Antiangiogenic Activities and Cisplatin-combined Antitumor Activities of BPR0L075

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Abstract. *Background: Antimitotic tubulin-binding BPR0L075 is structurally analogous to the vascular-disrupting combretastatin A-4. Materials and Methods: In vitro/in vivo models of endothelial cells cultures, Matrigel™ plug assay, tumor-bearing nude mice, and murine leukemia cells-inoculated mice were utilized to evaluate BPR0L075 for antiangiogenic and antitumoral activity spectra. Results: BPR0L075 concentration-dependently inhibited proliferation and migration of human umbilical vein endothelial cells (HUVECs), disrupted capillary tube formations of HUVECs and rat aorta endothelial cells, and suppressed in vivo VEGF-mediated angiogenesis in Matrigel™ plugs in mice. Besides inhibiting the colony growth of cancer cells, BPR0L075 suppressed growth of subcutaneously-xenografted human lung, colorectal, and cervical solid tumors in nude mice. Combination treatments of BPR0L075 plus cisplatin, compared to either agent alone, demonstrated a stronger growth inhibition against the tumor xenografts in nude mice and longer lifespan in the leukemia mice. Conclusion: BPR0L075 is an antitumoral and antiangiogenic agent and potentiates the anticancer activity of cisplatin.*

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Angiogenesis, the formation of new blood vessels from preexisting vessels, is a complex and tightly regulated physiological process (1) involved in the pathogenesis of many chronic diseases (2) and is a proven mechanism of tumor growth (3). Tumors, *via* angiogenesis, gain blood supplies providing nutrients and oxygen for tumor cell growth. The newly formed blood vessels surrounding the tumor tissues also provide a route for the metastasis of cancer cells (4). The first anti-angiogenic agent bevacizumab has been approved for the treatment of colorectal cancer and is active as a single agent or in combination with cytotoxic agents in pre-clinical and clinical studies (5, 6). Therefore, many efforts have been focused on finding anti-angiogenesis agents for cancer therapies (7). Combretastatin-A4 disodium phosphate (CA-4P) is a vascular-targeting anticancer agent with improved solubility (8, 9) modified from a naturally occurring stilbene combretastatin A-4 (CA-4) isolated from the South African tree *Combretum caffrum* (10). CA-4P inhibits tubulin polymerization into microtubules by binding to tubulin at the colchicine-binding sites (11), exhibits vascular targeting anti-angiogenic activities against tumors in animals and humans (8, 12) and cytotoxic effects against a broad spectrum of human cancer cell lines (11), and is now in the clinical trials (13, 14).

Microtubule is another proven molecular target and its interactions attribute to the mechanism of anticancer actions. Microtubules are important for a variety of subcellular functions, including the regulation of the mitotic apparatus (15). Disruption of microtubules or alteration in microtubules assembly kinetics leads to aberrations in cell cycle progression such as arrest of the mitotic phase, formation of abnormal mitotic spindles, and initiation of apoptotic signals (16). Several microtubule-targeting anticancer drugs have been shown effective and currently used in patients.

Available anticancer drugs targeting the microtubules are antimitotic microtubule-destabilizers, *Vinca* alkaloids vincristine, vinblastine, and vinorelbine (17) and microtubule-stabilizers, epothilone (18) and taxanes paclitaxel and docetaxel (19). Mutations and changed expression levels in tubulins have been associated with the resistance to *Vinca* alkaloids and taxanes (20, 21). Efforts to find new tubulin targeting agents with improved drug properties have been ongoing (22, 23). A number of new tubulin targeting agents are currently in pre-clinical and clinical developments. Most of them however have shown limited objective responses in patients (22). The colchicine-site binders are a novel class of promising anticancer agents in discovery and development such as CA-4P, a known vascular-disrupting agent.

BPR0L075, 6-methoxy-3-(3',4',5'-trimethoxy-benzoyl)-1*H*-indole (Figure 1A) is structurally analogous to the vascular-disrupting CA-4. We have previously demonstrated its tubulin-targeting antimitotic effects and cytotoxic activities against human cancer cells, including multidrug resistant phenotypes (24). In the present study, we examined BPR0L075 for anti-angiogenic activities against human and rat endothelial cells in *in vitro*, *ex vivo* and *in vivo* models and further explored its *in vivo* antitumor activity spectrum. Moreover, antitumor activities of the combination of BPR0L075 and cisplatin were also investigated.

