ORIGINAL ARTICLE

Germanicol induces selective growth inhibitory effects in human colon HCT-116 and HT29 cancer cells through induction of apoptosis, cell cycle arrest and inhibition of cell migration

Yi-Li Hu^{1*}, Xiao-Bo Wang^{1*}, Dian-Dian Chen^{1*}, Xiao-jun Guo², Quan-Jun Yang¹, Li-Hua Dong¹, Le Cheng¹

¹Department of Oncology, China Three Gorges University Renhe Hospital, Yichang, Hubei 443001, China; ²Department of Oncology, Tongchuan City People's Hospital, Tongchuan, Shanxi 727000, China.

*These authors contributed equally to this work

Summary

Purpose: The main aim of this research was to evaluate the anticancer and apoptotic effects of germanicol - a natural triterpene - in HCT-116 and HT29 human colon cancer cells and deciphering its mode of action by studying its effect on the cell cycle and cell migration.

Methods: Cell cytotoxicity was evaluated by MTT assay, while cell death was assessed by LDH assay. Fluorescence microscopy, using DAPI and acridine orange/ethidium bromide (AO-ETBR), was carried out to evaluate the effect of germanicol on cellular morphology and apoptosis induction. Apoptosis quantification was performed by Annexin V-FITC assay, while cell cycle analysis was performed by flow cytometry using propidium iodide (PI).

Results: The results revealed that germanicol showed selective, potent and dose-dependent cytotoxicity in HCT-116 and HT29 human colon cancer cells, while it showed lower cytotoxicity in normal colon cells (human colon fibroblast, CCD-18Co). LDH assay also showed that germanicol

induced dose-dependent cell death in HCT-116 and HT29 cells. Fluorescence microscopy revealed that germanicol induced apoptosis via chromatin condensation and DNA damage in HCT-116 colon cancer cells. It also revealed that the percentage of cells with orange and red fluorescence increased when adding a germanicol dose, indicating apoptosis. Germanicol also inhibited cancer cell migration.

Conclusion: The current findings reveal that germanicol exhibits selective antiproliferative activity against two human colon cancer cells. The normal cell line was less affected by the drug, as compared to the two cancer cell lines, indicating that germanicol will not target normal living cells. The antiproliferative effect was shown to be mediated through the induction of apoptosis and suppression of cell migration.

Key words: annexin-V, apoptosis, colon cancer, cytotoxic-ity, germanicol

Introduction

Cancer continues to be one of the major causes of death worldwide and only slight progress has been made in dropping the morbidity and mortality rates of this disease. Colon cancer, also known as colorectal carcinoma, is the development of a tumor in a part of the large bowell, known as colon or rectum. Various risk factors which could cause colon cancer have been recognized and include older age, inherited genetic factors and life style [1,2]. Colon cancer is recognized as the third principal cause of cancer-related deaths in the Western world. In the developing countries, the prevalence of colon cancer is on the rise due to many key factors, including changing food habits

Correspondence to: Le Cheng, PhD. Department of Oncology, China Three Gorges University Renhe Hospital, No.410, Yiling Road, Yichang, Hubei 443001 P.R. China. Tel & Fax: + 86 0537 2253157, E-mail: lecheng778@hotmail.com Received: 15/10/2015; Accepted: 06/11/2015 coupled with increasing life expectancy. As such, colon cancer is a serious public health threat throughout the globe [3]. According to reports, cases of colorectal cancer in China have been increasing every year which is expected to grow within the next years. Colon cancer can be treated with either chemotherapy, radiation therapy, surgery or a combination of the above, depending on the type and stage of the tumor. Colon cancer that is limited within the colon wall can be cured by simply surgically removing the tumor. However, if the tumor is spread to other parts of the body, it is usually incurable [4].

Natural products, especially plant-based drugs, have always played key roles by furnishing quite a number of potential anticancer agents. These include taxanes (paclitaxel, docetaxel), camptothecin and its derivatives like irinotecan, topotecan, vinca alkaloids such as vincristine, vinblastine and vinorelbine, anthracyclines like doxorubicin, epirubicin, daunorubicin etc. It is estimated that around 65% of all the anticancer drugs approved, have been either natural products or their synthetic or semisynthetic derivatives [5-7]. Naturally occurring triterpenoids have been reported to exhibit potent anticancer and apoptotic activities against a range of cancer cells [8].

