

1,6-Bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane potentiates *in vitro* and *in vivo* antitumor effects of irinotecan on human colorectal cancer cells

PO-SHENG YANG^{1,2}, JANE-JEN WANG³, YEA-HWEY WANG³,
WOAN-CHING JAN⁴, SHIH-PING CHENG^{1,2} and YI-CHIUNG HSU^{5,6}

¹Department of Surgery, Mackay Memorial Hospital, Taipei 10449; ²Department of Medicine, Mackay Medical College, New Taipei 25245; ³Department of Nursing, National Taipei University of Nursing and Health Sciences, Taipei 11219;

⁴Department of Nursing, Mackay Junior College of Medicine, Nursing and Management, Taipei 11260;

⁵Institute of Statistical Science, Academia Sinica, Taipei 11529; ⁶Department of Horticulture and Biotechnology, Chinese Culture University, Taipei 11114, Taiwan R.O.C.

Received January 19, 2015; Accepted March 10, 2016

DOI: 10.3892/ol.2016.4430

Abstract. 1,6-Bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane (DPD), a diamantane derivative, was previously noted as an anticancer compound through anticancer drug screening with NCI-60 human tumor cells. Irinotecan (CPT-11), a semisynthetic derivative of camptothecin, is clinically active in the treatment of colorectal cancer, with no cross-resistance. The current study conducted a pharmacokinetic evaluation of DPD, an essential component of drug discovery. Subsequent pathway analysis of microarray gene expression data indicated that the anticancer mechanisms of DPD were associated with cell cycle progression and apoptosis. The combined effect of DPD and CPT-11 with regard to the mechanisms of apoptosis-related pathways in COLO 205 cells, and the antitumor effects in colon cancer xenograft mice, were investigated. The plasma concentration and pharmacokinetic parameters of DPD in male albino rats were analyzed following a single dose of DPD by injection. The protein expression of active caspase-3, procaspase-3 and poly ADP-ribose polymerase (PARP) in COLO 205 cells treated with DPD and CPT-11, alone or combined, was evaluated by western blotting. A trypan blue dye exclusion assay revealed that, whilst DPD alone demonstrated good antitumor effects,

this effect was potentiated when combined with CPT-11. Combined treatment with DPD and CPT-11 upregulated the expression of cleaved PARP, procaspase-3, caspase-3 and active caspase-3 in COLO 205 cells. In the colon cancer xenograft model, compared with the control (vehicle-treated) mice, the sizes of the tumors were significantly lower in mice treated with DPD and CPT-11, alone or in combination. Thus, DPD may be a potential therapeutic agent for the treatment of colorectal cancer via upregulating apoptosis-related pathways.

Introduction

Cancer is one of the most prevalent causes of mortality in humans worldwide, with colorectal cancer being the most common type of malignancy. The occurrence of colorectal cancer in Asia is increasing, possibly due to dietary habits and lifestyle factors (1,2). However, the diagnosis of progressive colorectal cancer is inefficient, and the majority of the presently offered therapeutic agents may be toxic, expensive and lacking in efficacy. Cytotoxic chemotherapy has been applied, with limited success, for treatment of patients with advanced colorectal cancer, often causing severe side effects (3). Thus, identifying new and effective chemotherapeutic agents is important for improving the treatment of this disease. Recent evidence involving targeted therapy recommends merging cancer prevention and cancer treatment, with inhibition of cancer being desired over treatment (4,5).

Irinotecan (CPT-11) is a water-soluble, semisynthetic derivative of camptothecin, which is converted *in vivo* to its active metabolite, SN-38. CPT-11 is clinically active in the treatment of colorectal cancer and exhibits no cross-resistance (6). CPT-11 demonstrates antitumor activity against a variety of human tumor xenografts when administered via intravenous, intraperitoneal (i.p.) or oral routes (7,8). Clinical studies have also revealed that CPT-11 has significant activity against a range of tumor types, including colon cancer (9-11). Although CPT-11 has been shown to be highly effective in the treatment of colon cancer, the dosage is limited by

Correspondence to: Dr Yi-Chiung Hsu, Institute of Statistical Science, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 11529, Taiwan R.O.C.
E-mail: syic@stat.sinica.edu.tw

Dr Po-Sheng Yang, Department of Surgery, Mackay Memorial Hospital, 92 Chung-Shan North Road, Section 2, Taipei 10449, Taiwan R.O.C.
E-mail: psyangd0039@gmail.com

Key words: pharmacokinetics, apoptosis, microarray, irinotecan, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane (DPD), COLO 205, NCI-60, colorectal cancer

toxicities, including diarrhea and neurotoxicity (12,13). Therefore, developing new anticancer drugs or establishing effective combinations of drugs would greatly improve colon cancer therapy.

Adamantane and diamantane are closely analogous polycyclic alkanes, which structurally comprise three and six fused cyclohexane rings, respectively (14). Diamantane derivatives have been extensively investigated by chemists; however, limited research regarding the biological activity of diamantane derivatives has been reported (15). A previous study characterized the anticancer activities of diamantane derivatives using 60 human cancer cell lines in the National Cancer Institute (NCI) Anticancer Drug Screening, and evaluated the structure-activity association. 1,6-Bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane (DPD) demonstrated significant anticancer activity on the sub-panel of cell lines (16). The antiproliferative and differentiation-inducing effects of DPD were observed in human colon cancer cells, and these effects were irreversible following the removal of DPD (17). DPD has also been reported to induce apoptosis in human leukemic cells via the elevation of reactive oxygen species (18). However, the molecular mechanisms of the combination of DPD and CPT-11, with special reference to apoptotic signaling, still warrant study. Therefore, the present study evaluated the effect of DPD and CPT-11, alone or in combination, on apoptotic pathways and its mechanisms in colon cancer cell lines. Its *in vivo* anticancer effect in colon cancer xenograft mice was also investigated.