Materials and Methods

Cell lines and chemicals. Murine leukemia P388 cells and human gastric MKN-45 cancer cells were purchased from Japanese Foundation for Cancer Research (Tokyo, Japan). Human cervical KB, lung H1299, and colorectal Colo205 and HCT-116 cancer cells were from the American Type Culture Collection (Manassas, VA, USA). Human cancer cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) from Invitrogen and P388 cells were cultured with additional 0.003% 2-mercaptoethanol. Human umbilical vein endothelial cells (HUVECs) were from Cascade Biologics (Portland, OR, USA). All cells were cultivated at 37˚C in a humidified 5% CO_2 atmosphere. BPR0L075, CA-4, CA-4P, and 6-methoxy-3-(3',4',5'-trimethoxy-benzoyl)-7-azaindole (BPR0L187) were in-house synthesized. Cisplatin and vascular endothelial growth factor (VEGF) was purchased from Sigma (St. Louis, MO, USA). All compounds were dissolved in a vehicle mixture of DMSO/Cremophor EL/water (5/20/75%: v/v/v) for dosing. Acetonitrile of HPLC grade was purchased from J.T Baker (Philipsburg, NJ, USA). All other chemicals and reagents were of analytical grade and were obtained commercially.

Clonogenic growth assay. The growth inhibition activity of BPR0L075 against cancer cells was assessed using the clonogenic growth assay as previously described (25). Cancer cells, KB and Colo205, were seeded at 150 and 1,000 cells/well, respectively, allowed to attach for 5 h, and exposed to various concentrations (1, 10 and 100 nM) of BPR0L075 for 24 h. The cells were allowed clonogenic proliferation in the medium in the absence of drug for 8-10 days followed by fixation and staining with 0.5% crystal violet in 6% glutaraldehyde. Clusters of at least 25 crystal violet-stained cells were counted and the IC_{50} values were determined using PRISM®4 from GraphPad Software, Inc. (San Diego, CA, USA).

HUVEC proliferation assay. Cytotoxic activity of BPR0L075 against HUVECs was measured according to previous reports (26, 27). HUVECs were cultured in M200 medium (Cascade Biologics, Portland, OR, USA) containing endothelial cell growth supplement (Millipore, Billerica, MA, USA), seeded at 6.000 cells/well in 96-well plates, and cultured in 200 μl/well media containing BPR0L075 of various concentrations for 72 h followed by a colorimetric MTS/PMS assay using a Zenyth 340 microplate reader from Anthos (Sulzburg, Austria) to measure the optical density values at 490nm for the number of viable HUVECs cells. The IC_{50}s, concentrations that inhibit the growth of 50% of the cultured cells, were estimated.

HUVEC migration assay. The migration ability of HUVECs was measured in the Transwell® system as previously reported (27, 28) with modifications. Transwells with a porous polycarbonate membrane of 8.0-μm pore size (Cat. #3422) from Corning (Corning, NY, USA) were coated with 20 μl/well of a mixture in 1:1 (v:v) ratio of Matrigel™ from BD Bioscience (Bedford, MA, USA) and M200 medium and incubated for 40 min at 37˚C. HUVECs were seeded onto the upper compartment of the Transwell at 1×10^5 cells/100 μl/well. Media containing BPR0L075 of various concentrations were added in 100 μl/well to the Transwell cultures. VEGF-containing (10 ng/ml) M200 media were added to the lower chambers (700 μl/well) of the culture plates and incubated in a CO_2 incubator at 37˚C for 6 h. The cultures were fixed with 4% formalin for 1 h and the HUVECs remained on the upper surface of the membrane were scraped off with a cotton bud. The migrated HUVECs attached on to the lower surface of the membrane facing the lower chamber and were stained with hematoxylin for 30 min. The blue color-stained migrated HUVECs were then visualized and counted from 5 different viewing areas under 100-fold magnification using a DM IRB inverted microscope from Leica Microsystems (Wetzlar, Germany). The IC_{50}, concentration inhibiting 50% of the HUVECs migrating through the porous membrane, was determined.

