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ORIGINAL ARTICLE

Cytotoxic diarylheptanoid induces cell cycle arrest and apoptosis via increasing ATF3 and stabilizing p53 in SH-SY5Y cells

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Abstract
Purpose The aim of the study is to dissect the cytotoxic mechanisms of 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-4*E*-en-3-heptanone (compound **1**) in SH-SY5Y cells and therefore to provide new insight into neuroblastoma chemotherapy.
Methods Nine diarylheptanoids were isolated from *Alpinia officinarum* by chromatography and their cytotoxicity was evaluated by an MTS assay. Flow cytometry, BrdU incorporation assay and fluorescence staining were employed to investigate cytostatic and apoptotic effects induced by the compound **1**. In addition, Western blot, qPCR and siRNA techniques were used to elucidate the molecular mechanisms of the cytotoxicity.
Results The study to elucidate the cytotoxic mechanisms of compound **1**, the most potent diarylheptanoid showed that cell cycle-related proteins, cyclins, CDKs and CDKIs, as well as two main apoptotic related families, caspase and Bcl 2 were involved in S phase arrest and apoptosis in neuroblastoma cell line SH-SY5Y. Furthermore, following the drug treatment, the protein expression of p53, phospho-p53 (Ser20) as well as the p53 transcriptional activated genes ATF3, puma and Apaf-1 were increased dramatically; MDM2 and Aurora A, the two p53 negative regulators were decreased; the p53 protein stability was enhanced, whereas the p53 mRNA expression level slightly decreased and ATF3 mRNA expression apparently increased. In addition, the knockdown of ATF3 gene by siRNA partially suppressed p53, caspase 3, S phase arrest and apoptosis triggered by compound **1**.
Conclusion These results suggest that compound **1** induces S phase arrest and apoptosis via up regulation of ATF3 and stabilization of p53 in SH-SY5Y cell line. Therefore, compound **1** might be a promising lead structure for neuroblastoma therapy.

Keywords Diarylheptanoid · Cell cycle arrest · Apoptosis · p53 · ATF3

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Introduction

Neuroblastoma, a tumor originating from the sympathetic nervous system, is the most frequently occurring extra-cranial malignancy in childhood [23]. Despite many advances in diagnosis and standard interventions in the past three decades, neuroblastoma has remained a formidable challenge to clinical and basic scientists [3]. In search for novel therapeutic strategies for neuroblastoma, natural product has attracted interest to achieve new approach for chemotherapy.

Alpinia officinarum Hance (Zingiberaceae), a pungent and aromatic rhizome, cultivated in southern China and Vietnam, is used as a spice ingredient for flavoring food throughout southeastern Asian countries [20, 21]. The dried rhizome of *A. officinarum* is a traditional Chinese medicine (TCM) with anti-inflammatory, antioxidant and analgesic activities and has been used for relieving stomachache, treating colds, invigorating the circulatory system, and reducing the swelling for a long time [1]. Recent studies on *A. officinarum* showed that MeOH and CH_2Cl_2 extractable fractions possess significant cytotoxicity against COR L23 human large-cell carcinoma with IC_{50} values of 13.3 and 5.4 μg/ml, respectively. A phenylpropanoid compound, 1-acetoxychavicol acetate is one of the active constituents in the herb with IC_{50} values of 5.8 and 8.6 μM against COR L23 and MCF-7 cells [16]. Phytochemical studies showed that of the many chemical constituents isolated from this plant, diarylheptanoids are among the characteristic compounds [36]. Multiple lines of evidence showed that diarylheptanoids are cytotoxic agents against many cancer cell lines. Curcumin, a well-known diarylheptanoid, has been postulated to be of potential use not only in cancer chemoprevention but also in chemotherapy [30]. A number of reports demonstrated that curcumin could inhibit chemical carcinogen or radiation-induced tumorigenesis and suppress the growth of mammary tumors via various pathways [2, 6].

Our previous screening study has shown that some diarylheptanoids possess good cytotoxicity in a series of cancer cell lines, including HepG2, MCF-7, SF-268 and SH-SY5Y with a similar IC_{50}, ranging from 6-10 μg/ml [1]. In addition, SH-SY5Y cells are more sensitive to the most potent diaryheptanoid named compound **1** in cell cycle analysis. Thus, it is of great interest to investigate the underlying mechanisms of compound **1** in the most potent cell line SH-SY5Y, and this will provide a new insight into neuroblastoma therapy.

