Ganodermanontriol, a lanostanoid triterpene from *Ganoderma lucidum*, suppresses growth of colon cancer cells through ß-catenin signaling

ANDREJ JEDINAK[1], ANITA THYAGARAJAN-SAHU[1], JIAHUA JIANG[1] and DANIEL SLIVA[1,2,3]

[1]Cancer Research Laboratory, Methodist Research Institute, 1800 N. Capitol Ave, E504, Indianapolis, IN 46202; [2]Department of Medicine, and [3]Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 00000, USA

Abstract. Colorectal cancer is one of the most common cancers in men and women in the world. Previous molecular studies have revealed that deregulation of the ß-catenin signaling pathway plays a crucial role in the progression of colorectal cancer. Therefore, modulation of the ß-catenin pathway offers a strategy to control colorectal cancer progression. The medicinal mushroom *Ganoderma lucidum* (GL) is a rich source of triterpenes with anticancer properties. Here, we show that ganodermanontriol (GNDT), a purified triterpene from GL, inhibited proliferation of HCT-116 and HT-29 colon cancer cells without a significant effect on cell viability. Moreover, GNDT inhibited transcriptional activity of ß-catenin and protein expression of its target gene cyclin D1 in a dose-dependent manner. A marked inhibition effect was also seen on Cdk-4 and PCNA expression, whereas expression of Cdk-2, p21 and cyclin E was not affected by the GNDT treatment. In addition, GNDT caused a dose-dependent increase in protein expression of E-cadherin and ß-catenin in HT-29 cells. Finally, GNDT suppressed tumor growth in a xenograft model of human colon adenocarcinoma cells HT-29 implanted in nude mice without any side-effects and inhibited expression of cyclin D1 in tumors. In conclusion, our data suggest that ganodermanontriol might be a potential chemotherapeutic agent for the treatment of cancer.

Introduction

Colorectal cancer (CRC) is the fourth most common non-cutaneous malignancy and the second most frequent cause of

Correspondence to: Dr D. Sliva, Cancer Research Laboratory, Methodist Research Institute, 1800 N Capitol Ave, E504, Indianapolis, IN 46202, USA
E-mail: dsliva@clarian.org

cancer deaths in the United States (1). In 2010, an estimated 142,570 cases of colorectal cancer will be diagnosed, and 51,370 people will die from the disease (2). Molecular studies have revealed that over 90% of CRC arises from mutation in Wnt signaling pathway (3). ß-catenin is a multi-function protein and plays a key role in Wnt signaling pathway. In addition, ß-catenin has two independent functions in the cell: one in the linking the cytoplasmic side of cadherin-mediated cell-cell contacts to the actin cytoskeleton, another in signaling that involves transactivation in complex with transcription factors of the lymphoid enhancing factor/T-cell factor (LEF-1/Tcf) family. Elevated ß-catenin levels in colorectal cancer caused by mutations in ß-catenin or by the adenomatous polyposis coli molecule (APC), which regulates ß-catenin degradation, result in the binding of ß-catenin to LEF/Tcf and increased transcriptional activation of target genes including cyclin D1, c-myc and proliferator-activated receptor δ, which play important role in colorectal cancer (4-7). Cyclin D1 is a critical oncogene involved in the regulation of progression through the G1 phase of the cell cycle, thereby contributing to cell proliferation (8). In addition, the expression of cyclin D1 is elevated in ~30% of human adenocarcinomas of the colon (9) and represents a good molecular target for cancer therapy or chemoprevention (10).

At the present time there is an increasing public interest in the secondary metabolites from higher fungi for discovering new drugs or lead compounds (11). The significance of bioactive molecules from higher fungi is that it might offer an alternative approach to synthetic drugs, which very often have side-effects and poor bioavailability. *Ganoderma lucidum* (Leyss. ex Fr.) Karst is a medicinal mushroom that has been utilized for centuries in East Asia to prevent or treat various diseases and to reduce the likelihood of cancer invasion and metastasis (12). The anticancer properties of *G. lucidum* have been attributed to a variety of bioactive compounds, including lanostane-based triterpenes (13-15). One of the biologically active molecules isolated from *Ganoderma* species is ganodermanontriol (GNDT) which showed cytotoxicity against cancer cells (13), and antioxidant, anti-HIV-1 protease, and anti-complement activities, respectively (16-18).