HUVEC capillary-like tube formation assay. A 2-dimensional capillary-like tube formation assay in HUVECs was conducted to evaluate the anti-angiogenic activity of BPR0L075 (26). HUVECs cultured in M200 medium containing endothelial cell growth supplement (Millipore, Billerica, MA, USA) were plated onto Matrigel™-coated 96-well plates at 4×10^4 cells/well with BPR0L075 (0.001-10 μg/ml) and incubated at 37˚C for 4 h. The capillary-like tube formations of HUVECs were visualized with 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2*H*-tetrazolium bromide (MTT) staining, images were acquired using a DMIRB inverted microscope from Leica Microsystems, Inc. (Wetzlar, Germany), and integrated area intensities of the capillary network were estimated by computer-aided image analysis.

Rat aorta tube formation assay. Rat aorta cultures were established as previously reported (29). Adult male Sprague-Dawley rats purchased from The National Laboratory Animal Center (NLAC) (Taipei, Taiwan) were intraperitoneally anesthetized with urethane (2 g/kg) and the thoracic aortas were harvested, cross-sectioned into rings of 1-mm thickness, and cultured at one ring/well in 48-well plates. One-day after culture, the aorta rings were treated with

BPR0L075 and cultured in CO_2 incubator at 37˚C for 5 days. The cultures were subjected to the MTS/PMS colorimetric reactions and the optical densities at 490nm were measured using a Zenyth 340 microplate reader. The concentration, IC_{50}, inhibiting 50% of 3-dimensional rat endothelial capillary network growth was determined.

Matrigel™ plug assay. The Matrigel™ plug assay was performed as previously described with modifications to evaluate the anti-angiogenic activity (30). Matrigel™ containing 50 ng/ml of vascular endothelial growth factor (VEGF) was subcutaneously implanted at 0.5 ml/mouse into 7- to 8-week-old male C57BL/6JNarl mice (NLAC, Taipei, Taiwan, ROC). The mice were intravenously administered once daily with BPR0L075 at doses indicated in 5 consecutive days per week for 2 weeks. The mice were euthanized on the 14th day after dosing and Matrigel™ plugs were harvested and extracted for hemoglobin content measurements.

Subcutaneously xenografted tumors in nude mice. Modified from previous reports (31), male BALB/c nude mice of 7-8 weeks old (NLAC; Taipei Taiwan, ROC) were subcutaneously inoculated with H1299 (1×10^6), HCT-116 (1×10^6), KB (1×10^6), or MKN-45 (5×10^5) cells/mouse. Animal body weight and the tumor dimensions of length (*L*), width (*W*), and height (*H*), *i.e*., tumor volume= $L\times W\times H\times\pi/6$, were measured twice a week after inoculation. The tumor-bearing mice were intravenously administered with test drugs *via* tail veins at the indicated dose regimens when the tumors achieved a size of approximately 100 mm^3 or >800 mm^3 for the large tumor growth regression studies.