Keeping this in view, the focus of this current study was to evaluate the anticancer and apoptotic activity of germanicol triterpene against human colon cancer cell lines HCT-116 (colon), HT29 (colon), by studying its effect on cell morphology, cell cycle arrest and cancer cell migration. The present report on germanicol is the first such study concerning these cell lines.

Methods

Reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin/ streptomycin solution, Hoechst 33342, acridine orange/ethidium bromide, PI and ethanol 96% were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Germanicol was purchased from Wuxi Gorunjie Natural-Pharma Co., Ltd. China. RPMI-1640 medium and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). DNA Flow Cytometric Analysis Kit and FITC Annexin V Apoptosis Detection Kit were purchased from KeyGEN, Nanjing, China.

Cell lines

Human colon cancer cell lines HCT-116 (colon) and HT29 (colon) and human colon normal cell line (Human colon fibroblast, CCD-18Co) were procured from Chinese Academy of Science, Shanghai, China. Cells were grown in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin and 100 μ g/mL streptomycin. Cell cultures were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were allowed to adhere for 24 hrs before treatment with germanicol.

Evaluation of cell viability and cell death

Cell viability testing was assessed by MTT assay while cell death was assessed by LDH assay. In brief, the cells were seeded in 96-well cell culture plates ($2x10^5$ cells/well) and subsequently treated with germanicol (0, 5, 10, 20, 40 and 100 µM) at 37 °C for 6 and 24 hrs. After the incubation, 20 µL of the MTT reagent (final concentration 1.0 mg/ml) were added to each well. The plate was incubated for 3 hrs in a humidified atmosphere. Then, 150 µl of the DMSO solution were added into each well. Finally, the plate was allowed to stand overnight. Absorbance of the samples was measured spectrophotometrically, using a microplate reader (Automated Microplated Reader, Bio-Tek, VT, USA) at a wavelength of 570 nm. A solution of RPMI-1640 medium (assay medium) acted as negative control.

LDH cell death assay was performed as per the manufacturer's protocol (Roche Diagnostics GmbH) given for the LDH cytotoxicity detection kit. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death, due to cell membrane damage [9]. The cells were seeded into 96-well plate and subjected to treatment with germanicol for 24 hrs. Next, LDH (10 µL) release reagent was added in each well. The cell culture plates were then centrifuged after 1 h at 1200 g for 20 min and 200 µL of the supernatant from each well was drawn out and placed into a new black 96-well plate. Afterwards, 50 μ L of LDH assay mixture were added to each well and the plate was incubated at 37 °C for 30 min. The absorbance was measured spectrophotometrically at 490 nm wavelength.

Fluorescence microscopic study of germanicol-induced apoptosis in colon cancer cells, using Hoechst 33342 staining

HCT-116 colon cancer cells were seeded into 12well plates at a density of 2x106 cells/well. Following treatment with 0, 10, 40 and 100 µM dose of germanicol for 48 hrs, cell apoptosis was assessed by the Hoechst staining kit as per instructions of the manufacturer. After compound treatment, the cells were fixed with 3.5% polyoxymethylene and then incubated in Hoechst 33342 solution for 20 min in the dark. The images were recorded using a fluorescence microscope (Olympus IX-70, Tokyo, Japan) at 200x magnification, to identify morphological signs of apoptosis.

Fluorescence microscopic study of germanicol-induced apoptosis in colon cancer cells, using acridine orange/ethidi-

um bromide (AO/EB) staining

HCT-116 cells, at a concentration of $2x10^5$ cells/ mL, were taken in a Petri dish and treated with 0, 10, 40 and 100 μ M dose of germanicol for 48 hrs. The cells were washed with PBS and stained with acridine orange (20 μ g/mL): ethidium bromide (20 μ g/mL) 1:1 ratio for 30 min. 10 μ L of the cell suspension were put on a slide and images were taken using a fluorescence microscope (200x magnification; Olympus IX-70, Tokyo, Japan).