In the present study, we examined the effect of GNDT on colon cancer cells *in vitro* and *in vivo*. Here, we show that

GNDT suppressed proliferation of human colon cancer cells and inhibited growth of tumors in a xenograft model of colon cancer. In summary, our data demonstrate a molecular mechanism responsible for the anti-cancer activity of GNDT and suggest the therapeutic use of GNDT for the treatment of colon cancer.

Materials and methods

Materials. Ganodermanontriol (GNDT) was purchased from ChromaDex (Santa Ana, CA, USA) and Planta Analytica (Danbury, CT, USA). Dimethylsulfoxide (DMSO), cremophor and alcohol were purchased from Sigma (St. Louis, MO, USA).

Cells. Human colon cancer cell lines HT-29 and HCT-116 were purchased from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 U/ml). The media and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). The cells were grown at 37˚C in 5% CO_2 and 95% humidity.

Proliferation. HT-29 and HCT-116 cells (2.5×10^5) were plated in 96-well plates. The cells were let to attach overnight, followed by the treatment with GNDT (0-80 μM) or vehicle (DMSO) at indicated times. The antiproliferative effect of GNDT was determined by tetrazolium salt method according to the manufacturer's instructions (Promega, Madison, WI, USA) as described before (19).

Viability. Cell viability of HT-29 and HCT-116 was determined after incubation with GNDT (0-80 μM) for 24 h by staining with trypan blue as described (20).

Western blot analysis. HT-29 cells (1×10^6) were treated with GNDT (0-80 μM) for 24 h and the whole cell extracts were prepared as described previously (21). Equal amounts of proteins (15 μg/lane) were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The protein expression was determined by corresponding primary antibodies: Cdk-2, Cdk-4, cyclin D1, cyclin E, PCNA and p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ß-catenin (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), E-cadherin (BD Biosciences, San Jose, CA, USA) diluted to 1:1000 in blocking solution. The membranes were striped and reprobed with ß-actin antibody (Santa Cruz Biotechnology).

DNA transfection and reporter gene assay. HT-29 and HCT-116 cells (5×10^5) were seeded into 6-well plates in DMEM medium containing 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 U/ml) prior to transfection. The cells were transfected with the TOP-FLASH or FOP-FLASH luciferase reporter constructs (0.5 μg), gift from R.T. Moon, and ß-galactosidase expression vector pCH110 (0.5 μg). Transient transfections were performed with the Lipofecta-mine 2000 and Plus reagent (Invitrogen) in DMEM medium without FBS according to the manufacturer's instructions.

Twenty-four hours after transfection, cells were washed with DPBS and the medium was replaced with DMEM with 2% FBS, followed by treatment with GNDT (0-80 μM) or vehicle for 24 h. Cells were harvested and cell extracts prepared as previously described (21). Luciferase activity was determined using the Lmax (Molecular Devices, Sunnyvale, CA, USA) and normalized for equal numbers ß-galactosidase units.

Human colon cancer xenograft experiments. Male nude immunodeficient mice (nu/nu), 6 weeks old, were purchased from Harlan (Indianapolis, IN, USA) and housed in accordance with protocol approved by the Institutional Laboratory Animal Care and Use Committee of the Methodist Research Institute. On day 0, human colon cancer HT-29 cells (5×10^6) suspended in 0.2 ml of DMEM medium were inoculated subcutaneously (s.c.) in the right flank of each mouse. Seven days after inoculation, mice were randomly divided into two groups: control (n=11) and treatment group (n=10) treated daily with intraperitoneal (i.p.) injection of 100 μl of a vehicle [10% DMSO, 70% cremophor/ethanol (3:1), and 20% PBS] or GNDT (3 mg/kg of body weight) dissolved in DMSO and adjusted to the final concentration of 10% DMSO, 70% mixture of ethanol and cremophor (3:1) and 20% PBS daily for 28 days. Tumor sizes were measured three times a week using calipers and their volumes were calculated using a standard formula: tumor volume (mm^3) = (W x L) 2x1/2, where L is the length and W is the width of the tumor. The animals were sacrificed after 28 days and dissected tumors were fixed in 10% neutral formalin. Body weight was measured twice weekly.

Immunohistochemistry. Formalin-fixed paraffin-embedded blocks were sectioned at 5 μm and placed on positive-charged slides. The expression of cyclin-D1 was evaluated after incubation with rabbit anti-cyclin D1 primary antibody (Santa Cruz Biotechnology) and quantified by counting cyclin D1 positive cells in 3 different fields from 3 animals of the control and GNDT group.