Measurements of BPR0L075 tissues concentrations in tumors subcutaneously xenografted in nude mice. A single intravenous dose (5, 10, or 50 mg/kg) of BPR0L075 was given at time zero *via* a tail vein to 6-8-week-old male BALB/c nude mouse (NLAC, Taipei, Taiwan, ROC), which harbored a subcutaneously growing human gastric MKN-45 tumor in a size of approximately 1 cm^3. Tumors were harvested from the euthanized animals at 2, 5, 8, 16, 24, and 48 h after the dosing. A 30-μl homogenate aliquot of each tumor was mixed with 60 μl of acetonitrile containing internal standard BPR0L187 (500 ng/ml) followed by centrifugation at 15,000×g for 20 min. As previously reported with modifications (32), the supernatants of 15-μl aliquot were subjected to LC-MS/MS analyses for BPR0L075 levels using a Zorbax Eclipse XDB-C8 reversed-phase column (5μm, 3.0mm×150mm) with mobile phase: water containing 0.1% formic acid (A) and acetonitrile (B) and gradient profile: 0.0-1.2/5, 1.3-3.9/95, and 4.0-5.0/5 (min/%B) at a flow rate of 1.5 ml/min. The retention times for BPR0L075 and internal standard BPR0L187 were 2.40 and 2.46 min, respectively. The LC-MS/MS system was composed of an Agilent 1100 series LC system (Palo Alto, CA, USA) and the column was interfaced to an API 3000™ tandem mass spectrometer equipped with an ESI in the positive scanning mode (Applied Biosystems, Foster City, CA, USA). Data acquisition was *via* multiple reactions monitoring. The employed collision energy and monitored MS/MS ions were 29.0 V and *m/z* 342.3/195.0 for the BPR0L075, or 30.0 V and *m/z* 357.0/ 195.0 for BPR0L187.

Leukemia mouse model. The *in vivo* antitumor activity of BPR0L075 was also evaluated in murine leukemia P388 cells-inoculated mice as previously reported (33). Female or male DBA/2JNarl mice of 20-25g obtained from NLAC (Taipei, Taiwan) were intravenously inoculated with 1×10^6 P388 leukemia cells, and intravenously administered with cisplatin, CA-4P, and/or BPR0L075 *via* tail veins one day after the P388 cells inoculation (day 1) as the regimens indicated. The time when 50% of the leukemic mice still survived was defined as the medium survival time and its increase in percentage in relation to the control were compared for treatment responses.

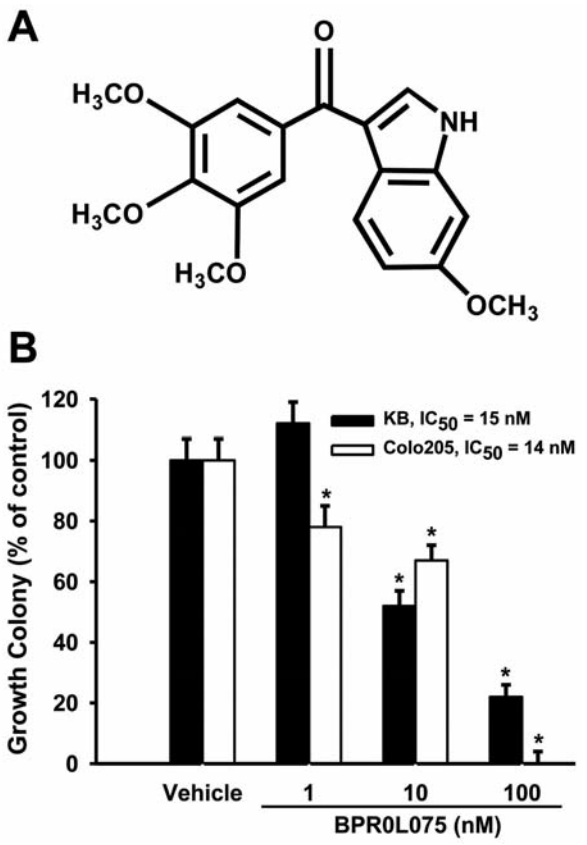

Figure 1. *BPR0L075 inhibited colony growth in human cancer cells. (A), Chemical structure of BPR0L075. (B), BPR0L075 concentration-dependently inhibited clonogenic activity of human cervical KB and colorectal Colo205 cancer cells. Data are means±SEM, n=3. *: p<0.05 vs. vehicle.*

Statistical analysis. Data were subjected to t-test or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test using SPSS software (SPSS Inc., Chicago, IL, USA). A *p* value of <0.05 was considered statistically significant. Data are presented as means±SEM.

Results

BPR0L075 inhibits clonogenic abilities of human cancer cells. After 24-h treatments, BPR0L075 concentration-dependently inhibited the clonogenic growths of human colorectal Colo205 and cervical KB cancer cells (Figure 1B). The IC_{50} values of BPR0L075 against Colo205 and KB cells were estimated at 14±3 and 15±1 nM (n=3), respectively.