Materials and methods

DPD assay and pharmacokinetic evaluation. DPD was synthesized and provided by Professor Yaw-Terng Chern (National Taiwan University of Science and Technology, Taipei, Taiwan). DPD was weighed and dissolved in dimethyl sulfoxide (DMSO) to produce 1 mg/ml stock solutions, which were stored at -20°C when not in use. The standard solution was prepared by dilution of the stock solution to 10 µg/ml, followed by further serial dilutions with rat plasma obtained from Wistar rats. A total of 8 standard solutions of DPD at 1,000, 500, 100, 50, 10, 5, 1 and 0.5 ng/ml were prepared. The internal standard (IS) 2,2-Bis (4-(4-amino-3-hydroxyphenoxy) phenyl) adamantane (DPA) stock solution, obtained from Professor Yaw-Terng Chern (National Taiwan University of Science and Technology, Taipei, Taiwan), was prepared in DMSO at a concentration of 1 mg/ml and was stored at 4°C when not in use. The IS working solution was prepared by dilution of the IS stock solution to 0.5 µg/ml with acetonitrile.

The dynamic range of the calibration curve was 0.5-1,000 ng/ml. The calibration standards were freshly prepared and run on the day when the samples were analyzed. Blank plasma spiked with known amounts of DPD was prepared and analyzed along with study samples and plasma standards on the day of analysis. The quality control (QC) samples (800, 400, 80 and 4 ng/ml) were prepared from 10, 0.8, 0.4 and 0.08 µg/ml standard solutions in duplicate. The plasma sample (25 µl) was mixed with 50 µl of acetonitrile containing the IS, and was then capped, vortexed and centrifuged at 21,000 x g at ambient temperature for 20 min. The supernatant portion was transferred to a clean autosampler vial prior to injection (20 µl) into a liquid chromatography-mass spectrometry (LC-MS) system. The high

performance LC (HPLC) system consisted of an Agilent 1100 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA) and a Waters C8 Column (5 µm; 3x150 mm; Waters, Elstree, UK), interfaced to the Agilent HPLC System with ESI Positive Ion Spray (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mobile phase consisted of 10 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, USA) and acetonitrile (Sigma-Aldrich) at a ratio of 30:70 (v/v). The flow rate was 0.6 ml/min (total running time, 10 min). The retention times of DPD and DPA (IS) were 6.6 and 6.8 min, respectively. Nitrogen was used as the nebulizing gas. The electrospray needle was maintained at 4.5 kV and heated-capillary temperature was set at 400°C. Data acquisition was via multiple reactions monitoring. Ions representing the [M+H]⁺ species for the analyte and IS were selected in MS1 and collisionally dissociated with nitrogen gas to form specific product ions, which were subsequently monitored by MS2. The mass (M+1) for DPD (analyte) and DPA (IS) were 587 and 536 amu, respectively. Plasma samples that had concentrations above the upper limit of quantitation (1000 ng/ml) were diluted proportionally with blank plasma prior to extraction with acetonitrile. The calibration curve was calculated and plotted based on the spiked drug concentration per sample. The plasma calibration curve was generated from drug concentration vs. peak area ratio, followed by a quadratic or linear regression. The regression parameters of slope, intercept and correlation coefficient were calculated by weight (1/x) linear regression in Analyst[®] version 1.3 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The calibration curves require a correlation coefficient $r > 0.999$. The plasma concentrations in the QC and unknown samples were calculated from the calibration curve. The lower limit of quantitation was 0.5 ng/ml, calculated from the lowest concentration of the calibration curve.

Rats. In total, 8 male Wistar albino rats, weighing 250-350 g each (8-10 weeks old), were obtained from BioLASCO (Taipei, Taiwan). The rats were housed individually and fed a laboratory standard diet (Purina Laboratory Rodent Chow 5001; Ralston Purina Co., St. Louis, MO, USA) *ad libitum*. Animals were handled according to The Guide for the Care and Use of Laboratory Animals (19). A single 2 mg/kg (intravenous to the tail vein) or 20 mg/kg (orally) dose of DPD was separately administered to two groups (4 rats/group). At 0 (prior to dosing), 0.033, 0.083, 0.25, 0.67, 1, 1.5, 2, 4, 6, 9, 24 and 27 h following dosing, a blood sample (~150 µl) was collected from each animal via the jugular-vein cannula and stored on ice (0-4°C). Plasma was separated from the blood by centrifugation (14,000 x g for 15 min at 4°C; Allegra[®] 6R; Beckman Coulter, Inc., Brea, CA, USA) and stored in a freezer (-60°C). All samples were analyzed for the parent compound by LC-MS. Data were acquired via multiple reaction monitoring. Plasma concentration data were analyzed using a standard non-compartmental method with Phoenix WinNonLin version 3.1 software (Pharsight; Certara, L.P., Princeton, NJ, USA).