Statistical and densitometric analysis. Data represent the mean ± SD of three independent experiments. The significance was evaluated by ANOVA or Student's t-test. Tumor volume measurements were summarized by treatment group using descriptive statistics (mean ± SD). The comparison of the change in tumor volume and immunohistochemical staining for cyclin D1 between groups was performed using a two-sample Student t-test. The value of $p < 0.05$ was considered significant. Quantification of Western blot data was performed by measuring optical densities of autoradiograms with HP-Scanjet 550c and analyzed by UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The ratios of specific proteins to ß-actin were calculated by standardizing the ratios of each control to the unit value.

Results

Effect of GNDT on colon cancer cell growth and viability. We have recently demonstrated that an extract containing lanostane-type triterpenes from *Ganoderma lucidum* suppressed growth of human colon cancer cells *in vitro* and in animals
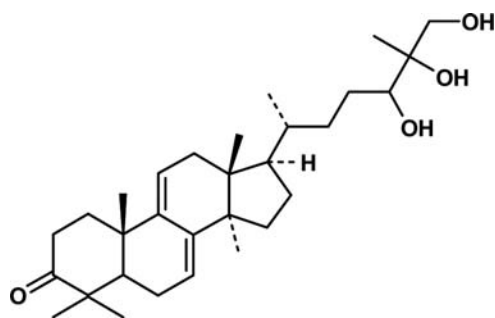
Figure 1. Structure of ganodermanontriol (GNDT).

(15). Therefore, we investigated whether GNDT (Fig. 1), a purified lanostanoid triterpene from *G. lucidum*, has the potency to suppress proliferation of colon cancer cells. As seen in Fig. 2A and B, GNDT significantly suppressed growth of HCT-116 and HT-29 colon cancer cell lines in a time- and dose-dependent manner, respectively. Moreover, the growth-inhibitory effect of GNDT was stronger in HT-29 than in HCT-116 cells. To determine whether this antiprolife-

rative effect was cytotoxic or cytostatic we performed a viability assay (Fig. 2C and D) and we found that GNDT did not affect viability of HCT-116 or in HT-29 cells. Therefore, the data suggest that the effect of GNDT on colon cancer was mainly cytostatic.

