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Gallic acid induces mitotic catastrophe and inhibits centrosomal clustering in HeLa cells

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ABSTRACT

Cancer cells divide rapidly, providing medical targets for anticancer agents. The polyphenolic gallic acid (GA) is known to be toxic for certain cancer cells. However, the cellular mode of action has not been elucidated. Therefore, the current study addressed a potential effect of GA on the mitosis of cancer cells. GA inhibited viability of HeLa cells in a dose-dependent and time-dependent manner. We could show, using fluorescence-activated cell sorting (FACS), that this inhibition was accompanied by elevated frequency of cells arrested at the G2/M transition. This cell-cycle arrest was accompanied by mitotic catastrophe, and formation of cells with multiple nuclei. These aberrations were preceded by impaired centrosomal clustering. We arrive at a model of action, where GA inhibits the progression of the cell cycle at the G2/M phase by impairing centrosomal clustering which will stimulate mitotic catastrophe. Thus, GA has potential as compound against cervical cancer.

1. Introduction

Cancer is one of the major public health problems worldwide and the leading cause of death in economically developed countries (Ferlay et al., 2010). For instance, in the United States, one in four deaths is due to cancer (data of 2013 from American Cancer Society) (Siegel et al., 2013). Also in developing countries, the burden of cancer is increasing due to aging populations, and meanwhile developing countries account for more than half of the global cancer incidence (Jemal et al., 2011). So far, cancer therapy has been based on surgery, radiation and chemotherapy (Portenoy, 2011). The success of chemotherapy depends on the specificity of the compound and still is accompanied by unpleasant side effects for the well-being of the patient. In addition, especially under prolonged treatments, drug-resistance can evolve and seriously reduce the efficacy of the treatment. For this reason, the identification of novel compounds with anti-cancer activity is highly warranted.

One strategy to get access to such molecules is to screen natural compounds that have evolved by organisms to manipulate or impair the cell division of intruders. Plants as sessile organisms generate an estimated million of secondary compounds (Saito and Matsuda, 2010). Many of these compounds specifically block the development of feeding insects, and in addition to substances that corrupt development or interfere with neural signaling, inhibition of cell division represents an important target for plant-derived allelochemicals (Rattan, 2010). In fact, some of the most important anti-cancer drugs, such as paclitaxel/taxol or the *Vinca* alkaloids, originate from plants (Owellen et al., 1976; Wani et al., 1971). However, also in addition to the alkaloids, there is enough space to mine for compounds with anti-cancer activity.

Epidemiologic studies have shown that certain polyphenols, which are especially abundant in fruits and vegetables, correlate with a reduced risk for certain types of cancers (Manach et al., 2004). For instance, polyphenols from apple were shown to inhibit protein kinase C in a human colon cancer cell line by inducing apoptotic cell death (Kern et al., 2007). Polyphenols comprise a variety of bioactive compounds which are commonly divided into several classes, hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonoids, stilbenes and lignans (Manach et al., 2005). So far, only a small number of polyphenols has been evaluated for their potential effect on carcinogenesis. Especially, gallic acid (GA) has already been reported to inhibit the proliferation of numerous types of cancer cells such as leukemia, prostate cancer, cervical, lung, gastric, colon, breast, brain, melanoma and esophageal cancer cell lines (Inoue et al., 1995; Lu et al., 2010; Pan et al., 2014; Park and Kim, 2013). These anti-cancer activities of GA seem to be related with the induction of apoptosis through different signal pathways that depend on the respective cell type. For instance, in human brain cancer cells, GA impairs cell viability, proliferation, invasion and angiogenesis partly through the phosphatidylinositol 3-kinase/protein kinase B and

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; GA, gallic acid; HeLa, Human cervical cancer cell line; HepG2, Human hepatoma cell line; MAPs, microtubule-associated proteins; LD_{50}, median lethal dose; MT, microtubule; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; $PBS^{-}/^{-}$, Phosphate-buffered saline without calcium and magnesium.

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Ras–mitogen-activated protein kinase signaling pathways (Lu et al., 2010), while in human prostate cancer cells, GA inactivates phosphorylation of cell division cycle 25A/C (Cdc 25A/C) and cell division cycle 2 (Cdc 2), leading to cell cycle arrest and induction of apoptosis (Agarwal et al., 2006). Moreover, by modulating cell cycle regulators, GA inhibited prostate cancer growth and progression in a mouse model (Raina et al., 2008).

Mitotic catastrophe is a form of aberrant mitosis. Chromosomes mis-segregate leading to daughter cells with multiple nuclei. This is often followed by impaired viability (Roninson et al., 2001). Mitotic catastrophe can be induced by agents that disturb the mitotic spindle (Vakifahmetoglu et al., 2008). For example, taxol (paclitaxel) was reported to induce mitotic catastrophe in human colon and cervical cancer cells, respectively (Fujie et al., 2005; Jordan et al., 1996). In addition to induction of apoptosis, drug-induced senescence, and lytic necrosis, mitotic catastrophe can provide another target for cancer therapy (Morse et al., 2005).