Materials and methods

Extraction and isolation

The dried rhizomes of *A. officinarum* (28 kg) were extracted with EtOH at room temperature. The extract yielded a residue of 2.2 kg, which was suspended in H_2O and extracted with petrol ether, $CHCl_3$, EtOAc and *n*-BuOH, respectively. The dried $CHCl_3$ part (150 g) was subjected to Si-gel, polyamide and Sephadex LH-20 chromatography to give 9 diarylheptanoids, which were identified as 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-4*E*-en-3-heptanone(1), 5-hydroxy-1-(3-methoxy4-hydroxyphenyl)-7-phenyl-4*E*,6*E*-dien-3-heptanone(2), (5*S*)-5-hydroxy-1-phenyl-7-(3,4-dihydroxyphenyl)-3-heptanone(3), (5*R*)-5-hydroxy-1-phenyl-7-(4,5-dihydroxy-3-methoxyphenyl)-3-heptanone(4), (5*R*)-5-hydroxy-1-(3,4-dihydroxy phenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone (5), 1-(4-hydroxy-3-methoxyphenyl)-7-phenyl-3,5-heptandiol(6), (3*R*,5*R*)-1-(4-hydroxy-3-methoxyphenyl)-7-phenyl-3,5-heptanoidiol(7), (3*R*,5*R*)-1,7-*bis*(4-hydroxyphenyl)-3,5-heptanoidiol(8), (3*R*,5*R*)-1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)-3,5-heptanoidiol (9) based on their IR, NMR and MS.

Cell culture

SH-SY5Y cells (ATCC, Manassas, VA) were grown in DMEM (Gibco, Grand Island, New York, USA) containing 10% FBS (Gibco), 2% L-Glutamine, 1% penicillin sodium salt and streptomycin sulfate at 37°C in air with 5% CO_2 and passage regularly.

Cytotoxicity assay

SH-SY5Y cells were seeded in 96-well tissue culture plates and treated with the test compounds (100–3.125 μg/ml) at various concentrations or vehicle (0.1% DMSO). Cell viability in cell culture after compounds exposure was measured by a CellTiter 96® AQueous One Solution Cell proliferation assay (MTS) assay. The IC_{50} value was derived from the dose-response curve.

Cell cycle analysis

SH-SY5Y cells were treated with compound **1** at 2.5, 5 and 10 μg/ml for 48 h. At the end of treatment, cells were harvest and fixed in 70% cold ethanol (4°C) overnight. After washing twice with PBS, cells were resuspended. RNase A (0.5 mg/ml) and PI (2.5 μg/ml) were added to the fixed cells and incubated at 37°C for 30 min. The DNA content of cells was then analyzed with a FACSCalibur instrument (Becton Dickinson, San Jose, CA). The percentage of cells in different cell cycle phases was calculated by ModFit 3.0 (Verity Software House, Inc.).

BrdU incorporation assay

SH-SY5Y cells were seeded onto glass coverlips 24 h before drug treatment. After treatment with vehicle or compound **1** at 5 μg/ml for 24 h, cells were incubated with 20 mM Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) for another 1 h before fixation with acetone (−20°C) for 10 min. After DNA denaturation in 2 N HCl at 37°C for 30 min, the cells were blocked with PBSST for 20 to 30 min, followed by incubation with BrdU antibody (10 μl Brdu-Alexa 488/647 with DAPI 1 μl into 90 μl PBSST) for 1 h at room temperature. Pictures were taken under fluorescence microscopy.

Apoptotic morphology observation

Cells were plated in 6-well plates and treated with vehicle or compound **1** at 5 μg/ml for 48 h. After treatment, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then stained with Hochest33258 at 5 μg/ml for 10 min. Photographs were taken under an ECLIPSE TE2000-S fluorescence microscope (Nikon Instruments INC. Melville, NY).

Western blot

Following treatment, SH-SY5Y cells were washed three times with cold PBS and lysed with lysis buffer. Protein concentration was measured by the Bradford method. The lysates were subjected to electrophoresis on 10% polyacrylamide gels and then transferred onto a nitrocellulose sheet. The nitrocellulose membrane was then incubated with rabbit polycolonal anti-phospho-p53 (Ser20, #9287, Cell Signaling Technology, Danvers, MA); anti-Aurora A (07–648, Upstate, Billerica, MA); anti-p53, anti-MDM2, anti-cyclin A, anti-cyclin B, anti-cdc 2, anti-cdk4, anti-p21, anti-p27, anti-Bax, anti-PUMA a/b, anti-Apaf-1 anti-PARP antibodies; mouse monoclonal anti-cyclin E, anti-caspase-3, and anti-Bcl-2, (sc-6243, sc-7918, sc-596, sc-25764, sc-954, sc-260, sc-397, sc-528, sc-493, sc-28226, sc-8339, sc-7150, sc-247, sc7272, sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA); Mouse monoclonal GAPDH (MAB374, CHEMICON, Billerica, MA) was used as an internal control. Secondary antibody, IgG conjugated to horseradish peroxidase was used. Protein bands were visualized with the ECL Western blot detection system according to the manufacturer's instructions.

Caspase 9 activity assay

After drug treatment, SH-SY5Y cells were harvested and Caspase-9 activity was evaluated using caspase 9 detection kit (QIA 115, Calbiochem, Darmstadt, Germany), as described previously [31]. One-factor ANOVA was used, followed by Dunnett's test, $P < 0.05$ was considered significant.