Effect of GNDT on cell cycle regulatory proteins. In our previous study (15) we have seen that triterpene extract from *G. lucidum* caused cell cycle arrest at G0/G1 in HT-29 cells. Therefore, we performed a pilot cell cycle analysis of HT-29 cells treated with GNDT (50 μM) by flow cytometry and we have also found cell cycle arrest at G0/G1 phase (not shown). In the next step we wanted to determine which proteins involved in G0/G1 phase are affected by the GNDT treatment. HT-29 cell were treated with GNDT (0-80 μM) and Western blot analysis were performed in whole cell lysates. We observed that GNDT markedly suppressed the expression of cyclin D1, which was down-regulated in a dose-dependent manner (Fig. 3). Moreover, GNDT also down-regulated expression of cyclin-dependent kinase Cdk-4 and proliferating cell nuclear antigen (PCNA), whereas expression of Cdk-2, p21 and cyclin E in HT-29 cells was not affected by the GNDT
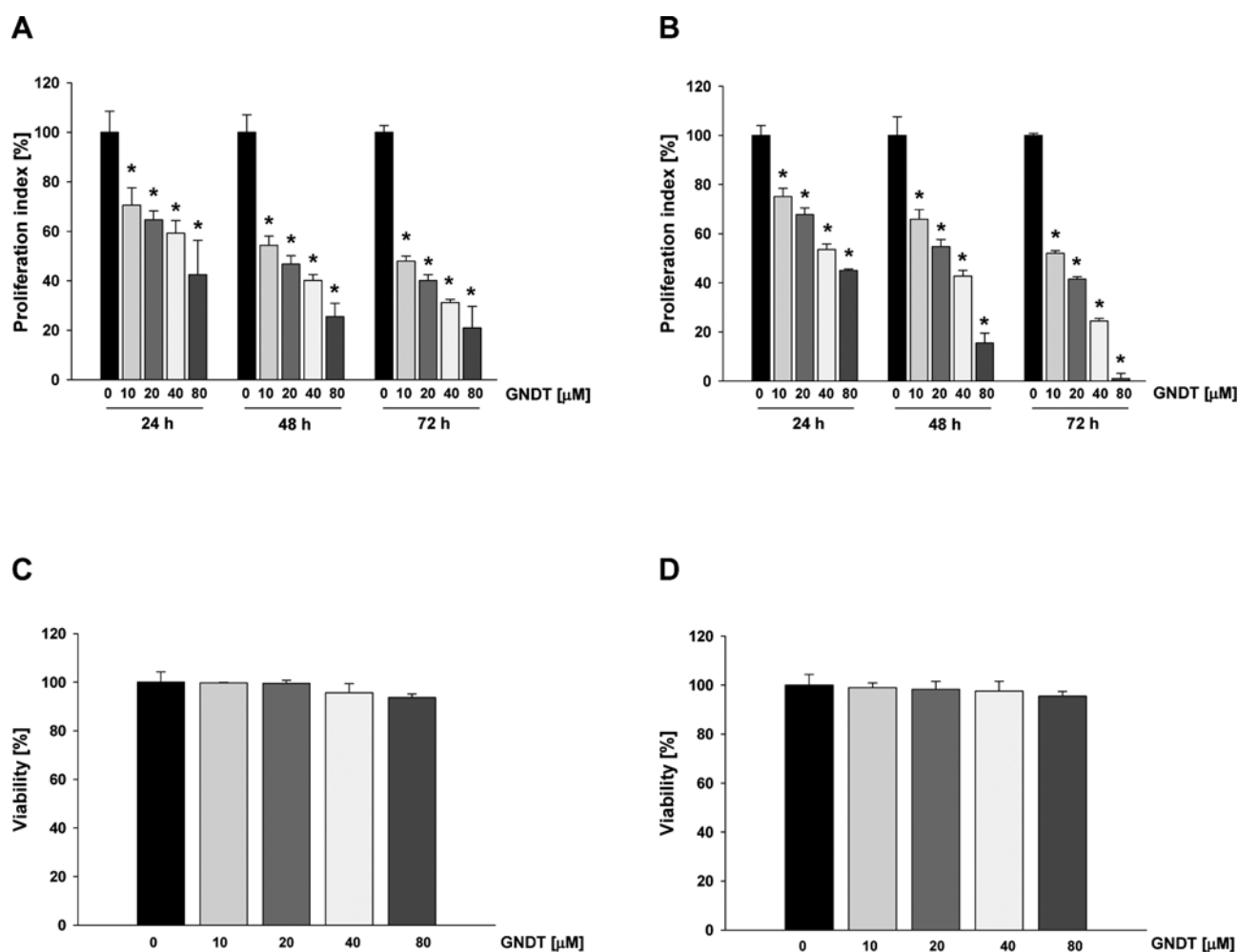