The bipolar character of the spindle and, therefore, the accurate segregation of chromosomes into the daughter cells depends on the correct localization and organization of the centrosomes (Fukasawa et al., 1996). In most cancer cells, supernumerary centrosomes are a common phenomenon indicating that these cells fail to control centrosome numbers (Kwon et al., 2008). This should lead to deleterious multipolar divisions and thus to the progressive loss of cancer vigor. However, this is frequently circumvented by clustering of these excessive centrosomes during mitosis, such that most cells are still able to generate a functional bipolar spindle. Centrosomal clustering has been recognized as important mechanism sustaining proliferation of cancer cells (Drosopoulos et al., 2014; Quintyne et al., 2005) and therefore represents a promising target for the therapy of cancers.

Although many reports support a specific activity of GA against malignant transformation and cancer progression, a potential effect of this compound for mitotic catastrophe and centrosome clustering has not been addressed so far. In the current study, we therefore investigated the cellular mechanisms of GA-induced cell death in the HeLa cell line, a classical cervical cancer cell model. We find that the cytotoxicity of GA is linked with a specific block of cell-cycle progression at the G2/M transition, which is preceded by reduced centrosome clustering and consequently the elevated incidence of multinucleated cells.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid (GA), dimethyl sulfoxide (DMSO), Triton X-100, fetal calf serum (FCS), Hoechst 333258, ATTO 488 and anti-γ-tubulin antibody were obtained from Sigma Chemical Co. (Neu-Ulm, Germany). Antibiotics, phosphate-buffered saline without calcium and magnesium ($PBS^{-}/^{-}$), Dulbecco's modified Eagle's medium (DMEM), and trypsin-EDTA were purchased from Gibco (Eggenstein, Germany), DRAQ5™ from Biostatus (Leicestershire, UK), secondary antibody and Cell Light Tubulin-GFP BacMam 2.0 baculoviral particles from Invitrogen Life Technologies (Karlsruhe, Germany).

2.2. Cell culture

Human cervical cancer cell line, HeLa and Human hepatoma cell line, HepG2, were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% FCS and 1% antibiotics (60 μg/mL penicillin and 100 μg/mL streptomycin) at 37 °C with 5% CO_2.

2.3. Cell viability assay

For the evaluation of cell viability, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was conducted using the Cell Titer 96 kit (Promega Corp., Madison, USA) as previously described (Hida et al., 2000). In brief, cells were seeded in 96-well plates containing 100 μL medium at a density of 10,000 cells per well. After being incubated overnight, the cells were treated with either 0.01% DMSO (control) or with GA in concentrations from 10 μM to 400 μM for 24, 48 or 72 h. MTT was added to each well and incubated for 4 h at 37 °C, and 100 μL stop solution were added to stop the reaction by solubilizing the formazan. The absorption was then measured at a wavelength of 595 nm using an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, USA). Three independent biological replicates were carried out.

2.4. Flow cytometry for cell cycle analysis

Cell cycle progression was quantified by flow cytometry as described previously (Yun and Hiom, 2009). Briefly, 1×10^6 cells were seeded in 50 mL culture flasks and treated with different concentrations of GA (10 μM, 50 μM and 100 μM) for 24 h. Control was treated with 0.01% DMSO. To harvest cells for flow cytometric DNA analysis, cells were washed with $PBS^{-}/^{-}$ before being trypsinized (0.25% trypsin-EDTA). The supernatant containing non-adherent cells was also collected and pooled with the detached, trypsinized cells. All the cells were sedimented by centrifugation at 200 *g* for 3 min, washed twice with $PBS^{-}/^{-}$, fixed in ice-cold 70% ethanol overnight, stained with DRAQ5, and then analyzed by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany). 5×10^4 cells were counted for each sample and three independent biological replicates were carried out.

2.5. Phenotyping of cells

To follow the cell microtubular response to GA by microscopy, 5×10^4 HeLa cells per well were seeded into a μ-slide 8 well of ibiTreat microscopy chambers (ibidi GmbH, Munich, Germany). The cells were incubated with 0.01% DMSO as control or indicated concentration of GA after transfection with 3 μL Cell Light Tubulin-GFP BacMam 2.0 baculovirus particles (Cellular lights 2.0, Life Technologies) per well for 24, 48 or 72 h at 37 °C. Confocal z-stacks were recorded with an AxioObserver.Z1 (Zeiss, Jena, Germany) using an oil immersion 63× LCI-NeofluarImmCorr DIC objective (NA 1.3), the 488-nm emission line of an Ar-Kr laser, and a spinning disc device (YOKOGAWA CSU-X1 5000), and a cooled digital CCD camera (AxioCam MRm; Zeiss, Jena, Germany). All images were processed with Zen 2012 (Blue edition) software or Adobe Photoshop (version CS4; Adobe, San Jose, CA). To classify the different cell phenotypes, more than 1000 cells were sampled randomly, and at least three independent experiments were carried out for each treatment.