Cell culture and DPD treatment. The colon cancer cell line COLO 205 (CCL-222[™]) was purchased from the American Type Culture Collection (Manassas, VA, USA). COLO 205 cells were cultured in Hyclone RPMI-1640 with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA). Cells were incubated in a humidified atmosphere of

5% CO₂ in air at 37°C. DPD was dissolved in DMSO at a stock concentration of 10 mM and added to culture media at a final concentration of 0.5–8 μ M. Cells were seeded at 6x10⁵ cells/60-mm dish in the growth medium. The following day, the cells were replenished with a medium containing DPD. Cells were harvested and counted by hemocytometer at 24, 48 and 72 h after treatment with DPD and used for further analysis.

Microarray analysis. COLO 205 cells were treated with DPD (2 μ M) for 24 h. Cells were harvested and 0.2 μ g of total RNA was purified using a Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) and labeled with Cy3 (GE Healthcare Life Sciences) during the *in vitro* transcription process. Cy3-labeled cRNA (0.6 μ g) was fragmented to an average size of 50–100 nucleotides by incubation with a fragmentation buffer (Asia BioInnovations Corporation, Taipei, Taiwan) at 60°C for 30 min. Corresponding fragmented and labeled cRNA was then pooled and hybridized to the ABC Human UniversoChip 20K Microarray (Asia BioInnovations Corporation) at 65°C for 17 h. After washing and drying via nitrogen gun (Asia BioInnovations Corporation), microarrays were scanned using a GenePix 4000B Microarray Scanner (Molecular Devices, LLC, Sunnyvale, CA, USA) at 535 nm for Cy3. Scanned images were analyzed by GenePix Pro Microarray Acquisition & Analysis Software v3.0.5.56 (Molecular Devices, LLC); the image analysis and normalization software was used to quantify signal and background intensities for each feature. Following the acquisition and initial quantification of array images, raw array data were normalized per chip and per gene and filtered based on raw signal intensity and detection call. Genes with an expression fold change of ≥ 2 between a treatment (cells treated with 2 μ M DPD for 24 h) and a control (cells treated with DMSO for 24 h) were considered to be significant. To determine the potential mechanistic network, transcripts with differential expression were studied using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).

Chemosensitivity data. Pearson's correlation coefficients of DPD (#NSC-706831) with Food and Drug Administration-approved chemotherapy compounds whose mechanism of action is presumptively known and published on the NCI Developmental Therapeutics Program (DTP) (20) were used. These data sets were downloaded from the NCI DTP's NCI-60 screen (discover.nci.nih.gov/cellminer/). Seven compounds that were inactive in all cell lines were excluded. A total of 99 drugs were analyzed. Drug sensitivity was measured by the negative of log₁₀ GI₅₀ (50% growth inhibition).

Trypan blue dye exclusion method. Viability of cells was determined using an exclusion assay based on trypan blue dye [0.4% in phosphate-buffered saline (PBS)]. Cultured COLO 205 cells were washed with Hanks' Balanced Salt Solution (HBSS; Gibco; Thermo Fisher Scientific, Inc.), incubated with a solution of 0.125% trypsin, 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.05% glucose dissolved in HBSS (pH 7.2) for 2 min, and then incubated with trypan blue solution (1:1 dilution) for 5 min. Finally, cells were transferred to a Bürker counting chamber and counted by microscopy (Observer-A1; Carl Zeiss, Oberkochen, Germany). Dead cells were defined as those stained with the dye. The percentage of living cells was calculated as the number of viable cells out of the total number of cells counted.

Western blot analysis. Cells were washed twice with ice-cold PBS and lysed in a lysis buffer [0.5% proteinase inhibitors cocktail (Calbiochem; EMD Millipore, Billerica, MA, USA) in 1 ml M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Inc.)]. Cell lysates were centrifuged at 12,000 x g for 30 min at 4°C, and supernatants were separated. Protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). After boiling for 5 min in the presence of 2-mercaptoethanol (MP Biomedicals, LLC, Santa Ana, CA, USA), samples containing cell lysate protein were electrophoretically separated on 10% or 7.5% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto equilibrated nitrocellulose membranes. Following blocking in skimmed-milk, the membranes were incubated with the following primary antibodies: rabbit polyclonal anti-caspase-3 and anti-procaspase-3 (catalog no. 14-264; 1:1,000) (Upstate™; EMD Millipore); mouse monoclonal anti-poly ADP-ribose polymerase (PARP; catalog no. P248; 1:1,000) and mouse monoclonal anti- β -actin (catalog no. A3854; 1:2,500) (Sigma-Aldrich). The bound antibodies were detected with horseradish peroxidase-labeled rabbit polyclonal anti-mouse IgG secondary antibody (catalog no. A9044; 1:10,000; Sigma-Aldrich) and an enhanced chemiluminescence detection kit (EMD Millipore).