Figure 2. Effect of GNDT on proliferation and viability of HCT-116 and HT-29 cells. (A) HCT-116 and (B) HT-29 cells were treated with ganodermanontriol (0-80 μM) for 24-72 h and cell proliferation was assessed as described in Materials and methods. (C) HCT-116 and (D) HT-29 cells were treated with GNDT (0-80 μM) for 24 h and cell viability was determined as described in Materials and methods. The data are means ± S.D. of triplicate measurements. *Statistically different from control group, p<0.05, analyzed by ANOVA.
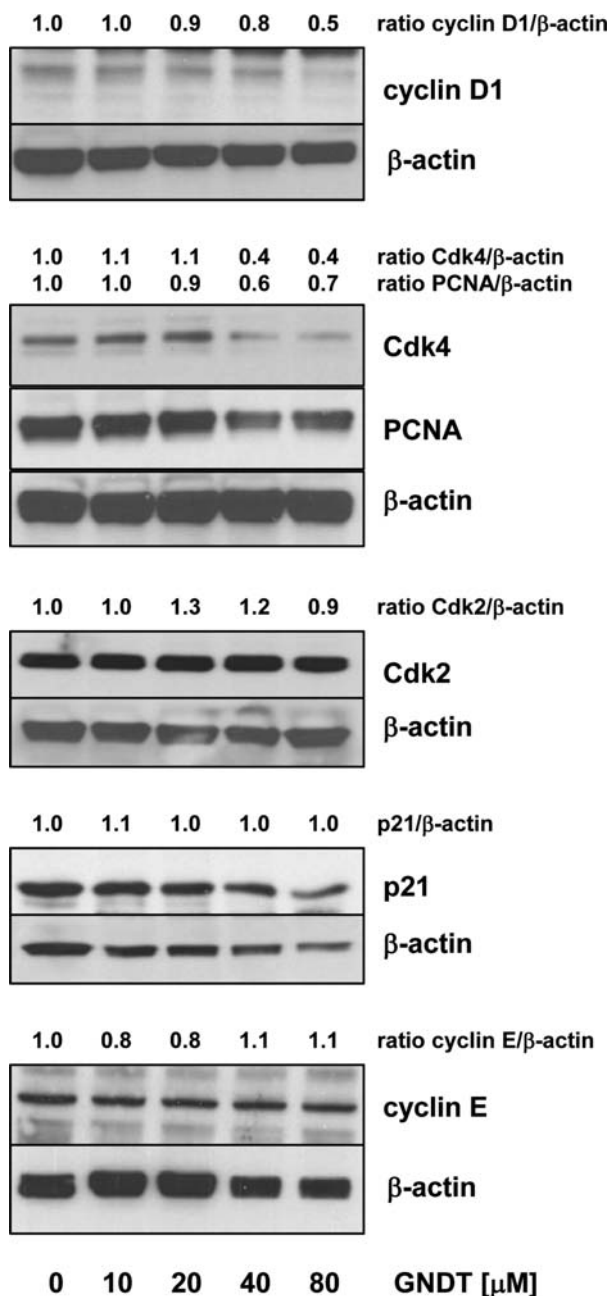
Figure 3. Effect of GNDT on expression of cell cycle regulatory proteins in HT-29 cells. HT-29 cells were treated with GNDT (0-80 μM) for 24 h. The expression of cyclin D1, Cdk-4, PCNA, Cdk-2, p21 and cyclin E was evaluated in whole cell extracts as described in Materials and methods. The equivalent amount of protein loading was verified by reprobing the blot with anti-ß-actin antibody. The expression levels were quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments.