RT-qPCR

Gene expression level of ATF3 and p53 was evaluated using real-time PCR. Primers, designed using Primer 3, were as follows: ATF3 forward primer: *gccattggagagctgtctt,* reverse primer: *gggccatctggaacataag*; p53 forward primer: *gcccacttcaccgtactaa*, reverse primer: *tggtttcaaggccagatgt*; GAPDH forward: *gagtcaacggatttggtcgt*, reverse: *ttgattttggagggatctcg*. Briefly, total RNA was prepared after drug treatment using an RNeasy® Mini Kit (Qiagen, Maryland, USA) according to the protocol. One microgram RNA of each sample was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) with RNAse H^+, following the instruction. Real Time PCR was performed on the iQ5 Real-Time PCR detection system with the iQ SYBR Green Supermix (Bio-RAD) and GAPDH was used as an internal control. The relative quantification of mRNA expression was calculated according to the literature [26].

Cycloheximide chase assay

Following treatment with vehicle or compound **1** at 5 μg/ml for 48 h, we treated SH-SY5Y cells with 50 μg/ml cycloheximide, harvested the cells at indicated time points and subjected cell lysates to Western blotting.

Transient transfection

Silencer 1 Negative Control No. 1 siRNA (Cat No. 4635) and ATF3 siRNA (Cat No. AM16708A) were obtained from Ambion (Austin, TX). The sequence of siRNA duplex targeting ATF3 is as follows: #241437 sense, 5′-AAGU GCCGAAACAAGAAGAtt-3′; antisense, 5′-UCUUCUU GUUUCGGCACUUtg-3′; #115224 sense, 5′-CGAGAAG CAGCAUUUGAUAtt-3′; antisense, 5′-UAUCAAAUGC UGCUUCUCGtt-3′. SH-SY5Y cells were plated in 6-well plates in antibiotic-free medium for 24 h before transfection and transfected at 70% confluence. Transfection was done with 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 50 nmol/l of ATF3 siRNA mixed in serum-free OPTI-MEM (Invitrogen, Carlsbad, CA). The medium was changed to DMEM with 10% FBS without antibiotics, 6 h post-transfection. Forty-eight hours post-transfection, cells were treated with compound **1** for an additional 24 h and harvested for Western blotting and cell cycle analysis.

Results

Cytotoxicity of diarylheptanoids in SH-SY5Y cells

The cytotoxicity of a series of diarylheptanoids was determined by MTS assay and curcumin, a well-known cytotoxic diarylheptanoid was used as positive control. The diarylheptanoids could inhibit the proliferation of SH-SY5Y cells in a dose-dependent manner (Fig. 1b). Among them, compounds **1** and **2** are the most potent compounds, and even more efficacious than curcumin.

Compound **1** induces S phase arrest in SH-SY5Y cells

We chose the most potent compound **1** to elucidate its cytotoxic mechanism in SH-SY5Y cells. Following treatment