BALB/c-nu mouse tumor xenograft model. All *in vivo* experiments were conducted with ethics committee approval (Animal Research Committee of the National Research Institute of Chinese Medicine; approval no. A-100-1), and met the standards required by the UK Co-ordinating Committee on Cancer Research guidelines (21). The 8-week-old male BALB/c-nu mice were obtained from the National Laboratory Animal Center of National Applied Research Laboratories (Taipei, Taiwan) and housed in a laminar flow room under sterilized conditions with a temperature maintained at 25°C and light controlled at a 12 h light and 12 h dark cycle. COLO 205 cells were harvested and resuspended in serum-free RPMI-1640 medium. Cells were adjusted to a concentration of 1x10⁷ cells/ml, and 0.1 ml was inoculated into each mouse. Each experimental group included 6–7 mice bearing tumors. DPD and CPT-11 were dissolved in DMSO and normal saline, respectively. Treatment was initiated when tumor size reached 3–5 mm. DPD (18.75 mg/kg), CPT-11 (15 mg/kg) or a combination of DPD (18.75 mg/kg) and CPT-11 (15 mg/kg) were administered via i.p. injection twice per week (volume of injection, 0.1 ml/20 g body weight). The control group received DMSO vehicle. Tumor size and body weight were monitored twice per week throughout the experiment. The tumor size was measured using a vernier caliper twice per week. Tumor size (V) was calculated according to the formula $V \text{ (mm}^3\text{)} = 0.4 \times A \times B^2$, where A and B are the longest diameter and the shortest diameter of the tumor, respectively (22). At the end of the experiment, all mice were sacrificed using CO₂ gas. The tumors, livers, kidneys and lungs were collected, fixed, embedded and stained with hematoxylin and eosin for pathological analysis.

Statistical analysis. All data are expressed as the mean \pm standard error (SE). The differences between the drug treatment

Table I. Plasma pharmacokinetic parameters after intravenous administration of 2 mg/kg of DPD to rats (n=4).

Parameter	Mean \pm standard error
Clearance rate	2.71 \pm 0.52 l/h/kg
Steady state volume of distribution	4.51 \pm 1.28 l/kg
T _{1/2}	8.0 \pm 1.8 h
Maximum plasma concentration	1.51 \pm 0.16 μ g
Area under the curve	0.75 \pm 0.13 μ g/ml x h
DPD, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane; T _{1/2} , elimination of half-life.	

groups and control group were assessed using a Student's t-test with SigmaPlot version 12.5 software (Systat Software, Inc., San Jose, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DPD assay and pharmacokinetic profile. Following intravenous administration of DPD (2 mg/kg) to rats, the plasma concentration-time profiles of DPD (Fig. 1) and the respective pharmacokinetic parameters were calculated (Table I). DPD exhibited a rapid distribution phase and could not be detected at 27 h after injection. As shown in Table I, the area under the curve (AUC), elimination of half-life (T_{1/2}), maximum plasma concentration (C_{max}), volume of distribution at steady state (V_{ss}) and clearance rate of DPD were 0.75 \pm 0.13 μ g/ml x h, 8.0 \pm 1.8 h, 1.51 \pm 0.16 μ g/ml, 4.51 \pm 1.28 l/kg, and 2.71 \pm 0.52 l/h/kg, respectively.

Pathway analysis of gene expression associated with DPD treatment. To identify gene expression signatures that are associated with biological functions of DPD, microarray and pathway enrichment analysis was conducted to compare expression patterns in COLO 205 cells treated with DPD (2 μ M) for 24 h. The top ten pathways identified by the pathway enrichment analysis are presented in Table II. Notably, the most prominent transcriptional changes in COLO 205 cells treated with DPD (2 μ M) were enriched for cell cycle and apoptosis pathways (7/10). The most common pathways associated with DPD treatment include apoptosis and cell adhesion; other pathways are related to cancer signaling. The results indicate that DPD may regulate cell cycle progression and cancer cell apoptosis in COLO 205 cells.

Chemosensitivity data. The significant associations between the DPD sensitivity profile and each of the 99 drug sensitivity profiles in NCI-60 cell lines are listed in Table III. There were 10 chemotherapy drugs that significantly correlated with DPD sensitivity profiles in NCI-60 cell lines. A significant negative correlation was found for capecitabine and anastrozole, whilst a significant positive correlation was observed for floxuridine, topotecan, nitrogen mustard, gemcitabine, pemetrexed, megestrol acetate, hydroxyurea and methotrexate. From this data, an analog of topotecan (a topoisomerase I inhibitor), CPT-11, was selected to determine

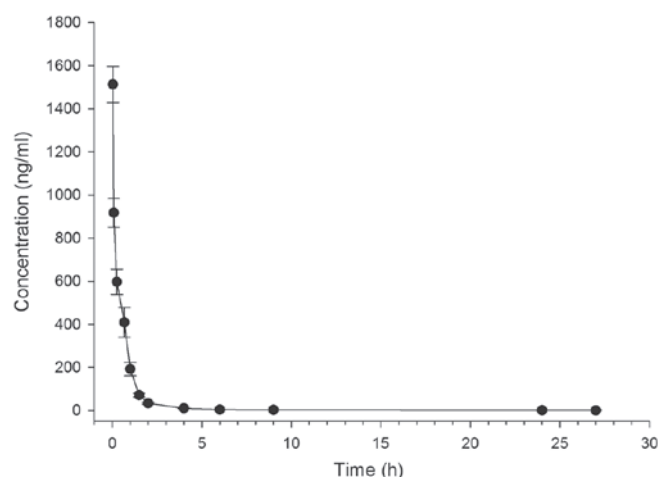


Figure 1. Plasma concentration-time profile of 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane following its intravenous administration at a dose of 2.0 mg/kg to rats (mean \pm standard error; n=4).

whether its combination with DPD had a multiplied antitumor effect in colon cancer.