treatment (Fig. 3). Thus, these results suggest that cytostatic effect of GNDT could be specific to the down-regulation of cyclin D1 expression.

Effect of GNDT on ß-catenin transcription activity and ß-catenin expression. Cyclin D1 is one of the main target genes regulated by ß-catenin pathway in colorectal cancer. Therefore, we investigated whether GNDT regulates transcription activity of ß-catenin in colon cancer cells. HCT-116 and HT-29 cells were transfected with reporter gene constructs

containing wild-type LEF/Tcf (TOP-Flash) or mutated LEF/Tcf (FOP-Flash) ß-catenin binding sites and treated with GNDT as described in Materials and methods. As seen in Fig. 4A and B, GNDT significantly decreased specific ß-catenin/LEF/Tcf activity in a dose-dependent manner in HCT-116 and HT-29 cells, respectively. On the other hand, there was no effect on ß-catenin/LEF/Tcf activity in cells transfected with reporter gene containing mutated LEF/Tcf binding sites, except the 80-μM dose of GNDT in HCT-116-transfected cells (Fig. 4A). Thus, our data confirm that GNDT suppresses transcription activity of ß-catenin resulting in the down-regulation of expression of cyclin D1 in colon cancer cells.

As mentioned above, ß-catenin possesses binary function in cell signaling. ß-catenin associates with the cytoplasmic tail of E-cadherin in the cell membrane (22), and is therefore involved in the E-cadherin-mediated cell junction. In order to evaluate the effect of GNDT on the expression of ß-catenin and E-cadherin in colon cancer cells, we treated HT-29 cells with GNDT and performed Western blot analysis as described in Materials and methods. As seen in Fig. 4C, GNDT markedly induced expression of ß-catenin and E-cadherin in a dose-response manner in HT-29 cells.

Effect of GNDT in vivo. Our *in vitro* data suggest that GNDT regulates cell cycle of human colon adenocarcinoma cells HT-29 through down-regulation of cell cycle regulatory proteins, mainly cyclin D1. Therefore, in the next step, we evaluated the effect of GNDT *in vivo* by using xenograft model of human colon adenocarcinoma cells HT-29 subcutaneosly implanted into nude mice. Intraperitoneal administration of GNDT (3.0 mg ganodermanontriol/kg of body weight/day) for 28 days significantly suppressed tumor volume in nude mice (Fig. 5A). As seen in Fig. 5A and B, GNDT suppressed tumor volume and tumor weight; however, tumor weight reduction was not significant (Fig. 5B). Moreover, we did not observe any side-effect of GNDT and there were no differences in body weight between control and treatment groups (Fig. 5C), suggesting that GNDT is not toxic. The immuno-histochemistry revealed that expression of cyclin D1 in tumors was significantly reduced in mice treated with GNDT (Fig. 6).