Table I. *Lifespan of the leukemia mice treated with BPR0L075, CA-4P, and cisplatin alone and the combinations.*

Treatment	Number of mice†	Median lifespan (Range), Day	Lifespan, %‡
Vehicle Control	45(6)	8(6-9)	100±21
Cisplatin (5 mg/kg, once)	24(4)	15(14-20)	170±13§
CA-4P (25 mg/kg/day, daily)	11(2)	9(8-11)	126±5
BPR0L075 (25 mg/kg/day, daily)	45(6)	9(7-12)	118±11
Cisplatin (5 mg/kg, once) + CA-4P (25 mg/kg/day, days 1-5, 8-12)	12(2)	17(16-18)	211±46§
Cisplatin (5 mg/kg, once) + BPR0L075 (25 mg/kg/day, days 1-5, 8-12)	24(4)	29(18-33)	311±51§,*,¶

†: The total numbers of mice used in several individual experiments were pooled, number of repeats is in the parentheses. ‡: % of the lifespan of vehicle control expressed in mean±SD and calculated using an equation: Lifespan in %=T/C×100%, where T and C are the median lifespans of drug-treated and vehicle control groups, respectively. §: $p<0.05$ *vs*. Vehicle Control. *: $p<0.05$ *vs*. Cisplatin. ¶: $p=0.05$ *vs*. Cisplatin+CA-4P.