Apoptosis quantification by Annexin V-FITC assay

Annexin V-FITC apoptosis detection kit was used to evaluate the apoptosis induced by germanicol in HCT-116 colon cancer cells. In brief, HCT-116 cells, treated or not with germanicol (0, 10, 40 and 100 μ M) were stained with propidium iodide and Annexin V-FITC as per the guidelines from the manufacturer. After staining, flow cytometry of the HCT-116 cells was carried out using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software to analyze the data for quantifying the fraction of necrotic, apoptotic and viable cells.

In vitro wound healing assay for cell migration

HCT-116 cells $(2x10^5 \text{ cells/ml})$ were seeded in a 6-well plate and incubated at 37 °C until 100 % full confluent monolayer of cells was attained. A straight cell-free wound was made by a 50 ml pipette tip after 12-h starvation. Each well was washed three times with PBS, to eliminate any cellular debris, and then exposed to a number of doses of germanicol (0, 10, 40 and 100 μ M) in a medium. After 48 hrs of incubation, the cells were fixed and stained with 5% ethanol containing 0.5% crystal violet powder for 30 min, and randomly selected fields were captured by a light microscope (inverted light microscope (IX71; Olympus, Tokyo, Japan). The lengths of the wound were determined by Image J (version 1.46) software.

Statistics

Data are shown as the mean \pm standard error of the mean (SEM) of the control. All experiments were performed at least in triplicate. The differences between groups were analyzed by one-way ANOVA, and significance of difference was indicated as *p<0.05, **p<0.01.

Results

Selective cytotoxicity and cell death induced by germanicol against HCT-116 and HT29 colon cancer cells

The chemical structure of germanicol is shown in Figure 1. The cytotoxicity of germanicol against two cancer cell lines (HCT-116 and



Figure 1. Chemical structure of germanicol.

HT29) and one normal cell line (human colon fibroblast, CCD-18Co) was evaluated by using MTT assay. The results which are shown in Figure 2 indicates that germanicol at low concentrations (5,10 and 20 μ M) did not show significant effect on cell cytotoxicity. However, at higher doses (40, 80 and 100 μ M), germanicol exhibited potent and dose-dependent cytotoxic effect on both HCT-116 and HT29 colon cancer cells. Interestingly, germanicol showed less cytotoxicity against normal colon cell line even at higher doses. These results are interesting since germanicol has shown selectivity against cancer cells without, however, affecting normal cells to that extent.

The results of the LDH assay are shown in Figure 3. Germanicol in low doses did not induce significant release of LDH from the cell culture, while, at higher doses, significant LDH release was detected from the cell cultures indicating cell death. The LDH release induced by germanicol at 100 μ M was much more significant. It was noticed that LDH release from HCT-116 was much more pronounced than that from HT-29 colon cancer cells.

Effect of germanicol on the apoptosis induction, via chromatin condensation and DNA damage in HCT-116 colon cancer cells

In order to evaluate the effect of germanicol on chromatin condensation and DNA damage, HCT-116 colon cancer cells were examined under fluorescence microscope. HCT-116 cells were treated with various doses of germanicol for 48



Figure 2. Dose-dependent and selective cytotoxic effect of germanicol in two human colon cancer cell lines (HCT-116 and HT-29) and a normal cell line (human colon fibroblast, CCD-18Co). Data is shown as the mean \pm SD of three independent experiments. * p<0.05, ** p<0.01, vs 0 μ M (control).



Figure 3. Germanicol induced cell death in HCT-116 and HT-29 colon cancer cells in a dose-dependent manner. Data is shown as the mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01, vs 0 μ M (control). The percentage of LDH release is an indication of cell death which was found to be greater in HCT-116 cells as compared to HT-29 cells.

hrs and then stained with DAPI. The results which are shown in Figure 4 indicate that germanicol induced potent and dose-dependent chromatin condensation, accompanied by subsequent DNA damage. The drug treatment also led to a decrease in the number of viable cells. The signs of apoptosis could be easily seen from the bright fluorescence in higher germanicol-dose treated cells.