2.6. Immunofluorescence microscopy

Immunofluorescence was performed as described in Saunders et al. (Saunders et al., 2000). Specifically, cells were grown for 24 h in microchambers (μ-Slide; ibidi GmbH, Munich, Germany) in the presence of specified concentrations of GA. Cells treated with 0.01% DMSO were used as control. Cells were fixed for 20 min in ice-cold 4% paraformaldehyde which was dissolved in $PBS^{-}/^{-}$ at 60 °C and titrated to pH 7.4, and then treated with 0.1% Triton X-100 for 3 min. After washing in $PBS^{-}/^{-}$ for three times, cells were incubated with blocking buffer (1% bovine serum in $PBS^{-}/^{-}$) at room temperature for 10 min and then with primary antibody, monoclonal mouse anti-γ-tubulin (Clone GTU-88) diluted at 1:1000 in blocking buffer for 3 h at room temperature. Subsequently, cells were washed in $PBS^{-}/^{-}$, incubated with the secondary antibody, Alexa Fluor 546 goat anti-mouse IgG (A11030) diluted at 1:500 in blocking buffer at room temperature for 1 h. Cells were washed again in $PBS^{-}/^{-}$ and incubated with Hoechst 333258 (500 ng/mL) at 37 °C for 5 min. Confocal z-stacks were recorded with an AxioObserver.Z1 (Zeiss, Jena, Germany) using an oil immersion

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63× LCI-NeofluarImmCorr DIC objective (NA 1.3), the 561-nm emission line of an Ar-Kr laser, and a spinning disc device (YOKOGAWA CSU-X1 5000). All images were processed with Zen 2012 (Blue edition) software or Adobe Photoshop (version CS4; Adobe, San Jose, CA). Three independent biological replicates were carried out.

2.7. In vitro polymerization of microtubules (MTs)

Tubulin was purified from fresh porcine brains obtained from pigs within 2 h after slaughtering by two cycles of an assembly–disassembly procedure following the classical protocol by Shelanski et al. (Shelanski et al., 1973). The protein concentrations of the final preparation were adjusted to 10 mg/mL. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed to determine the purity and quality of each tubulin preparation. Then, a part of the purified tubulin was conjugated with the fluorophore ATTO-488 according to Portran et al. (Portran et al., 2013). It should be noted that this neurotubulin was not subjected to ion-exchange chromatography and therefore, in addition to tubulin itself, was still containing microtubule-associated proteins (MAPs). The tubulin was polymerized as the method described by Altshuler (Altshuler et al., 2013). Briefly, purified bovine brain tubulin and ATTO-488-labeled tubulin were polymerized as follows: 12 μL of 10 mg/mL tubulin and 4 μL of 10 mg/mL of fluorescent tubulin were mixed with 0.8 μL of 100 mM GTP and 40 μL PME buffer (100 mM Pipes/KOH pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA), then the mixture was complemented with either 100 μM GA or 0.01% DMSO as control and incubated at 37 °C for 60 min. MTs were diluted 50 times with PME buffer, and 5 μL of the dilution was used for microscopy immediately (Apotome; Carl Zeiss, Jena, Germany). Images were captured under fixed exposure time and number and length of MTs were quantified automatically using ImageJ software (http://rsb.info.nih.gov/ij/). First, images were transformed into binary images using the thresholding function (default parameters and B/W option). After inversion, MTs were automatically selected by the "analyze particle" tool eliminating background by setting the lower limit to 10 square pixels and fitting particles by the "fit ellipse" command. Three independent biological replicates were carried out.

3. Results

3.1. Effects of GA on viability of HeLa and HepG2 cell line

To evaluate the effects of GA on the viability and proliferation of tumor cells, such as HeLa and HepG2 cells, we performed MTT assays (Fig. 1). As indicated in Fig. 1A, B and C, treatment of HeLa cells with GA resulted in a dose-and time-dependent reduction of cell viability. After 72 h GA showed a moderate toxicity with a median lethal dose (LD_{50}) ~121 μM in HeLa cells.

A similar dose-dependent reduction of the cell viability by GA was also observed in HepG2 cells as shown in Fig. 1D, E and F. However, compared to HeLa, the HepG2 cells were found to be slightly less susceptible to GA with an LD_{50} ~143 μM.

3.2. Effects of GA on the cell cycle of HeLa cells

To understand the mechanism behind the GA-induced toxicity, the effect of GA on the cell cycle of HeLa cells was analyzed by flow cytometry (Fig. 2). A typical cell cycle distribution of 28.8% in G2/M phase is shown in Fig. 2A. Lower concentrations (10 μM) of GA had no significant influence on cell cycle of HeLa cells (Fig. 2B). However, treatments of cells with 50 μM GA and 100 μM GA increased the number of cells in G2/M phase significantly to 33.7% and 51.4%, respectively (Fig. 2C and D) leading to a characteristic dose-dependent accumulation of cells in G2/M phase (Fig. 2E).

3.3. GA leads to aberrant mitosis and multinucleated cells in HeLa

By labeling non-transformed HeLa cells with Cell Light Tubulin-GFP virus, the spindle could be clearly visualized (Fig. 3). Aberrant *e.g.* central or multipolar spindles (Fig. 3B, C and D) were induced after incubating with 50 μM or 100 μM GA. This was accompanied by an increased frequency of multinucleated cells with two nuclei (Fig. 3F), four nuclei (Fig. 3G), and even with five nuclei (Fig. 3H). To quantify those aberrant mitotic cells and multinucleated cells, more than 1000 cells were scored under the microscope. As shown in Fig. 4, GA treatment resulted in a dose-and time-dependent increase in proportions of aberrant mitosis and multinucleated cells. Both, 50 μM and 100 μM, GA can significantly increase the percentage of aberrant mitosis of cells compared to the