DPD enhances *in vitro* antitumor effects of CPT-11 in human colon cancer. The *in vitro* antitumor effects of DPD and CPT-11 were tested by trypan blue dye exclusion assay. COLO 205 cells were treated with vehicle control, DPD (1 μ M, 2 μ M), CPT-11 (6.25 μ g/ml) or DPD in combination with CPT-11 for 24, 48 and 72 h. As shown in Fig. 2A, DPD alone had a positive antitumor effect (1 μ M, $P = 0.04$ at 72 hr; 2 μ M, $P = 0.009$ at 72 hr), and combination with CPT-11 had a markedly greater antitumor effect than that of CPT-11 alone. Western blotting showed that DPD alone induced the active caspase-3 expression. In addition, DPD combined with CPT-11 increased the active caspase-3 expression significantly (Fig. 2B). The result suggest that caspase-3 activity is a possible mechanism of this antitumor effect. The results indicated that PARP and caspase-3 may contribute to these antitumor effects.

DPD enhances *in vivo* antitumor effects of CPT-11 in human colon cancer xenografts. To further investigate whether DPD could enhance the antitumoral activity of the chemotherapeutic agent CPT-11 *in vivo*, COLO 205 cells were transplanted into BALB/c-nu mice. When the tumors were palpable (3-5 mm), the mice were treated with vehicle control, DPD (18.75 mg/kg, i.p., twice per week), CPT-11 (15 mg/kg, i.p., twice per week), or DPD in combination with CPT-11. As shown in Fig. 3A, the mean (\pm SE) tumor size in the control animals was 280.4 \pm 67.6 mm³ at the end of the study. By contrast, the mean tumor size in the DPD plus CPT-11 combination treatment group was 12.9 \pm 3.1 mm³ ($P = 0.028$). The mean tumor sizes of DPD and CPT-11 single-treatment animals were 69.2 \pm 23.6 ($P = 0.036$) and 119.4 \pm 23.6 mm³ ($P = 0.080$), respectively. The antitumoral activity of DPD in combination with CPT-11 showed 5-fold and 9-fold increases as compared with DPD and CPT-11 alone, respectively. These results clearly demonstrated that DPD enhances the antitumoral activity of CPT-11.

Treatment with DPD (18.75 mg/kg, i.p., twice per week) or DPD in combination with CPT-11 in nude mice produced no obvious acute toxicity. No significant reduction in body weight

Table II. Top ten pathways identified by pathway enrichment analysis of differentially expressed genes in COLO 205 human colon cancer cells following treatment with DPD (2 μ M) for 24 h.

Pathway name	P-value	Input nodes	Total nodes	Ratio
Cell cycle control of chromosomal replication	1.37x10 ⁻⁸	11	34	11/34
Hereditary breast cancer signaling	1.84x10 ⁻⁷	20	134	20/134
Ataxia-telangiectasia mutated signaling	2.77x10 ⁻⁷	14	66	14/66
Mitotic roles of polo-like kinase	1.17x10 ⁻⁶	14	74	14/74
Cell cycle: G2/M DNA damage checkpoint regulation	2.54x10 ⁻⁶	11	49	11/49
DNA damage-induced 14-3-3 σ signaling	1.06x10 ⁻⁵	7	22	7/22
GADD45 signaling	1.57x10 ⁻⁵	7	24	7/24
Role of CHK proteins in cell cycle checkpoint control	2.53x10 ⁻⁵	11	59	11/59
Molecular mechanisms of cancer	6.41x10 ⁻⁵	32	388	32/388
Salvage pathways of pyrimidine ribonucleotides	4.11x10 ⁻⁴	14	96	14/96

DPD, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane; GADD45, growth arrest and DNA-damage-inducible; CHK, checkpoint kinase.

Table III. Correlation between the invasion profile and each of the 99 drug sensitivity profiles of NCI-60 cell lines.

NSC#	Name	Mechanism	Correlation	P-value
27640	Floxuridine	Antimetabolite/nucleoside	0.321165	0.013130
609699	Topotecan	Anthracycline/topoisomerase poison	0.288541	0.026673
762	Nitrogen mustard	DNA damaging agent	0.28534	0.028481
613327	Gemcitabine	Antimetabolite/nucleoside	0.295385	0.028566
712807	Capecitabine	Antimetabolite/nucleoside	-0.2889	0.032425
698037	Pemetrexed	Antimetabolite/nucleoside	0.279781	0.035049
71423	Megestrol acetate	Hormonal agent	0.273379	0.039627
32065	Hydroxyurea	Antimetabolite/nucleoside	0.267059	0.040882
740	Methotrexate	Antimetabolite/nucleoside	0.266899	0.041007
719344	Anastrozole	Hormonal agent	-0.27687	0.042682

was observed in DPD-treated mice (Fig. 3B). In addition, no tissue damage was observed in the liver, lungs or kidneys after examination of the hematoxylin and eosin-stained tissue slices (data not shown).

Discussion

The current study demonstrated, for the first time, that DPD potentiates the anticancer effects of CPT-11 by the stimulation of caspase-3 and PARP signaling *in vitro*, thus reducing the tumor size of colon cancer xenografts implanted in mice. A previous study showed that co-treatment with DPA and CPT-11 increased the p53-independent induction of p21/Cip1 and p27/Kip1 in the tumor tissue of nude mice, and that DPA induced the elevation of p21/Cip1 and p27/Kip1 in COLO 205 cells *in vitro* (23). However, to the best of our knowledge, no studies have reported a chemotherapeutic effect of DPD or its apoptosis-related mechanisms. Thus, the present findings provide novel insight into the potential role and mechanisms of DPD in the treatment of colon cancer.

