krepela E. Cysteine proteinases in tumor cell growth and apoptosis. Neoplasma. 2001;48:332–49.
33. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998;281:1322–6.
34. Auyeung KK, Ko JK. Novel herbal flavonoids promote apoptosis but differentially induce cell cycle arrest in human colon cancer cell. Invest New Drugs. 2010;28:1–13.
35. Moon DO, Kim MO, Lee JD, Choi YH, Kim GY. Butein suppresses c-Myc-dependent transcription and Akt-dependent phosphorylation of hTERT in human leukemia cells. Cancer Lett. 2009;286:172–9.
36. Shin SY, Yoon H, Hwang D, Ahn S, Kim DW, Koh D, et al. Benzochalcones bearing pyrazoline moieties show anti-colorectal cancer activities and selective inhibitory effects on aurora kinases. Bioorg Med Chem. 2013;21:7018–24.
37. de Vasconcelos A, Campos VF, Nedel F, Seixas FK, Dellagostin OA, Smith KR, et al. Cytotoxic and apoptotic effects of chalcone derivatives of 2-acetyl thiophene on human colon adenocarcinoma cells. Cell Biochem Funct. 2013;31:289–97.
38. Lin E, Lin WH, Wang SY, Chen CS, Liao JW, Chang HW, et al. Flavokawain B inhibits growth of human squamous carcinoma cells: Involvement of apoptosis and cell cycle dysregulation in vitro and in vivo. J Nutr Biochem. 2012;23:368–78.
39. Xiao XY, Hao M, Yang XY, Ba Q, Li M, Ni SJ, et al. Licochalcone A inhibits growth of gastric cancer cells by arresting cell cycle progression and inducing apoptosis. Cancer Lett. 2011;302:69–75.
40. Ducki S. Antimitotic chalcones and related compounds as inhibitors of tubulin assembly. Anticancer Agents Med Chem. 2009;9:336–47.
41. Wang G, Peng F, Cao D, Yang Z, Han X, Liu J, et al. Design, synthesis and biological evaluation of millepachine derivatives as a new class of tubulin polymerization inhibitors. Bioorg Med Chem. 2013;21:6844–54.
42. Ducki S, Forrest R, Hadfield JA, Kendall A, Lawrence NJ, McGown AT, et al. Potent antimitotic and cell growth inhibitory properties of substituted chalcones. Bioorg Med Chem Lett. 1998;8:1051–6.
43. Jordan A, Hadfield JA, Lawrence NJ, McGown AT. Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle. Med Res Rev. 1998;18:259–96.
44. Pasquier E, Andre N, Braguer D. Targeting microtubules to inhibit angiogenesis and disrupt tumour vasculature: implications for cancer treatment. Curr Cancer Drug Targets. 2007;7:566–81.
45. Odlo K, Hentzen J. dit Chabert JF, Ducki S, Gani OA, Sylte I, et al. 1, 5-Disubstituted 1,2,3-triazoles as cis-restricted analogues of combretastatin A-4: synthesis, molecular modeling and evaluation as cytotoxic agents and inhibitors of tubulin. Bioorg Med Chem. 2008;16:4829–38.
46. Kinner A, Wu W, Staudt C, Iliakis G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res. 2008;36:5678–94.
47. Lavin MF, Birrell G, Chen P, Kozlov S, Scott S, Gueven N. ATM signaling and genomic stability in response to DNA damage. Mutat Res. 2005;569:123–32.
48. Sluss HK, Davis RJ. H2AX is a target of the JNK signaling pathway that is required for apoptotic DNA fragmentation. Mol Cell. 2006;23: 152–3.
49. Li GX, Chen YK, Hou Z, Xiao H, Jin H, Lu G, et al. Pro-oxidative activities and dose-response relationship of (-)-epigallocatechin-3-gallate in the inhibition of lung cancer cell growth: a comparative study in vivo and in vitro. Carcinogenesis. 2010;31:902–10.