Apoptotic detection of HCT-116 colon cancer cells by fluorescence microscopy, using AO/ETBR double staining

The results from this assay are shown in Fig-

ure 5 and reveal that viable cells with undamaged DNA have a round and green nucleus, while apoptotic cells have damaged or fragmented DNA and stain as orange and red. Germanicol treatment of HCT-116 cells revealed that it was able to reduce the number of viable cells significantly and in a dose-dependent manner. With an increase in the dose of germanicol, an increasing number of cells were stained orange and red indicating apoptotic process taking place within the cells. Germanicol-treated cells displayed characteristic features of apoptosis, including membrane blebbing, chromatin condensation and apoptotic body forma-



Figure 4. Germanicol induced chromatin condensation in HCT-116 human colon cancer cells. The cells were treated with 0 (**A**), 10 (**B**), 40 (**C**) and 100 (**D**) μ M of germanicol for 48 hrs and then harvested, treated with DAPI and finally analyzed using fluorescence microscopy (magnification x400). Arrows indicate apoptotic cells with chromatin condensation and cell shrinkage.



Figure 6. Germanicol induced apoptosis in human HCT colon carcinoma cells analyzed and quantified by flow cytometry, using Annexin V-FITC-/PI. The Q1, Q2, Q3 and Q4 represent necrotic, late apoptotic, early apoptotic and viable cells respectively. **A, B, C** and **D** represent HCT-116 cells treated with 0, 10, 40 and 100 µM of germanicol for 48 hrs.

tion, which was not detected in untreated cells.

Quantification of apoptosis induced by germanicol

In order to quantify the extent of apoptosis induced by germanicol, Annexin V-FITC assay was performed. In brief, HCT-116 cells were treated with different concentrations of germanicol, stained with Annexin V-FITC/PI and observed by



Figure 5. Induction of apoptosis by germanicol in HCT-116 human colon cancer cells. The cells were treated with 0 (**A**), 10 (**B**), 40 (**C**) and 100 (**D**) μ M of germanicol for 48 hrs and then harvested, treated with AO/ETBR double stain and finally analyzed using fluorescence microscopy (magnification x400). Arrows indicate apoptotic cells which increased with increasing dose of germanicol while cells without arrows are mostly viable and necrotic cells.

flow cytometry for signs of apoptosis. The results indicated that germanicol induced a dose-dependent increase in the percentage of apoptotic cells. The results are shown in Figure 6, which indicate that, when the cells were treated with 0, 10, 40 and 100 μ M dose of germanicol, the average proportion of Annexin V-staining positive cells (apoptotic cells) increased from 7.2% in the control to 18.5, 26.7 and 45.2%, respectively.

Germanicol induced suppression of cell migration

Figure 7 shows the effect of germanicol on the cell migration ability in HCT-116 colon carcinoma cells at different time intervals and different doses of the compound. It was observed that after different time intervals, the cell migration tendency decreased significantly following treatment with 0, 10, 40 and 100 μ M dose of the compound. As compared to the untreated control which exhibited a small wound area, the germanicol-treated cells showed a larger wound area which became bigger and bigger when increasing the dose.

Discussion

Natural products have always been used as anticancer agents for many decades. They have played an important role in the design and devel-



Figure 7. Germanicol suppressed cell migration in HCT-116 human colon cancer cells. The cells were treated with 0, 10, 40 and 100 μ M dose of germanicol for 0, 12, 24 and 48 hrs. * p<0.05, ** p<0.01, vs 0 μ M (control).

opment of more than 60% of the clinically used anticancer drugs. In addition, there are several natural products, or their analogs, which are currently in preclinical and clinical stage. The World Health Organization (WHO) has assessed that about 75-80% of the world population depends mainly on traditional medicines for their health care [10,11]. Natural products, which are highly effective and provoke less side-effects, are a promising substitute for chemotherapy, which has deleterious side-effects. These non-cytotoxic bioactive molecules have great potential for use against cancer, because most of these natural products exhibit pleiotropic properties [12]. The multistep nature of cancer development provides a rationale for cancer prevention. Activation of oncogenes, inactivation of tumor suppressor genes and modulation of mitogenic signal transduction pathways are critical in cancer progression and present attractive targets for cancer prevention/ intervention [13,14].

Numerous anticancer agents work primarily to induce apoptosis in cancer cells and prevent tumor development. The morphological changes of apoptosis, detected in most cell types, primarily start with a decrease in cell volume and condensation of the nucleus [15,16]. Tumor invasion and cell migration are collective features of various malignant tumors, leading to high morbidity and mortality, due to their high growth rate, invasive potential, and resistance to drug treatment. Migration and invasion are the significant features of cancer progression and metastasis [17]. As a result, therapeutic approaches for preventing or suppressing cancer invasion, migration and metastasis can significantly improve the survival of patients. After migration, cancer cells need to attack the cellular membranes to launch the metastasis process successfully at a remote site.