Discussion

Multiple molecular mechanisms seem to be involved in the tumor suppressive effects of triterpenes from *G. lucidum*. Therefore, we examined the cellular and molecular effects of ganodermanontriol (GNDT), a biologically active triterpene isolated from *G. lucidum* on the growth of human colon cancer cells. In this study, we demonstrate, for the first time, that GNDT inhibits proliferation of colon cancer cells by down-regulating expression of cyclin D1 via modulation of ß-catenin pathway.

Colorectal cancer remains one of the most common cancers in the Western world and amongst the top three causes of cancer morbidity and death (23). Although chemotherapy and radiation therapy have been attempted in adjuvant and palliative treatments, a more effective adjuvant therapy is needed for colon cancer patients. Nearly half of all patients
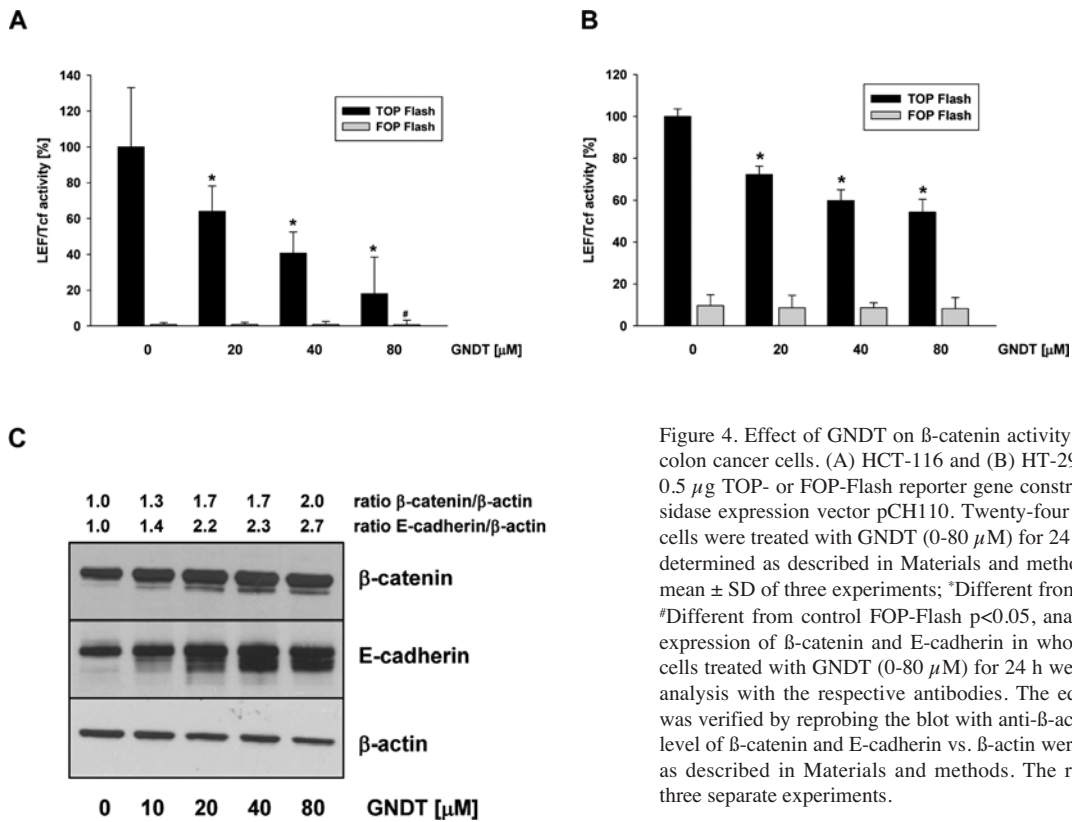
Figure 4. Effect of GNDT on ß-catenin activity and ß-catenin expression in colon cancer cells. (A) HCT-116 and (B) HT-29 cells were transfected with 0.5 μg TOP- or FOP-Flash reporter gene construct and 0.5 μg of ß-galacto-sidase expression vector pCH110. Twenty-four hours after transfection, the cells were treated with GNDT (0-80 μM) for 24 h and ß-catenin activity was determined as described in Materials and methods. Each bar represents the mean ± SD of three experiments; *Different from control TOP-Flash $p<0.05$. #Different from control FOP-Flash $p<0.05$, analyzed by ANOVA. (C) The expression of ß-catenin and E-cadherin in whole cell extracts from HT-29 cells treated with GNDT (0-80 μM) for 24 h were analyzed by Western blot analysis with the respective antibodies. The equivalent amount of protein was verified by reprobing the blot with anti-ß-actin antibody. The expression level of ß-catenin and E-cadherin vs. ß-actin were quantified by densitometry as described in Materials and methods. The results are representative of three separate experiments.
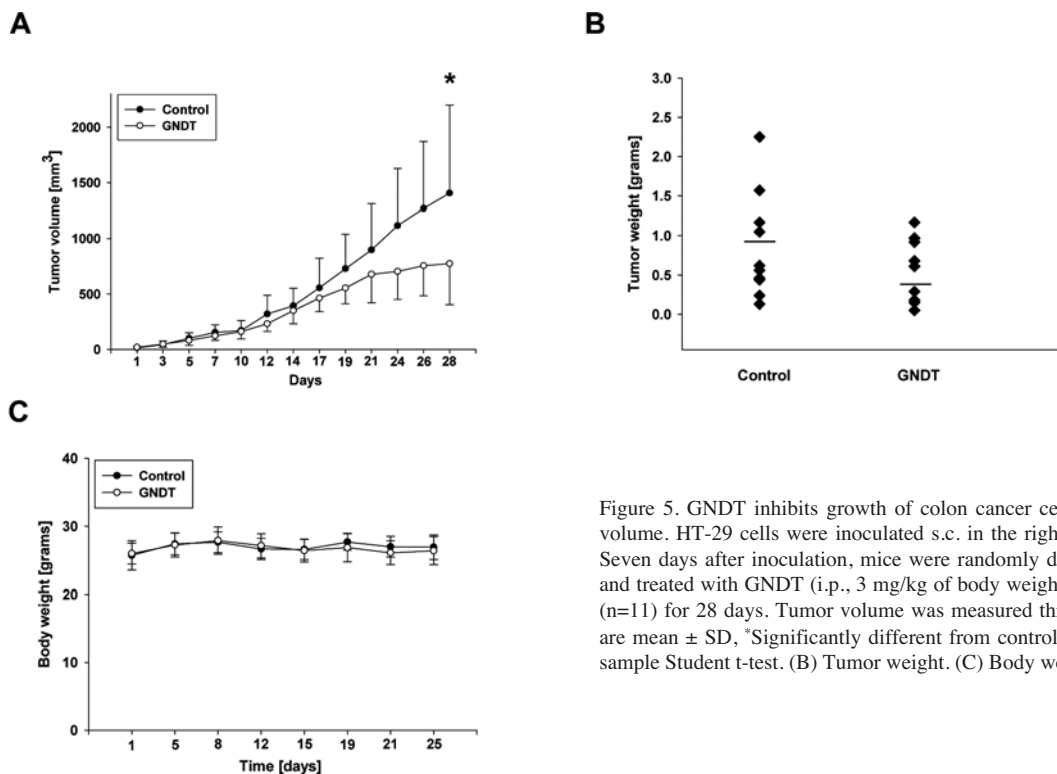