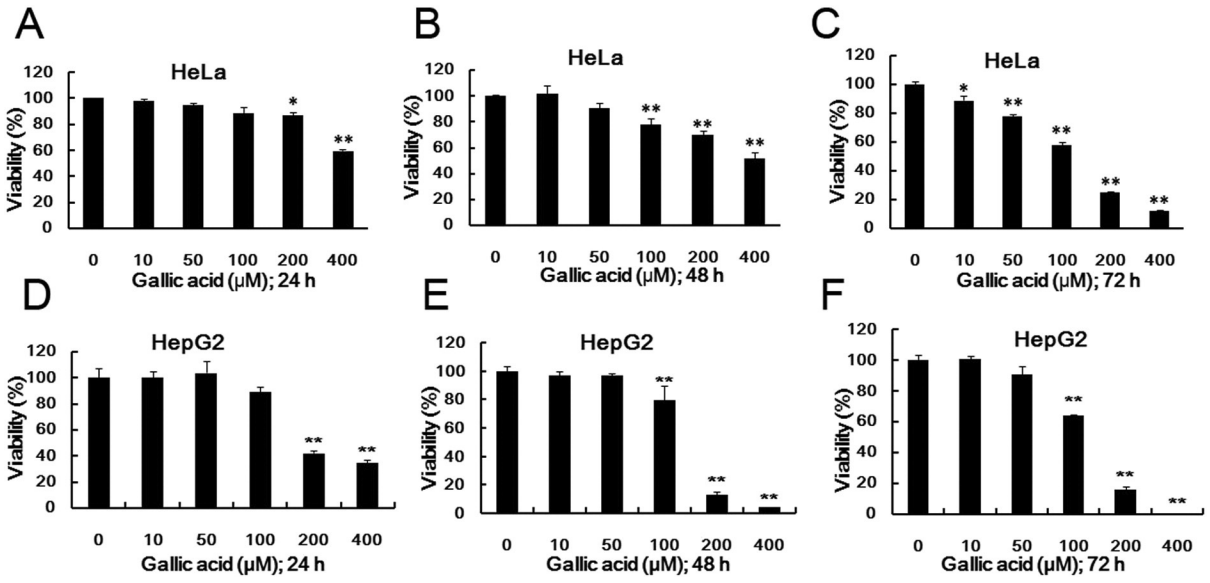

Fig. 1. GA inhibits the cell viability in HeLa and HepG2 cells. Cells were treated with the indicated concentrations of GA for 24, 48 and 72 h and the cell viability was detected using an MTT assay. (A) Viability of HeLa cells at 24 h. (B) Viability of HeLa cells at 48 h. (C) Viability of HeLa cells at 72 h. (D) Viability of HepG2 cells at 24 h. (E) Viability of HepG2 cells at 48 h. (F) Viability of HepG2 cells at 72 h. Three independent experiments were performed. Values are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$ compared with the control).

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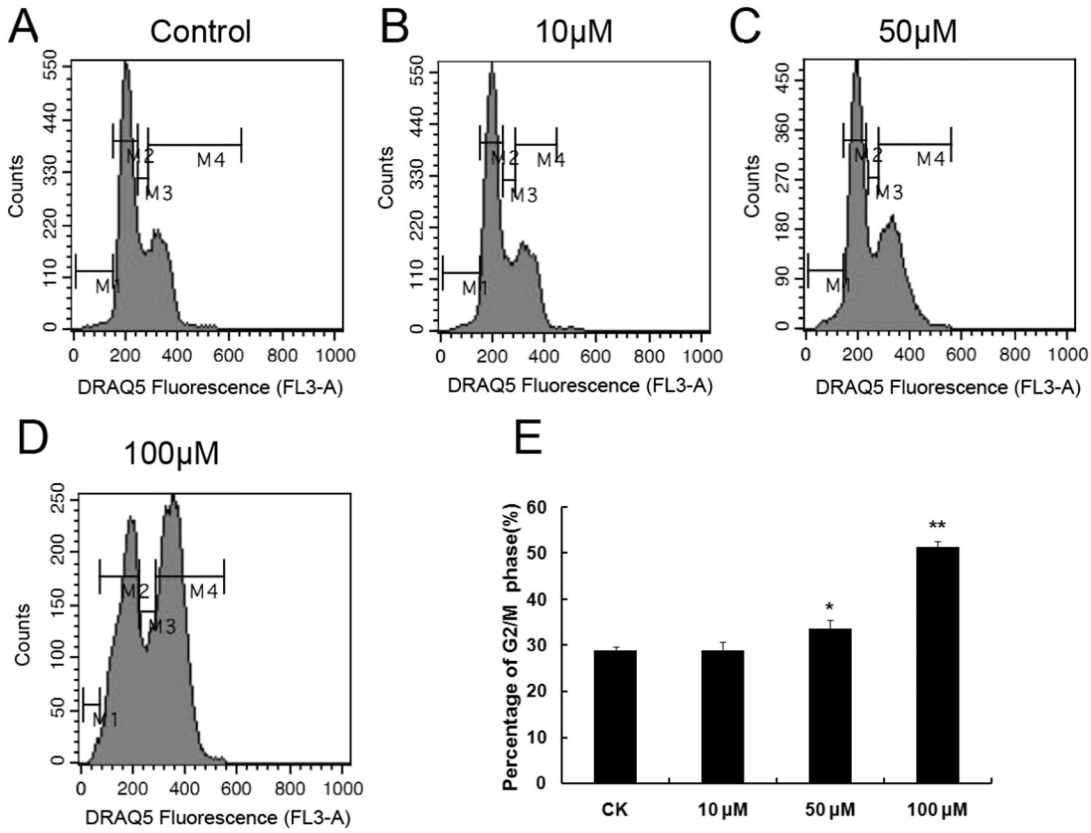