Apoptosis is a common and intricate cell suicide pathway, and is an effective way for the body to eliminate damaged

cells (24). Studies have demonstrated that components of apoptotic signaling pathways may be promising targets for the development of novel anticancer agents (25-28). A number of plant-derived, bio-active substances have been shown to act as chemopreventive agents via the induction of apoptosis in various experimental models of carcinogenesis (29). At present, it is generally accepted that agents able to induce apoptosis in cancer cells may have applications in the development of mechanism-based preventions and treatments for cancer (30). Thus, further elucidation of the mechanism of action of DPD and CPT-11 may be of significance for the development for cancer prevention and/or therapy.

Knowledge of the mechanisms of caspase regulation may aid in the manipulation of apoptosis for therapeutic applications in human cancer. Caspases, which are constitutively expressed as latent proenzymes in living cells, affect apoptosis in a manner dependent upon the specific tissue or cell type, or the presence of a particular death signal (31). A previous study demonstrated that cells in which caspase-3 was disrupted, and which were highly resistant to apoptosis induced by ultraviolet irradiation or osmotic shock, remained sensitive to apoptosis induced by γ -irradiation or heat shock (32), suggesting that

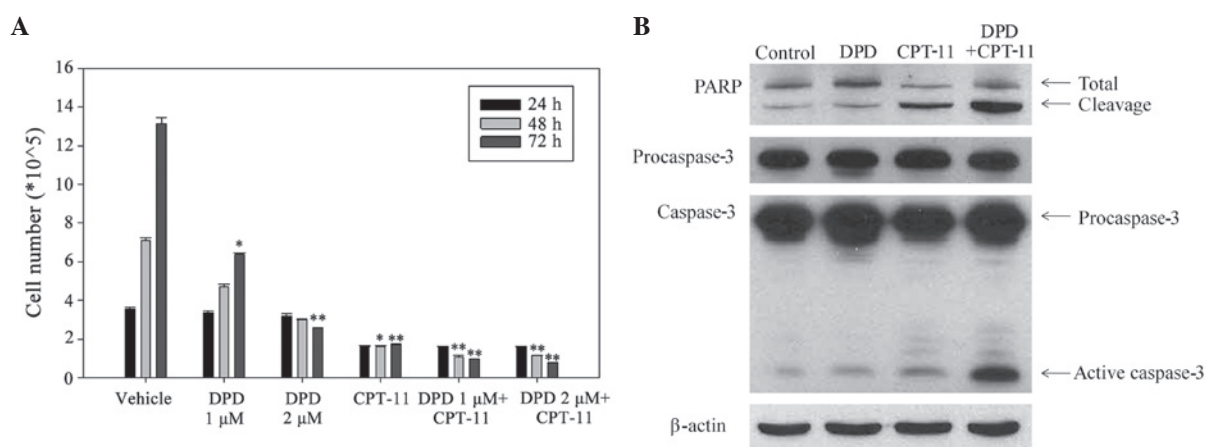


Figure 2. Enhancement of the anticancer activity of CPT-11 by DPD *in vitro*. (A) Antiproliferative effect of DPD and CPT-11 (6.25 μ g/ml), alone or combined, in COLO 205 cells assessed by trypan blue dye exclusion assay. * $P < 0.05$; ** $P < 0.01$ vs. vehicle control. (B) Western blot analysis of caspase 3 activity in COLO 205 cells treated with DPD (1 μ M) and CPT-11, alone or combined. CPT-11, irinotecan; DPD, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane; PARP, poly ADP-ribose polymerase.

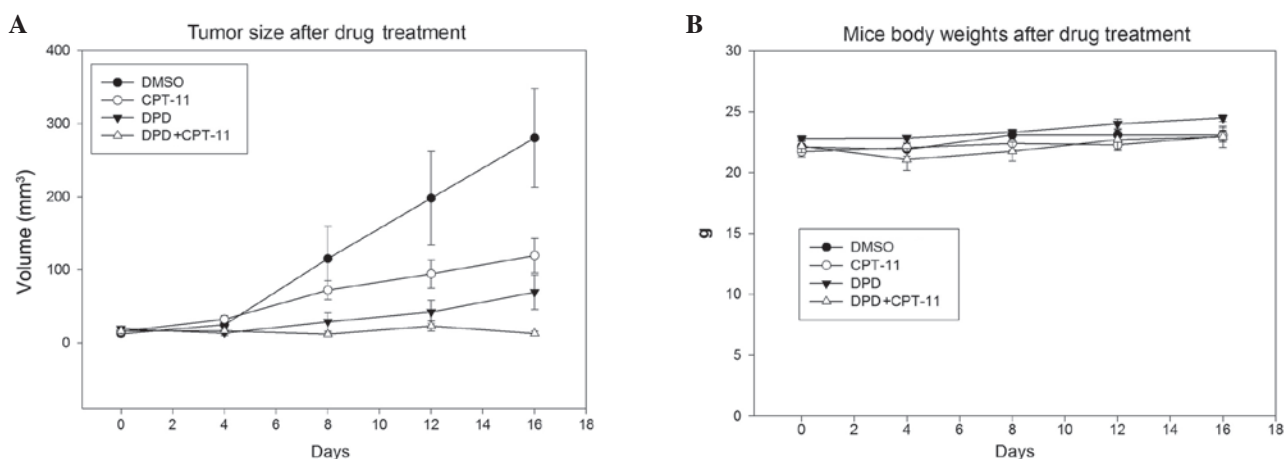


Figure 3. Enhancement of the anticancer activity of CPT-11 by DPD *in vivo*. (A) Effects on tumor volume of DPD and CPT-11, alone or combined, on human colon cancer xenograft. (B) Body weights of mice treated with, DPD and CPT-11, alone or combined. Data is presented as the mean \pm standard error. CPT-11, irinotecan; DPD, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane. DPD (18.75 mg/kg) and CPT-11 (15 mg/kg).