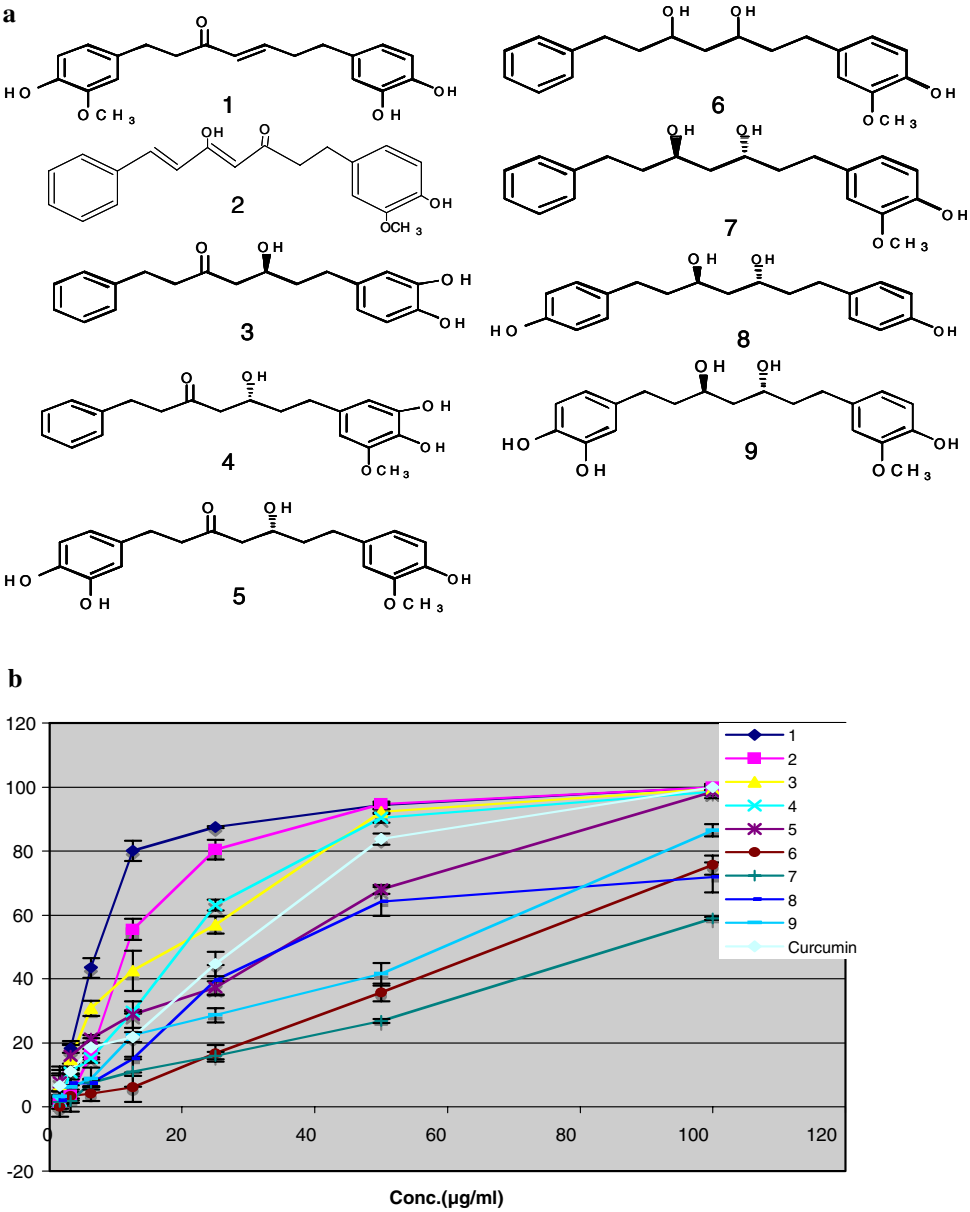

Fig. 1 Cytotoxicity of the diarylheptanoids in SH-SY5Y cell line. **a** Structures of diarylheptanoids. **b** Antiproliferation activity of diarylheptanoids in SH-SY5Y cells. Data shown represent the mean ± SD of four replicates

with compound **1** at various dosages for 48 h, consistent accumulations of S phase and apoptotic cells were observed in a dose-dependent manner (Fig. 2a). Since compound **1** can induce significant S phase arrest, we therefore performed BrdU incorporation assay to check the specific influence of compound **1** in DNA synthesis. After treatment with compound **1** at 5 μg/ml for 24 h, the green signal from Brdu was less than that in the control (Fig. 2b). This indicates that compound **1** inhibited DNA synthesis. Cell cycle is tightly governed by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). After drug treatment at different dosages for 48 h, cyclin A, cyclin B, cyclin E, CDK4 and Cdc2 were downregulated, whereas p21 and p27 were upregulated (Fig. 2c).

Compound **1** induces apoptosis in SH-SY5Y cells

Following treatment with compound **1** at 5 μg/ml for 48 h, chromatin aggregation, nuclear and cytoplasmic condensation and partition of cytoplasm and nucleus into membrane-bound vesicles were observed (Fig. 3a). Caspases and Bcl 2 are two main apoptosis-related families. After treatment with compound **1** at different dosages for 48 h, procaspase 3 was downregulated, which meant that caspase 3 was upregulated and caspase 9 activity was significantly increased in a dose-dependent manner. PARP, the substrate of caspase 3, which serves as the hallmark of apoptosis, was cleaved into two bands. Meanwhile, Bcl 2 was decreased and bax was increased. In addition, the other two p53 transcriptional activated proteins, Apaf-1 and puma were upregulated (Fig. 3b, c).