Fig. 2. GA induced enrichment in the G2/M phase of HeLa cell. (A–D) cell cycle distribution of HeLa cells: cells were treated with 10 μM, 50 μM and 100 μM GA, and 0.01% DMSO as control for 24 h, respectively. The cells were harvested, stained with DRAQ5, and analyzed using a flow cytometry. M1 represented sub-G1; M2 represented G1 phase; M3 represented S phase; M4 represented G2/M phase. (E) Proportions of HeLa cells in G2/M phase were calculated from their DNA histograms. Three independent experiments were performed. Values are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$ compared with the control).

control. With increasing incubation, the proportion of cells with aberrant mitosis increased (Fig. 4A, B and C). For incubation with 100 μM GA for 72 h, more than 50% mitotic cells with aberrant spindles were observed, accompanied by an increase in the frequency of multinucleated cells (Fig. 4D, E and F). Compared to the frequency of cells with aberrant spindles, the percentage of multinucleated cells was much lower, however, with 0.7% in the non-treated control and 3.2% after incubation with 100 μM GA for 72 h. This difference has to be seen in the context with the high incidence of apoptotic cells after treatment with GA (Fig. 1).

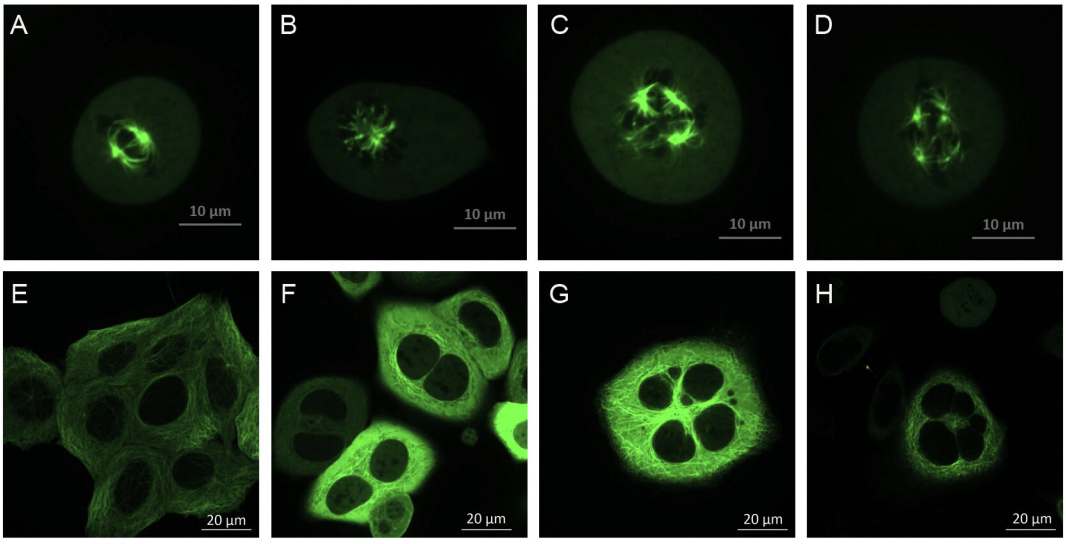

Fig. 3. Representative aberrant mitosis and multinucleated HeLa cells induced by GA. The cells were incubated with 0.01% DMSO as control, 50 μM or 100 μM GA. After incubation for 24 h, cells were observed under the spinning disc confocal system. (A) The normal spindle in mitosis; (B) aberrant mitosis-monopolar spindle; (C–D) aberrant mitosis-multipolar spindles; (E) mononuclear cells; (F–H) multinucleate giant cells with two, four or five nuclei respectively. A and E show untreated control cells, (B–C, F–H) cells which had been incubated with 100 μM GA for 24 h.

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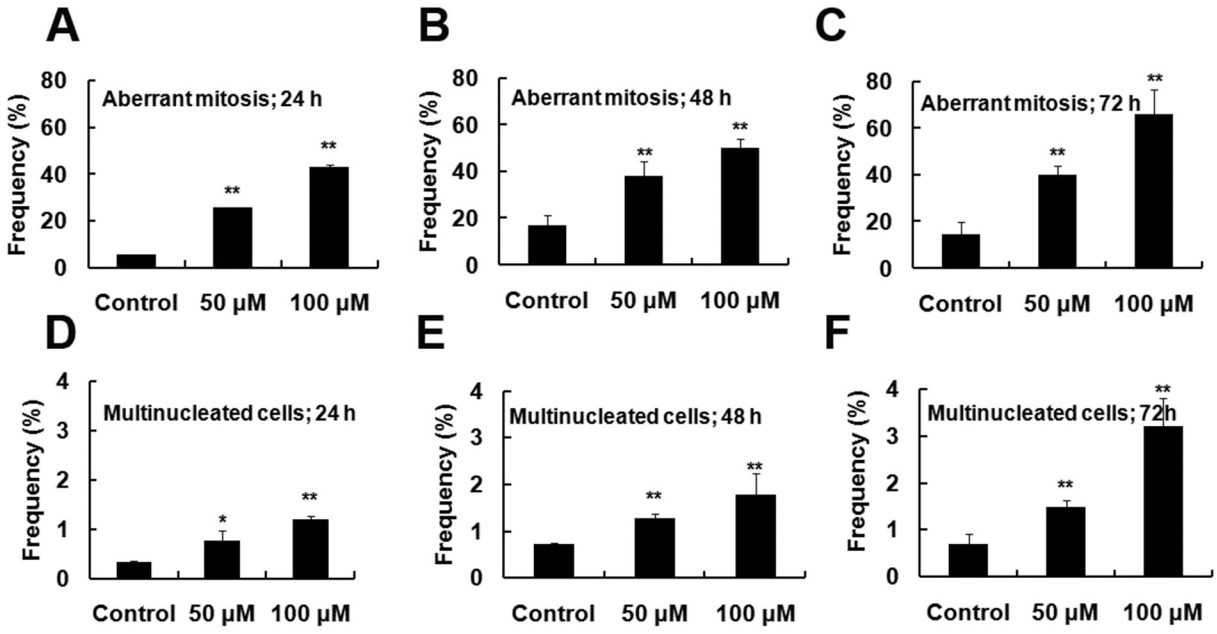