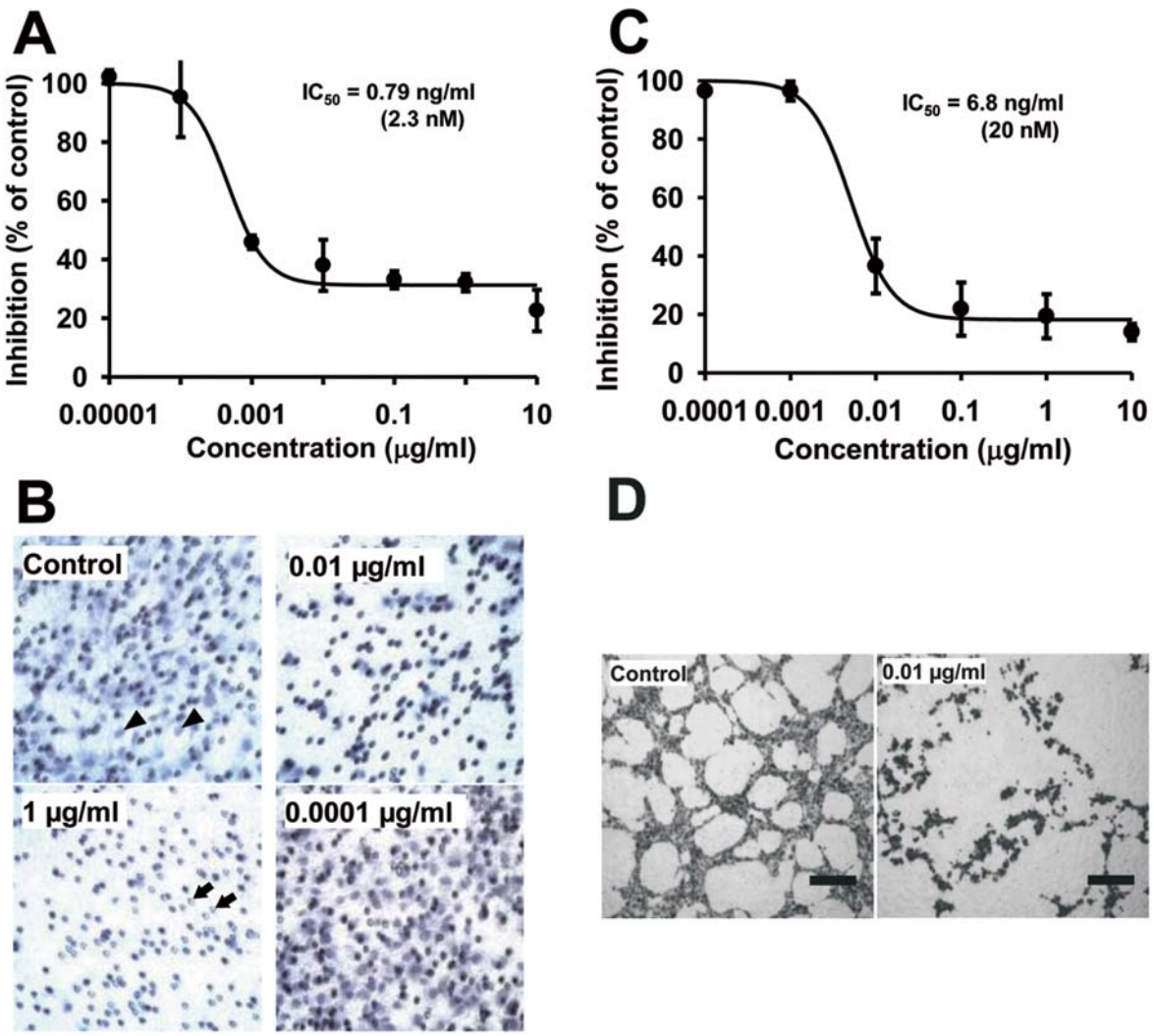

Figure 2. *Antiangiogenic activities of BPR0L075. The anti-angiogenic activities of BPR0L075 against HUVECs growth (A) and migration (B, representative images showing blue-staining nuclei of the migrated HUVECs as the two arrows indicated and 8-μm pores as the two arrowheads indicated) in a concentration-dependent manner are shown. (C), BPR0L075 inhibited the 3-dimensional endothelial capillary-like network formation outgrowing from the rat aorta ring cultures. BPR0L075 disrupted 2-dimensional tube formation of HUVECs, as shown (D, Control and 0.01 μg/ml). Scale bars=100 μm.*

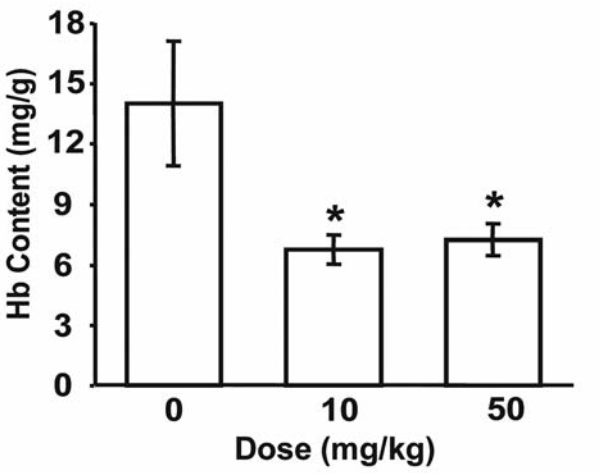

Figure 3. *BPR0L075 inhibited VEGF-mediated angiogenic activity in mice. Intravenously administered BPR0L075 at 10 and 50 mg/kg/day for 5 consecutive days decreased the hemoglobin (Hb) content in the VEGF-containing Matrigel™ plugs of the treated mice measured on day 7. Data are means±SEM, n=7-8. *: p<0.05 vs. control.*

In vitro antiangiogenic effects of BPR0L075. BPR0L075 inhibited the growth of HUVECs with an IC_{50} of 2.3±0.3 nM (n=3) as shown in Figure 2A. HUVECs migration ability was suppressed by BPR0L075 treatments with an IC_{50} of 64±7 nM (n=3) and representative images for vehicle control and BPR0L075-treated (0.0001, 0.01, and 1 μg/ml) are shown in Figure 2B. BPR0L075 (Figure 2C), CA-4 and CA-4P displayed a concentration-dependent inhibition activity on the 3-dimensional rat endothelial tube formation in the *ex vivo* aorta ring cultures with IC_{50}s of 20, 23, and 70 nM (n=3), respectively. BPR0L075 also disrupted the MTT-stained 2-dimensional capillary-like tube formation of HUVECs at a low concentration of 0.01 μg/ml (29 nM) as shown in Figure 2D with an estimated IC_{50} at 109±53 nM (n=3).