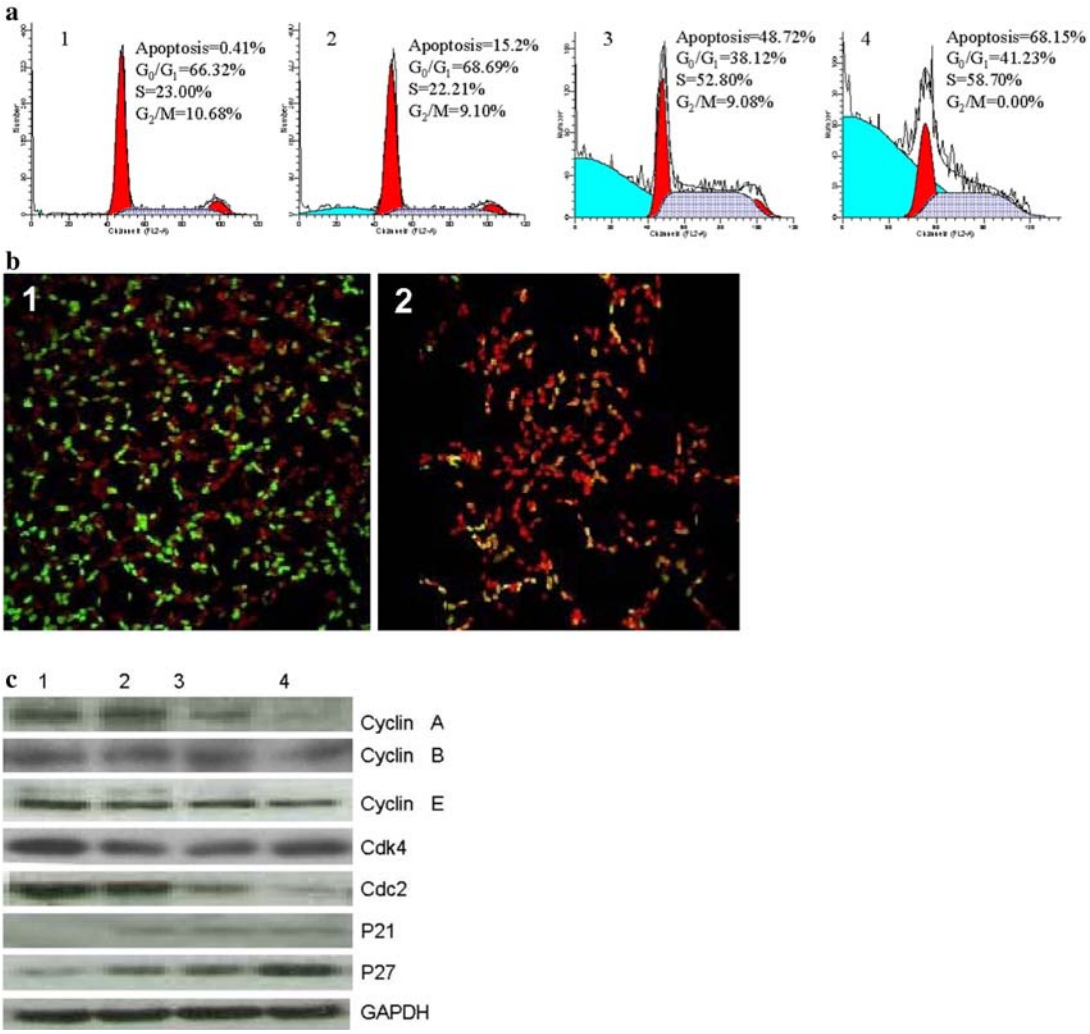

Fig. 2 Effects of compound **1** on cell cycle progression in SH-SY5Y cells. **a** Compound **1** induces S phase cell cycle arrest in SH-SY5Y cells. *1* Control SH-SY5Y cells, *2–4* SH-SY5Y cells treatment with compound **1** at 2.5, 5 and 10 μg/ml for 48 h respectively. NB: according to ModFit 3.0, cell cycle calculation does not include apoptosis population. Apoptosis percentage is calculated against all cell population including apoptotic cells and live cells in cell cycle phases. **b** Compound **1** inhibits DNA synthesis. *1* Control SH-SY5Y cells, *2* SH-SY5Y cells treatment with compound **1** at 5 μg/ml for 24 h. **c** Alteration of cell cycle related proteins following treatment with compound **1**. *Lane 1* Control SH-SY5Y cells, *Lanes 2–4* SH-SY5Y cells treatment with compound **1** at 2.5, 5 and 10 μg/ml for 48 h. The data shown are from one of the three independent experiments

Influence of compound **1** on p53 and ATF3

P53 plays an important role in neuroblastoma cell death and ATF3 is always coincidently induced with p53 by many kinds of stimulations, we therefore checked the influence of compound **1** on ATF3 and p53. After treatment with compound **1** at different dosages for 48 h, the protein expression level of ATF3, p53, as well as that of p-p53 was upregulated. However, only ATF3 increased at the gene expression level, whereas p53 gene expression level has no significant increase; in contrast, slightly decreased after treatment (Fig. 4).

Compound **1** increases p53 stability

Since compound **1** could not increase the gene expression level of p53, the up regulation of p53 protein by compound **1** might be due to post-transcriptional regulation. Thereby, we conducted cycloheximide chase assay to investigate the influence of compound **1** on p53 protein stability. In our experiment, compound **1** can increase the stability of p53 protein. In addition, two p53 protein negative regulators MDM2 and Aurora A were downregulated upon compound **1** treatment (Fig. 5).

ATF3 knockdown reduces apoptosis and cell cycle arrest triggered by compound **1**

The role of ATF3 in tumorigenesis remains a controversy. In order to address the function of ATF3 in compound **1**-triggered SH-SY5Y cell death, we knocked down ATF3 gene by ATF3 siRNA. ATF3 protein level was dramatically knocked down after transfection with ATF3 siRNA