Fig. 4. GA increased the aberrant mitosis and multinucleated cells of HeLa cells. Cells were treated with the indicated concentrations of GA for 24, 48 or 72 h and the frequency of either cells with aberrant mitosis after 24 h (A), 48 h (B), and 72 h (C) or the frequency multinucleated cells after 24 h (D), 48 h (E), and 72 h (F) was determined. More than 1000 cells were counted randomly, and at least three independent experiments were carried out. Values are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$ compared with the control).

3.4. Effects of GA on centrosomal clustering

The centrosome plays a crucial role in the equal division of chromosomes by contributing to bipolar spindle assembling during mitosis. We labeled the centrosome using anti-γ-tubulin antibody to see whether GA had effects on centrosome clustering (Fig. 5). As shown in Fig. 5A, untreated cancer cells successfully divide because centrosomal clustering allows for bipolar chromosome segregation and the suppression of multipolar mitosis. However, in the GA treated cells, impaired centrosomal clustering resulted in multipolar chromosome segregation. As shown in Fig. 5B and C, the chromosomes were separated into three or four directions under the control of multipolar centrosomes. In addition, we also observed cells, where the centrosomes were located in the cell center, such that chromosomes remained aggregated in the cell center without proper segregation (Fig. 5D). The frequency of these aberrant centrosomal localizations was significantly increased after treatment with 50 μM and 100 μM GA (Fig. 5E).

3.5. GA interferes with tubulin polymerization in vitro

To address, whether the cellular effect of GA correlates with a direct interaction with tubulin, microtubule polymerization *in vitro* from tubulin isolated from porcine brain was tested (Fig. 6). Under control conditions (0.01% DMSO as solvent control), numerous long MTs were formed (Fig. 6A). For 100 μM GA, MTs polymerization was clearly inhibited (Fig. 6B). A quantification of the effect (Fig. 6C and D) showed a significant reduction of both MT number and length of MTs after GA treatment.

4. Discussion

Numerous secondary plant compounds prevent carcinogenesis. These include bioactive molecules that are common in food products, which provide an inexpensive, convenient, and pragmatic approach to prevent cancer (Lee et al., 2011; Surh, 2003). GA is widely distributed in many fruits and vegetables, and it is also found in red wine. Besides being commonly used as food supplement and for cosmetics, it is also pharmaceutically interesting as a radical scavenger, and seems to provide antimicrobial, anti-inflammatory and antimutagenic activities (Chanwitheesuk et al., 2007; Chen et al., 2009; Kroes et al., 1992; Salcedo et al., 2014). GA also shows strong cytotoxicity against several cancer cells. However, the mechanism, by which GA can induce the death of cancer cells, has remained far from understood. Therefore, in the present study, we analyze the cellular mechanisms of GA induced cytotoxicity in HeLa cells.

Inhibition of cell-cycle progression and induction of cancer-cell specific cell death is a very effective strategy to inhibit the proliferation of tumors (Hartwell and Kastan, 1994; Kastan and Bartek, 2004). Our results show that GA significantly reduces cell viability in a dose- and time-dependent manner. We also found that GA induces arrest at the G2/M transition in HeLa, consistent with previous studies in breast cancer MCF-7 cells and lung xenograft cancer NCI-H460 cells (Hsu et al., 2011; Ji et al., 2009). Also, in a bladder transitional cancer cell line, GA induced G2/M phase arrest through Chk2-mediated phosphorylation of cell division cycle 25C (Ou et al., 2010). However, for some cancer cell lines, GA was reported to leave the cell cycle unaltered (Faried et al., 2007; Hsu et al., 2006). This cell-type dependence indicates that the effect of GA involves a specific target that may differ depending on cell type. In this study, we report for the first time that GA inhibits cell-cycle progression in the important cell model HeLa.

Arrest of cell-cycle progression in G2/M can be caused by the so-called mitotic catastrophe, a phenomenon, where multipolar spindles lead to aberrant or abortive mitosis (Roninson et al., 2001). Mitotic catastrophe is often followed by (programmed) cell death and underlies the mode of action for several anticancer agents (Fujie et al., 2005; Jordan et al., 1996). For example, docetaxel induced cell death *via* causing mitotic catastrophe in human breast cancer cells (Morse et al., 2005). Also casticin-induced apoptosis of leukemic cells is caused by mitotic catastrophe (Shen et al., 2009). In the current study, we show that both the incidence of aberrant mitosis and the mortality of HeLa cells increase when the dose of GA increases, or when the incubation with GA is prolonged. The progressive effect of GA is correlated with the appearance of multinucleate giant cells indicative of mitotic catastrophe (Okada and Mak, 2004). The incidence of these multinucleate cells is much lower than that of cells with aberrant spindles, consistent with the hypothesis that mitotic catastrophe is responsible for GA-induced cell death in HeLa.