In vivo antiangiogenic effects of BPR0L075. The *in vivo* Matrigel™ plug assays in mice were carried out and we observed a much lesser density of infiltrated angiogenic blood vessels in those VEGF-containing plugs harvested from the mice treated with BPR0L075 than those with vehicle. Hemoglobin contents in the plugs harvested from the mice treated with BPR0L075 at 10 and 50 mg/kg/day, *i.v.*, were decreased to 49 and 52% of those from the vehicle-treated control, respectively, after a 1-week treatment period (Figure 3). The results indicate that BPR0L075 is active *in vivo* against VEGF-mediated angiogenesis in mice.

BPR0L075 alone and combined with cisplatin suppress tumor growths in nude mice. Intravenously administered BPR0L075 exerted significant growth suppressive activities against the subcutaneously xenografted human tumors in nude mice. As shown in Figure 4A and Figure 4B. The growth of H1299 tumors was greatly inhibited by BPR0L075 (50 mg/kg/day, *i.v.,* on days 1-5, 8-12, 15-17, n=6) in which the initial tumor sizes on day 1 were not different from those on day 18 (p=0.240) and the growth of colorectal HCT-166 tumors was suppressed by BPR0L075 (50 mg/kg/day, *i.v.,* on days 1-5, 8-12, n=12), respectively. Cisplatin (5 mg/kg/day, *i.v.,* on days 1, 5, and 9, n=10) combined with BPR0L075 (50 mg/kg/day, *i.v.,* on days 1-5 and 8-12, n=10) exhibited an enhanced antitumor activity in relation to the singly used cisplatin of higher dose (7.5 mg/kg/day, *i.v.,* on days 1, 5, and 9, n=12) as observed in Figure 4C. A significant difference in the tumor growth-suppressive efficacy on KB tumors was observed between cisplatin alone and the combination regimens. Likewise, the enhanced tumor growth-suppressive effect was also obser-ved in the same single verse combinational treatment regimens of cisplatin and BPR0L075 in gastric MKN-45 tumors (n=7 per group) in Figure 4D. On day 22 post-treatments, the combination of cisplatin and BPR0L075 decreased the MKN-45 tumor size by 78%, whereas the single-agent treatments resulted in only 53% and 19% decreases in the tumor size for BPR0L075 for cisplatin (7.5 mg/kg), respectively. Our findings indicate that BPR0L075 is not only active as a single agent with a broad activity spectrum against human tumors but also active in potentiating the antitumor activities of cisplatin.

BPR0L075 reduces the size of large growing tumors in nude mice. Human cervical tumors grew subcutaneously and reached a relatively large size of approximately 800 mm^3 or larger in nude mice at which the mice were intravenously treated with BPR0L075. BPR0L075, given intravenously at 25 and 50 mg/kg/day on days 23-25 and 29-31 after the tumor cell inoculation as the arrowheads indicated in Figure 5, significantly reduced the size of the large growing tumors in nude mice (n=7). A dose-dependent relationship of BPR0L075 treatments in shrinking the sizes of large growing tumors in nude mice was observed.

Tissue concentrations of BPR0L075 in tumors subcutaneously xenografted in nude mice. Tissue concentra-tions of BPR0L075 in the subcutaneously growing MKN-45 tumors in the nude mice intravenously administered with a single dose of BPR0L075 at 5, 10, and 50 mg/kg were measured. The concentration profiles were plotted against time as shown in Figure 6. A dose- and time-dependent manner of BPR0L075 tissue levels in the growing tumors was observed in the dosing range. BPR0L075 in tumor tissues was only detectable in the initial 8 h after dosing in the animals of low dose (5 mg/kg, *i.v.* once). The results showed that BPR0L075, intravenously given at a dose of 10 mg/kg or higher, maintains a tissue level of 16.7 ng/g (approximately 50 nM) in the tumors for at least 48 h after the intravenous administration.