caspase-3 is important in cell apoptosis. Caspase-3 is considered to be the major effector caspase among the known execution caspases, which include caspases-3, -6 and -7 (33). In a previous study using caspase-3-defective MCF-7 human breast cancer cells, induction of apoptosis was accompanied by cleavage of PARP without the corresponding appearance of characteristic DNA internucleosomal degradation and typical morphological changes associated with apoptosis (34). Therefore, additional investigation into the cell morphological changes following DPD treatment are required. The current findings indicate that pro- and active caspase-3 are major contributors to apoptosis in COLO 205 cells. In addition, the results demonstrated that stimulation of caspase-3 and procaspase activities by DPD and CPT-11 strongly activated induction of apoptosis with concomitant stimulation of PARP cleavage.

The observed involvement of the caspase cascade in DPD- and CPT-11-treated COLO 205 cells provides important insights to further examine the mechanism. Circumvention of apoptosis is one of the hallmarks of cancer, and induction of apoptosis during tumor development is a critical step in

chemoprevention (35). In addition to inducing apoptosis in colon cancer cells, the present study revealed that DPD potentiates the effects of CPT-11 when administered in combination via preventing colonic tumor growth in a xenograft model; this suggests that it has the potential for utilization as a chemotherapeutic agent for colon cancer.

The current study also identified that combination treatment with DPD and CPT-11 suppressed tumor growth more prominently than treatments with either of these drugs alone. Additionally, pharmacokinetic studies have revealed that CPT-11 was eliminated faster ($T_{1/2}$, 1.87 ± 0.43 h) (36) than DPD ($T_{1/2}$, 8.0 ± 1.8 h). By contrast, DPD exhibited a markedly smaller distribution volume (V_{ss} , 4.51 ± 1.28 l/kg) than CPT-11 (V_{ss} , 12.50 ± 1.46 l/kg) (36). This finding suggests that DPD may have a higher protein binding rate in the circulation, or may exhibit specific accumulation *in vivo*. The C_{max} (1.51 ± 0.16 μ g/ml/ml \times h) of DPD was comparable with that of CPT-11 (C_{max} , 1.22 ± 0.13 μ g/ml \times h) identified by a previous study (36). However, the AUC of DPD (0.75 ± 0.13 μ g/ml \times h) was ~ 1.77 -fold lower than that of CPT-11 (2.19 ± 0.55 μ g/ml \times h). This

could be explained by the fact that a 5-fold lower dose of DPD (2 mg/kg) was administered than that of CPT-11 (10 mg/kg) (36). These results indicate that DPD may cause fewer side effects than CPT-11 whilst achieving a comparable C_{max} with that of CPT-11, by applying a relatively lower administration dose.

In conclusion, the current study demonstrates that DPD potentiates the anticancer effects of CPT-11 by inducing apoptosis in colon cancer cells. Results from a xenograft model suggest that the anticancer effects of DPD may arise from induction of apoptosis. Taken together, these results indicate that DPD is a potential therapeutic agent for the treatment of colorectal cancer.

Acknowledgements

The authors would like to thank Professor Yaw-Terng Chern (National Taiwan University of Science and Technology, Taipei, Taiwan), who provided the DPD compound used in these experiments, Professor Chin-Wen Chi (Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan) and Dr Shen Yuh-Chiang (National Research Institute of Chinese Medicine, Taipei, Taiwan) for arranging the animal study.