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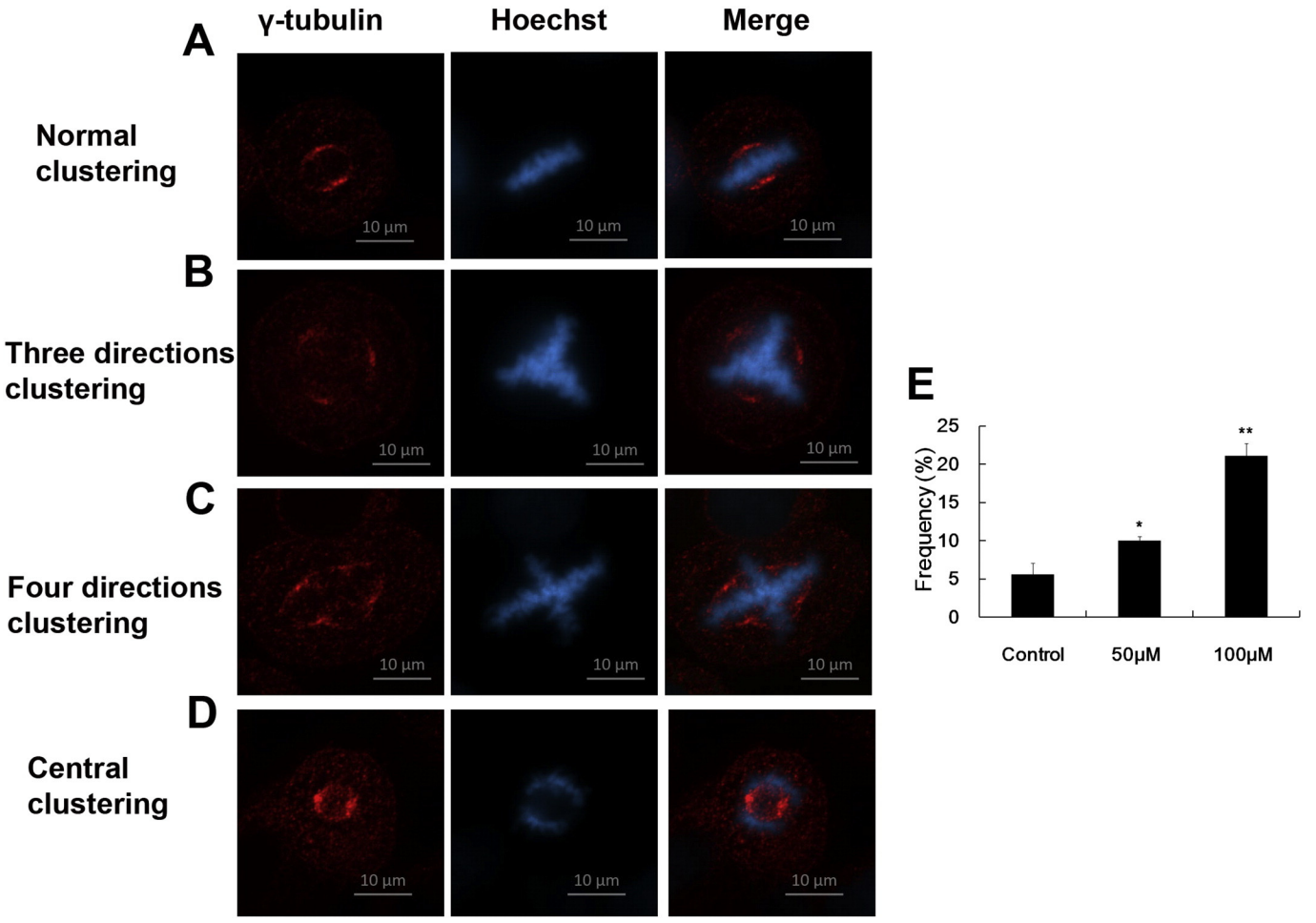

Fig. 5. GA influences the centrosome clustering in HeLa cells. Cells were incubated with 0.01% DMSO, 50 μM or 100 μM GA for 24 h. Then, all the cells were fixed and stained with anti-γ-tubulin antibody. (A) Representative confocal images of normal centrosome clustering in untreated HeLa cells; (B–D) representative images of the aberrant clustering of centrosome in GA-treated HeLa cells; picture A was taken from control cells, picture B, C and D were taken from those cells which had been incubated with 100 μM GA for 24 h. (E) The frequency of aberrant clustering of centrosome. More than one hundred mitotic cells were counted in each group, and three independent experiments were performed. Values are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$ compared with the control group).