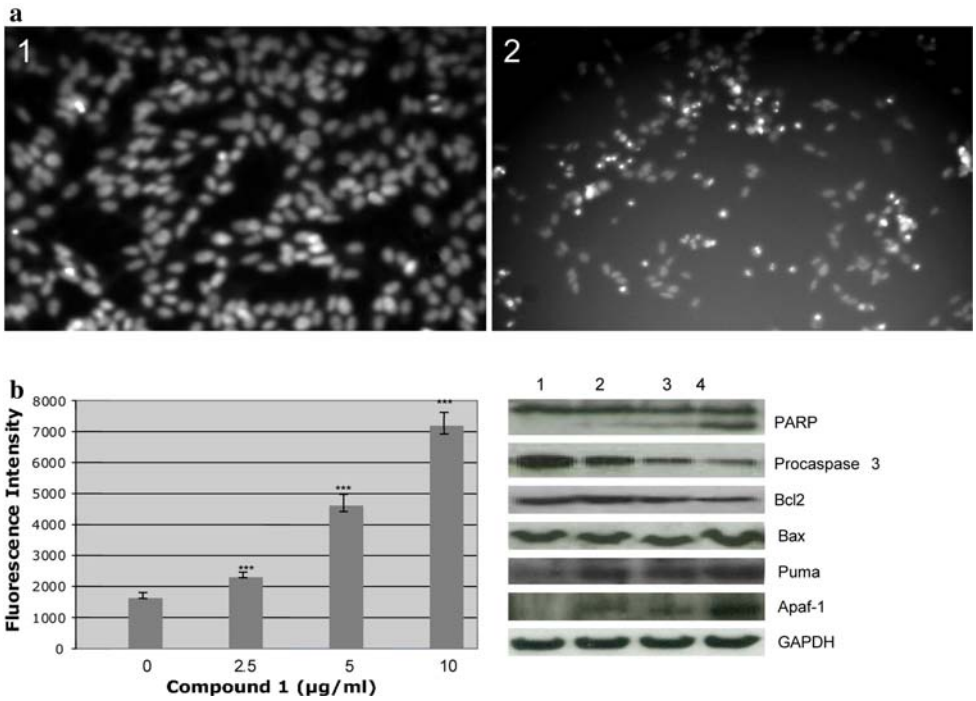

Fig. 3 Compound **1** induces apoptosis in SH-SY5Y cells. **a** Apoptotic morphology changes in SH-SY5Y cells. *1* Control SH-SY5Y cells, *2* SH-SY5Y cells treatment with compound **1** at 5 μg/ml for 48 h. **b** Compound **1** induces caspase 9 activity in SH-SY5Y cells. **c** Alteration of apoptotic-related proteins. *Lane 1* Control SH-SY5Y cells, *Lanes 2–4*, SH-SY5Y cells treatment with compound **1** at 2.5, 5 and 10 μg/ml for 48 h

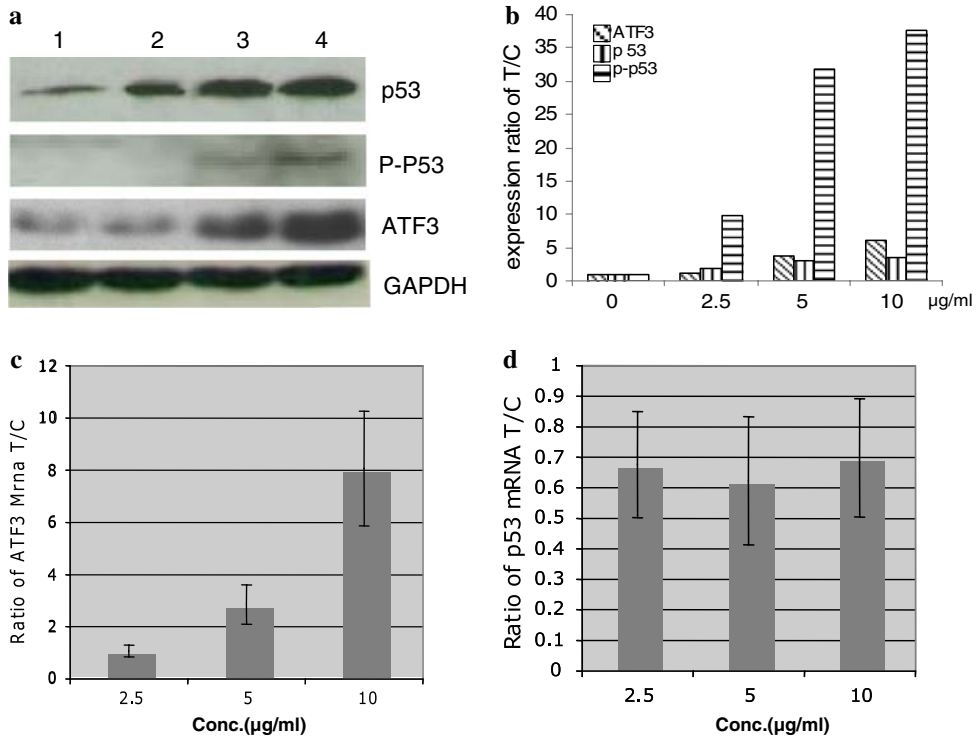

Fig. 4 Influence of compound **1** on ATF3 and p53. **a** Upregulation of protein expression of p53, p-p53 and ATF3 by compound **1**. *Lane 1* Control SH-SY5Y cells, *Lanes 2–4* SH-SY5Y cells treatment with compound **1** at 2.5, 5 and 10 μg/ml for 48 h. **b** Stimulation mRNA expression of ATF3 by compound **1**. Data shown are mean ± SD of three independent experiments. **c** Effect of compound **1** on mRNA expression of p53

for 48 h. Following transfection, cells were treated with compound **1** at 5 μg/ml for an additional 24 h. Since we cannot completely knockdown a gene by 100%, ATF3 protein was partially stimulated by compound **1** post-transfection with ATF3 siRNA. However, it was still less than ATF3 expression induced by compound **1** in the control siRNA sample. In addition, knockdown of ATF3 gene partially decreased the caspase 3 (increased procaspase 3) and p53 expression induced by compound **1**. As a result, cell cycle arrest and apoptosis induced by compound **1** were apparently inhibited by knockdown of ATF3 (Fig. 6).