References

- Byeon JS, Yang SK, Kim TI, Kim WH, Lau JY, Leung WK, Fujita R, Makharia GK, Abdullah M, Hilmi I, *et al*: Colorectal neoplasm in asymptomatic Asians: A prospective multinational multicenter colonoscopy survey. *Gastrointest Endosc* 65: 1015-1022, 2007.
- Pham NM, Mizoue T, Tanaka K, Tsuji I, Takamashi A, Matsuo K, Wakai K, Nagata C, Inoue M, Tsugane S, *et al*: Meat consumption and colorectal cancer risk: An evaluation based on a systematic review of epidemiologic evidence among the Japanese population. *Jpn J Clin Oncol* 44: 641-650, 2014.
- Wasserman E, Myara A, Lokiec F, Goldwasser F, Trivin F, Mahjoubi M, Misset JL and Cvitkovic E: Severe CPT-11 toxicity in patients with Gilbert's syndrome: Two case reports. *Ann Oncol* 8: 1049-1051, 1997.
- Saltz LB, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang TS, Rivera F, *et al*: Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: A randomized phase III study. *J Clin Oncol* 26: 2013-2019, 2008.
- Aggarwal BB, Takada Y and Oommen OV: From chemoprevention to chemotherapy: Common targets and common goals. *Expert Opin Invest Drugs* 13: 1327-1338, 2004.
- Robert J and Rivory L: Pharmacology of irinotecan. *Drugs Today (Barc)* 34: 777-803, 1998.
- Kawato Y, Furuta T, Aonuma M, Yasuoka M, Yokokura T and Matsumoto K: Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother Pharmacol* 28: 192-198, 1991.
- Houghton PJ, Cheshire PJ, Hallman JD II, Lutz L, Friedman HS, Danks MK and Houghton JA: Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother Pharmacol* 36: 393-403, 1995.
- Karato A, Sasaki Y, Shinkai T, Eguchi K, Tamura T, Ohe Y, Oshita F, Nishio M, Kunikane H, Arioka H, *et al*: Phase I study of CPT-11 and etoposide in patients with refractory solid tumors. *J Clin Oncol* 11: 2030-2035, 1993.
- Armand JP, Terret C, Couteau C and Rixe O: CPT-11. The European experience. *Ann NY Acad Sci* 803: 282-291, 1996.
- Rothenberg ML: Irinotecan (CPT-11): Recent developments and future directions - colorectal cancer and beyond. *Oncologist* 6: 66-80, 2001.
- Gamelin E, Gamelin L, Bossi L and Quasthoff S: Clinical aspects and molecular basis of oxaliplatin neurotoxicity: Current management and development of preventive measures. *Semin Oncol* 29 (5 Suppl 15): 21-33, 2002.
- Hecht JR: Gastrointestinal toxicity or irinotecan. *Oncology (Williston Park)* 12 (8 Suppl 6): 72-78, 1998.
- Chen CSH, Shen DM and Wentzek SE: Preparation of adamantane and diamantane derivatives as antivirals. Patent WO 9428885 A1, 1994.
- Chen CSHSD and Wentzek SE: Preparation of adamantane and diamantane derivatives as antivirals. WO 9428885 A1 (patent), 1994.
- Wang JJ, Huang KT and Chern YT: Induction of growth inhibition and G1 arrest in human cancer cell lines by relatively low-toxic diamantane derivatives. *Anticancer Drugs* 15: 277-286, 2004.
- Wang JJ, Chang YF, Chern YT and Chi CW: Study of *in vitro* and *in vivo* effects of 1,6-Bis [4-(4-amino-3-hydroxyphenoxy) phenyl] diamantane (DPD), a novel cytostatic and differentiation inducing agent, on human colon cancer cells. *Br J Cancer* 89: 1995-2003, 2003.
- Chang YF, Chi CW, Chern YT and Wang JJ: Effects of 1,6-Bis [4-(4-amino-3-hydroxyphenoxy) phenyl] diamantane (DPD), a reactive oxygen species and apoptosis inducing agent, on human leukemia cells *in vitro* and *in vivo*. *Toxicol Appl Pharmacol* 202: 1-12, 2005.
- National Research Council: Guide for the Care and Use of Laboratory Animals. 8th edition. The National Academies Press, Washington, DC, 2011.
- Holbeck SL, Collins JM and Doroshow JH: Analysis of food and drug administration-approved anticancer agents in the NCI60 panel of human tumor cell lines. *Mol Cancer Ther* 9: 1451-1460, 2010.
- United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br J Cancer* 77: 1-10, 1998.
- Attia MA and Weiss DW: Immunology of spontaneous mammary carcinomas in mice. V. Acquired tumor resistance and enhancement in strain A mice infected with mammary tumor virus. *Cancer Res* 26: 1787-1800, 1966.
- Wang JJ, Lee JY, Chen YC, Chern YT and Chi CW: The antitumor effect of a novel differentiation inducer, 2,2-Bis [4-(4-amino-3-hydroxyphenoxy) phenyl] adamantane (DPA), in combinatory therapy on human colon cancer. *Int J Oncol* 28: 1003-1012, 2006.
- Singh RP, Dhanalakshmi S and Agarwal R: Phytochemicals as cell cycle modulators-a less toxic approach in halting human cancers. *Cell Cycle* 1: 156-161, 2002.
- Kaufmann SH and Earnshaw WC: Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 256: 42-49, 2000.
- Makin G and Hickman JA: Apoptosis and cancer chemotherapy. *Cell Tissue Res* 301: 143-152, 2000.
- Cotter TG: Apoptosis and cancer: The genesis of a research field. *Nat Rev Cancer* 9: 501-507, 2009.
- Wang R, Ma L, Weng D, Yao J, Liu X and Jin F: Gallic acid induces apoptosis and enhances the anticancer effects of cisplatin in human small cell lung cancer H446 cell line via the ROS-dependent mitochondrial apoptotic pathway. *Oncol Rep* Mar 17, 2016 (Epub ahead of print).
- Khuda-Bukhsh AR, Das S and Saha SK: Molecular approaches toward targeted cancer prevention with some food plants and their products: Inflammatory and other signal pathways. *Nutr Cancer* 66: 194-205, 2014.
- Zhang L and Yu J: Role of apoptosis in colon cancer biology, therapy, and prevention. *Curr Colorectal Cancer Rep* 9: 2013.
- Lavrik IN, Golks A and Krammer PH: Caspases: Pharmacological manipulation of cell death. *J Clin Invest* 115: 2665-2672, 2005.
- Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kägi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, *et al*: Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev* 12: 806-819, 1998.
- Slee EA, Adrain C and Martin SJ: Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* 276: 7320-7326, 2001.
- Looi CY, Arya A, Cheah FK, *et al*: Induction of apoptosis in human breast cancer cells via caspase pathway by vernodalin isolated from *Centratherum anthelminticum* (L.) seeds. *PLoS One* 8: e56643, 2013.
- Lin Y, Bai L, Chen W and Xu S: The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. *Expert Opin Ther Targets* 14: 45-55, 2010.
- Zhou S, Li N, Wang X, *et al*: *In vitro* cytotoxicity, pharmacokinetics and tissue distribution in rats of MXN-004, a novel conjugate of polyethylene glycol and SN38. *Xenobiotica* 44: 562-569, 2014.