Figure 5. GNDT inhibits growth of colon cancer cells *in vivo*. (A) Tumor volume. HT-29 cells were inoculated s.c. in the right flank of each mouse. Seven days after inoculation, mice were randomly divided into two groups and treated with GNDT (i.p., 3 mg/kg of body weight/day, n=10) or vehicle (n=11) for 28 days. Tumor volume was measured three times a week. Data are mean ± SD, *Significantly different from control ($p<0.05$) using a two-sample Student t-test. (B) Tumor weight. (C) Body weight of mice.

with colon cancer still die of metastatic disease after curative surgery (24). Therefore, new therapeutic drugs with reduced side-effects and better bioavailability are needed. Triterpenoids are structurally diverse organic compounds with promising

anticancer and antimetastatic activity (25,26). The medicinal mushroom *G. lucidum* is a rich source of triterpenes with anticancer activity. Moreover, triterpenes from *G. lucidum* have been reported to also possess hepatoprotective, anti-
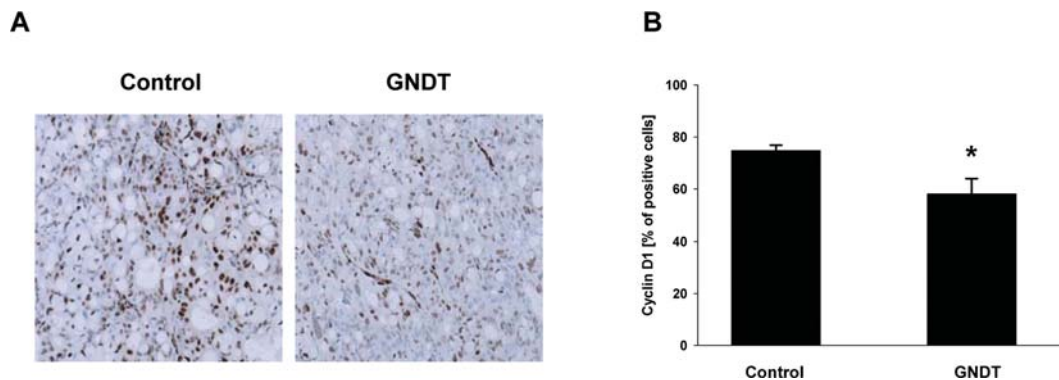
Figure 6. Effect of GNDT on cyclin D1 expression in tumors. (A) Tumors dissected from animals were weighed, sectioned and stained for cyclin D1 as described in Materials and methods. (B) The expression of cyclin D1 was quantified as described in Materials and methods. Data are the mean ± SD (n=3). *Significantly different from control (p<0.05) using a two-sample Student t-test.

hypertensive, hypocholesterolemic, anti-histaminic effects and anti-angiogenic activity (27). Furthermore, some of these triterpenes affected platelet aggregation, inhibited complement inhibition (28) and demonstrated good bioavailability (29,30).

In the present study, we demonstrate that GNDT inhibits proliferation of colon cancer cells and tumor growth by the down-regulation of expression of cyclin D1 and Cdk-4. We have previously demonstrated that other triterpenes, ganoderic acid A and H, suppressed growth of breast cancer cells through the inhibition of expression of Cdk-4 (31). Moreover, ganoderic acid DM suppressed proliferation of prostate cancer cells and inhibited 5α-reductase activity (32), whereas ganoderic acid T suppressed proliferation and tumor growth in mice by the up-regulation of expression of tumor suppressor p53 and by the induction of apoptosis (33), and ganoderic acid X suppressed proliferation of hepatocellular carcinoma cells through the inhibition of topoisomerase I and II (34). In addition, lucidenic acid A, C and N induced cell cycle arrest at G1 phase, whereas lucidenic acid B induced apoptosis of leukemia cells (35).

As mentioned above, the most prominent effect of GNDT was seen on expression of cyclin D1, a target gene of the ß-catenin/LEF-1/Tcf pathway (4). The function of ß-catenin is dual: i) as a structural component of the cell adhesion/actin cytoskeleton network and ii) as a signaling molecule when localized in the nucleus (36,37). After the release of ß-catenin from its intracellular binding partner, ß-catenin translocates to the nucleus, binds to the LEF/Tcf binding site in the promoter regions of target genes and participates in the transcriptional regulation of specific genes associated with differentiation, proliferation and malignant transformation (38-41). Here, we show that GNDT inhibits transcription activity of ß-catenin, finally resulting in the down-regulation of expression of cyclin D1 and inhibition of proliferation of colon cancer cells. On the other hand, GNDT increases total protein levels of ß-catenin in HT-29 cells. Thus, inhibition of ß-catenin transcription activity and induction of protein levels ß-catenin might suggest shift in the relocalization of ß-catenin to the cell membrane where it might bind to the cytoplasmic tail of E-cadherin (22). The adhesion protein E-cadherin is a well-recognized tumor and invasion suppressor that plays a

crucial suppressive role in the transition from adenoma to carcinoma in several epithelial cancers, including colorectal cancers (42). Indeed, here we show that GNDT also induces protein levels of E-cadherin in a dose-dependent manner in HT-29 cells. Therefore, our data are in concert with previous reports, demonstrating the suppression of ß-catenin signaling and associated increase in E-cadherin levels with other natural chemopreventive compounds: vitamin D, indole-3-carbinol and tangeretin (43-45). In agreement with our *in vitro* studies, GNDT also suppresses tumor growth in a xenograft model of colon cancer, and this inhibition is associated with the down-regulation of expression of cyclin D1 in tumors. Therefore, our study suggests that GNDT possesses antiproliferative activity through the modulation of ß-catenin signaling and its target gene cyclin D1 *in vitro* and *in vivo*.

In summary, our data indicate that ganodermanontriol, a purified triterpene from *G. lucidum*, could be a suitable candidate for the therapy of colon cancer. Further preclinical and clinical studies are warranted.

Acknowledgments

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