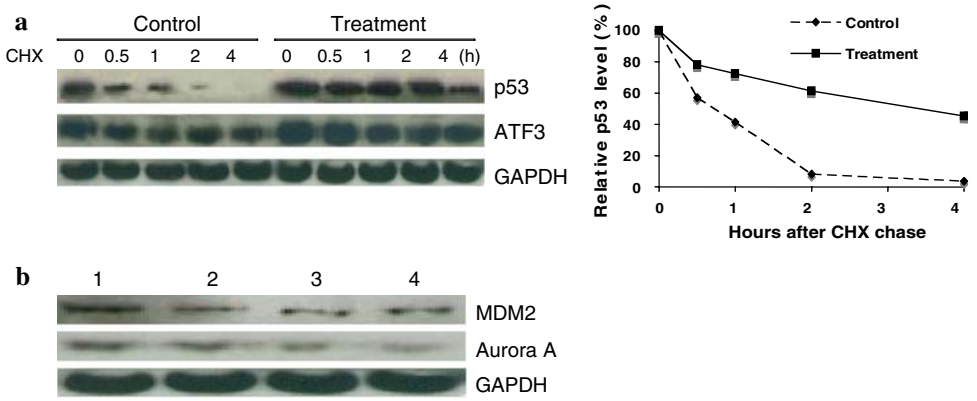

Fig. 5 Compound **1** increases p53 stability. **a** Influence of compound **1** on p53 stability using cycloheximide chase assay. Following treatment with vehicle or compound **1** at 5 μg/ml for 48 h, SH-SY5Y cells were then treated with cycloheximide at 50 μg/ml for the indicated times and harvested for Western blotting. **b** Down regulation of p53 negative regulators *MDM2* and *Aurora A* by compound **1**. *Lane 1* Control SH-SY5Y cells, Lanes *2–4* SH-SY5Y cells treatment with compound **1** at 2.5, 5 and 10 μg/ml for 48 h

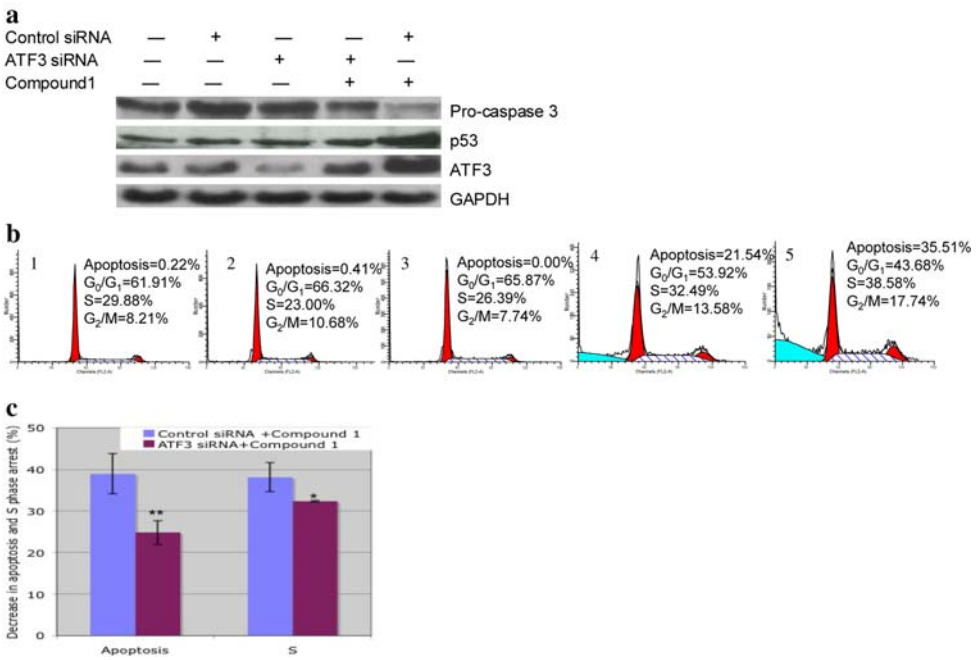

Fig. 6 ATF3 knockdown reduces apoptosis and cell cycle arrest triggered by compound **1**. **a** Knockdown of ATF3 gene partially decreased the caspase 3 and p53 expressions induced by compound **1**. Following 48 h transfection, SH-SY5Y cells were treated with vehicle or compound **1** for additional 24 h and harvested for Western blotting. **b** Knockdown of ATF3 genes partially reduced S phase arrest and apoptosis triggered by compound **1**. *1* Control SH-SY5Y cells, *2* SH-SY5Y cells transfected with control siRNA for 48 h, *3* SH-SY5Y cells transfected with ATF3 siRNA for 48 h, *4* SH-SY5Y cells transfected with ATF3 siRNA for 48 h and treatment with compound **1** at 5 μg/ml for additional 24 h, *5* SH-SY5Y cells transfected with control siRNA for 48 h and treatment with compound **1** at 5 μg/ml for additional 24 h. **c** S phase arrest and apoptosis induced by compound **1** were inhibited by knockdown of ATF3