The results of the present study indicate that germanicol has the potential to induce selective and dose-dependent cytotoxic effects in two human colon cancer cells HCT-116 and HT-29. The compound was also tested against the normal cell line (human colon fibroblast CCD-18Co), which showed that this cell line was less susceptible to the drug treatment. Furthermore, it was noted that these cytotoxic effects of germanicol were mediated via induction of apoptosis which was demonstrated by fluorescence microscopy using DAPI and AO/ETBR as staining agents. Germanicol induced apoptosis via chromatin condensation and DNA damage. When the cells were treated with 0, 10, 40 and 100 µM dose of germanicol, the average proportion of Annexin V-staining positive cells (apoptotic cells) increased from 7.2% in the control to 18.5, 26.7 and 45.2%, respectively. Germanicol also led to inhibition of the cancer cell migration.

In conclusion, the current findings reveal that germanicol exhibits selective antiproliferative activity against two human colon cancer cells lines. The normal cell line was less affected by the drug, as compared to the two cancer cell lines, indicating that germanicol will not target normal living cells. The antiproliferative effect was shown to be mediated through the induction of apoptosis and suppression of cell migration.

Conflict of interests

The authors declare no confict of interests.

References

- General Information about Colon Cancer. NCI 2014-05-12. Retrieved 29 June 2014.
- 2. Colorectal Cancer Prevention (PDQ). NCI 2014-02-27. Retrieved 29 June 2014.
- Lin JC, Ho YS, Lee JJ, Liu CL, Yang TL, Wu CH. Induction of apoptosis and cell-cycle arrest in human colon cancer cells by meclizine. Food Chem Toxicol 2007;45:935-944.
- 4. Dai Z, Zheng RS, Zou XN et al. Analysis and prediction of colorectal cancer incidence trend in China. Zhonghua Yu Fang Yi Xue Za Zhi 2012;46:598-603.
- 5. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007;70:461-477.
- 6. Butler MS. Natural products to drugs: natural product derived compounds in clinical trials. Nat Prod Rep 2005;22:162-195.
- 7. Bailly C. Ready for a comeback of natural products in oncology. Biochem Pharmacol 2009;77:1447-1457.
- 8. Petronelli A, Pannitteri G, Testa U. Triterpenoids as new promising anticancer drugs. Anticancer Drugs 2009;20:880-892.
- 9. Dong W, Simeonova PP, Gallucci R et al. Toxic metals stimulate inflammatory cytokines in hepatocytes through oxidative stress mechanisms. Toxicol Appl Pharmacol 1998;1551:359-366.

- 10. Mann J. Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer 2002;2:143-148.
- 11. Fulda S. Modulation of apoptosis by natural products for cancer therapy. Planta Medica 2010;76:1075-1079.
- 12. Slichenmyer WJ, Von Hoff DD. New Natural Products in Cancer Chemotherapy. J Clin Pharmacol 1990;30:770-788.
- 13. Chambers AF, Groom AC, MacDonald IC. Metastasis: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002;2:563-572.
- 14. Singh RP, Dhanalakshmi S, Agarwal R. Phytochemicals as cell cycle modulators a less toxic approach in halting human cancers. Cell Cycle 2002;3:156-161.
- 15. Li W, Wang J, Jiang HR et al. Combined effects of cyclooxygenase-1 and cyclooxygenase-2 selective inhibitors on ovarian carcinoma in vivo. Int J Mol Sci 2011;12:668-681.
- Khoo BY, Chua SL, Balaram P. Apoptotic effects of chrysin in human cancer cell lines. Int J Mol Sci 2010;11:2188-2199.
- 17. Lee WJ, Chen WK, Wang CJ, Lin WL, Tseng TH. Apigenin inhibits HGF-promoted invasive growth and metastasis involving blocking PI3K/Akt pathway and beta 4 integrin function in MDA-MB-231 breast cancer cells. Toxicol Appl Pharmacol 2008;226:178-191.