The mitotic spindle is organized by the centrosome (Rodionov et al., 1999), and the number of centrosomes is therefore under tight control in healthy cells. This control is frequently inactivated in cancer cells, leading to the formation of supernumerary centrosomes leading to multipolar spindles and cell death (Nigg, 2002; Sato et al., 2000). Several lines of cancer cells can circumvent the formation of multipolar spindles by clustering supernumerary centrosomes such that two functional spindle poles are produced (Drosopoulos et al., 2014; Sluder and Nordberg, 2004). Compounds which inhibit this centrosomal clustering are expected to promote the formation of multipolar spindle and cell death. This mode of action would be confined to cancer cells, since untreated cells are not affected due to their tight control of centrosome numbers (Ogden et al., 2012). In fact, a screen of a small molecule library for inhibition of centrosome clustering in human BT-549 breast cancer cells has identified the compound N2-(3-pyridylmethyl)-5-nitro-2-furamide that increases the incidence of multipolar spindles (Kawamura et al., 2013). Also long known compounds, such as griseofulvin from *Penicillium*, have been shown to induce multipolar mitosis by inhibiting centrosome clustering in N115 mouse neuroblastoma cells (Rebacz et al., 2007). Our study shows that GA inhibits the clustering of supernumerary centrosomes which is followed by mitotic catastrophe and cell death.

Until now, the mechanism of centrosomal clustering is far from understood. However, it is expected to rely on MAPs, that are essential for MT polymerization *in vivo*, where the concentration of free tubulin dimers is below the critical threshold (Karsenti and Vernos, 2001; Nigg, 2002). Recent work uncovered a requirement for cytoplasmic dynein, a minus end-directed MT motor, and for the spindle-associated MAP NuMA in centrosome clustering (Quintyne et al., 2005). Genetic analyses have further identified the integrin-linked kinase (ILK), a protein critically involved in actin and mitotic MT organization, as essential factor for centrosomal clustering (Fielding et al., 2010). Multiple asters (MAST)/Orbit, a member of a new family of non-motor MAPs, required for functional kinetochore attachment, and the maintenance of spindle bipolarity might be a further player (Maiato et al., 2002). Since the position of centrosome is controlled by the compression force of the MTs nucleated at the surface of the centrosome (Zyss and Gergely, 2009), even a mild inhibition of MT nucleation that would leave most of the (less dynamic) MT arrays untouched, should exert a conspicuous effect upon centrosomal clustering. To test, whether the effect of GA might be caused by inhibition of nucleation, we tested the effect of GA on tubulin polymerization *in vitro*. We find a reduced number of MTs indicative of impaired nucleation, and we also find a reduced length of MTs indicative of impaired organization of the plus end. Both effects would be expected for a compound that interferes with the GTPase function of tubulin (Akhmanova and Steinmetz, 2008). However, the exact molecular target needs to be further investigated.

In summary, the results of the present study demonstrate that GA inhibits HeLa cell growth, probably inhibiting centrosome clustering resulting in mitotic catastrophe and cell death. Since healthy cells do

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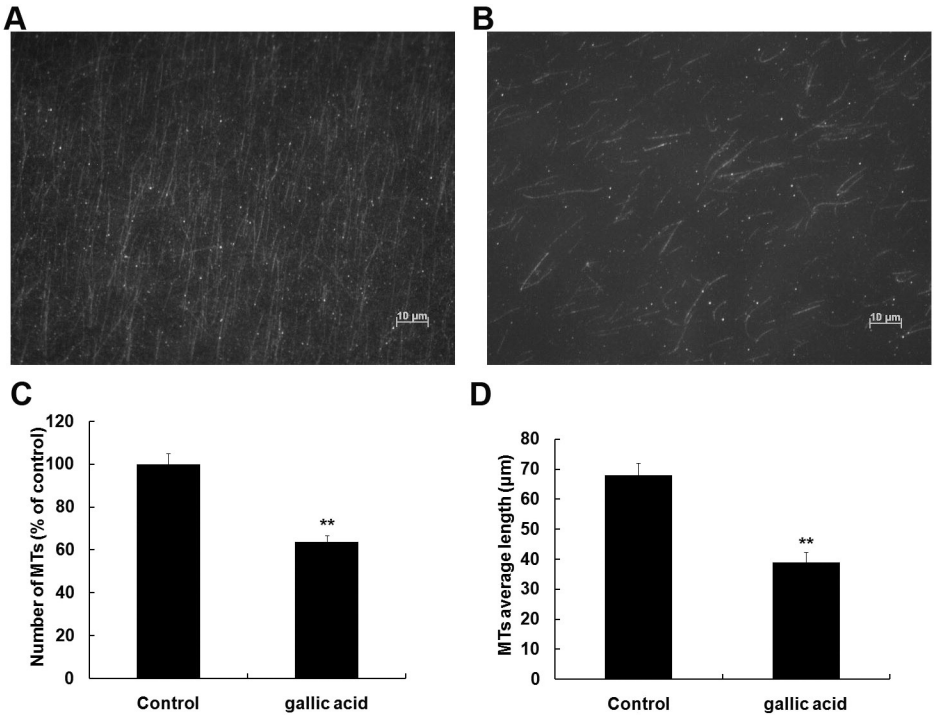

Fig. 6. Effects of GA on *in vitro* polymerization of tubulin. Tubulin was polymerized with 100 μM GA or 0.01% DMSO as a control. MTs were examined under the microscope. (A) Control; (B) GA treatment; (C) the number of MTs as measure for nucleation frequency and (D) the mean length of polymerized MTs as measure for elongation. Three independent experiments were performed. Values are presented as mean ± SEM (**$p < 0.01$ compared with the control).

not form supernumerary centrosomes, this mode of action makes GA interesting as potential agent for cervical cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

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