Discussion

Induction of cell cycle arrest and apoptosis has been implicated as an important mechanism underlying the cytotoxic activities of many cancer chemotherapeutic agents [9, 29, 32]. In our present study, we investigated the cytotoxicity and mechanisms of diarylheptanoids in SH-SY5Y cells. We found that compound **1**, the most effective diarylheptanoid could inhibit the proliferation of SH-SY5Y cells via inducing S phase arrest and apoptosis. It is well known that the cell cycle is governed by cyclins, CDKs and CDKIs. Among them, Cyclin A is required for both the initiation and elongation of DNA in the late G_1 and S phases, as well as is involved in the activation of cdc2-cyclin B complex [25, 34]. Meanwhile, p21 and p27 inhibit Cyclin A to block cell cycle progression from S phase to G_2/M phase. In our

experiments, following drug treatment, DNA synthesis was prominently inhibited; Cyclin A, Cyclin B, Cyclin E, cdc2, cdk4 were downregulated, whereas p21 and p27 were upregulated. These suggest that suppression of Cyclin A and an increase of p21 and p27 as well as inhibition of DNA synthesis may be implicated in the S phase arrest induced by compound **1**.

Unlike the majority of other human cancers, which is a defect in p53, neuroblastomas usually carry wild-type p53 [15, 27, 35]. Although cytoplasmic sequestration of p53 has been proposed as a mechanism for inactivation of the function of p53 in neuroblastoma cells, numerous studies reveal that the p53 signaling pathway is functional in the various neuroblastoma cell lines that were examined [8]. P53 plays a key role in genotoxicity-mediated apoptosis. In response to various stimuli-induced DNA damage, p53 protein accumulates rapidly through a post-transcriptional mechanism(s) and is also activated as a transcription factor, which leads to cytostasis or apoptosis. Phosphorylation of p53 at Ser20 leads to reduced interaction of p53 with its negative regulator, MDM2, and enhances its tetramerization, stability and activity [4, 12, 28]. MDM2 inhibits the accumulation of p53 by targeting it for ubiquitination and proteasomal degradation [13, 33]. In addition, Aurora A, another p53 negative regulator could suppress p53 function via at least two mechanisms: first, Aurora A phosphorylates Ser315 of p53 leading to its MDM2 association and degradation [14]; second, Aurora A also phosphorylates p53 at Ser215 resulting in inactivation of its transcriptional activity [18, 22]. In the light of our study, following drug treatment, the protein expressions of p53, phospho-p53 (Ser20), as well as that of the p53 transcriptional-activated genes, Bax, puma, Apaf-1 and ATF3 were increased dramatically; MDM2 and Aurora A, the two p53-negative regulators were decreased; the p53 protein stability was enhanced, whereas the p53 mRNA expression level slightly decreased. Our results suggest that compound **1** increases the p53 activity and stability via post-transcriptional regulation. The slight downregulation of p53 mRNA expression might be due to the negative feedback of over expression of p53 protein.

ATF3 (activating transcription factor 3), transcription factor of ATF/CREB family plays an important role in carcinogenesis via influence of cell death and cell cycle progression [5, 7, 10, 17]. Several lines of evidence demonstrated that ATF3 directly binds p53 and is coincidently induced with p53 by a broad spectrum of stimulations [11, 38]. In addition, P53 transcriptional activates ATF3 and in turn, ATF3 increases p53 stability [39]. However, there is still a controversy about the function of ATF3 in apoptotic cell death. Most approaches, both from gain-of-function and loss-of-function support a pro-apoptotic role of ATF3 [19]. In particular, ATF3 is a novel contributor to the proapoptotic effect of curcumin, which has similar structure as our tested compounds [37]. On the other hand, several reports suggest that ATF3 is anti-apoptotic [24]. In our study, compound **1** induced expression of ATF3 both on protein level and mRNA level. Furthermore, knockdown of ATF3 gene by siRNA partially suppressed p53, caspase 3 (increased procaspase 3), S phase arrest and apoptosis induced by compound **1**. These results indicate that ATF3 is functional as a pro-apoptotic protein in compound **1**-induced cell death.

Overall, compound **1** induces S phase arrest and apoptosis via up regulation of ATF3 and stabilization of p53 in SH-SY5Y cells. It might be a prospective neuroblastoma therapeutic agent after structure modification to increase the cytotoxicity and improve the solubility.

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