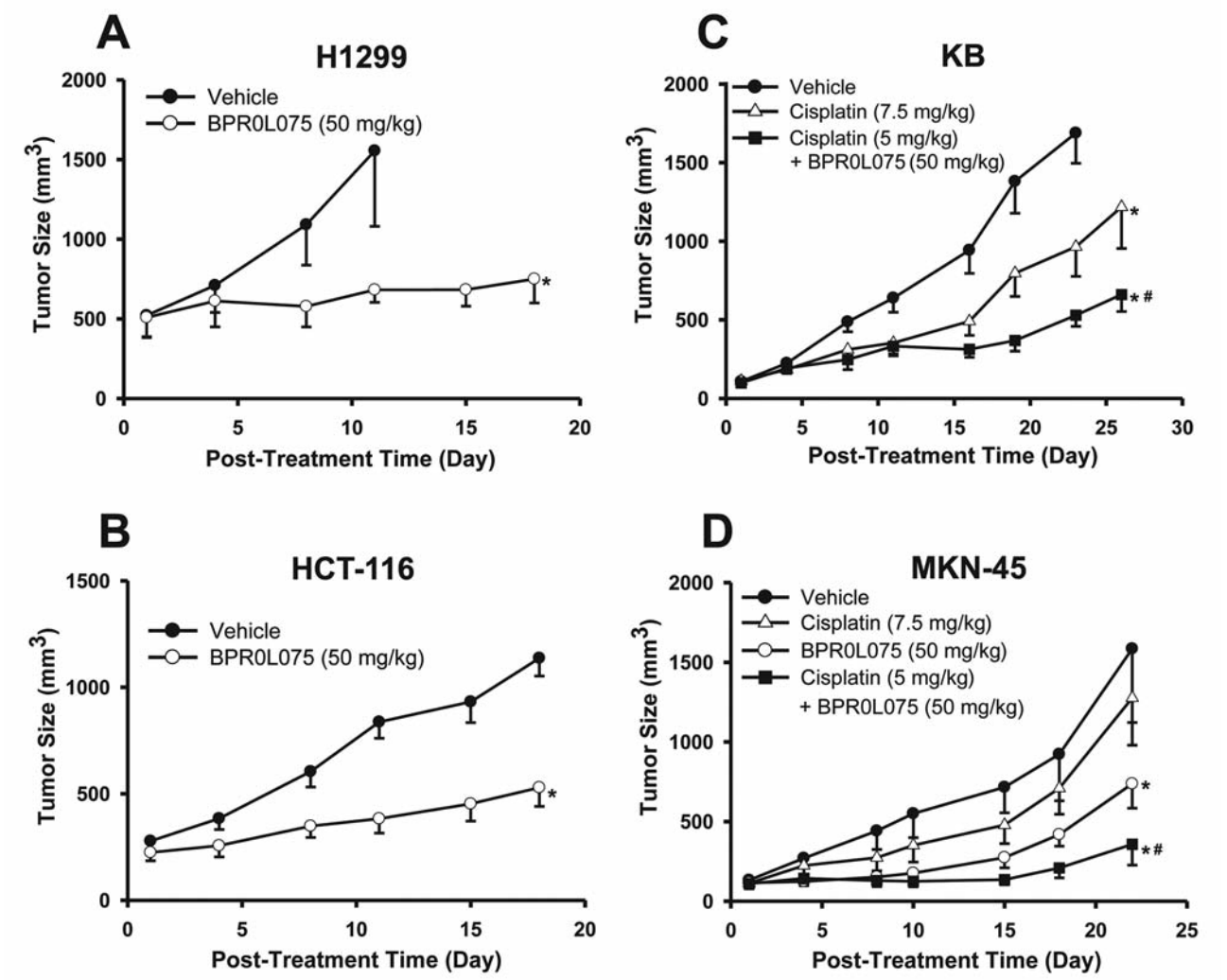

Figure 4. *BPR0L075 and cisplatin suppressed the growth of subcutaneously xenografted human tumors in nude mice. Human lung H1299 (A), colorectal HCT-116 (B), cervical KB (C), and gastric MKN-45 (D) tumor growths were inhibited by intravenous administrations of BPR0L075 or cisplatin alone, or the combination of BPR0L075 and cisplatin at the dose regimens indicated. Data of tumor sizes are means±SEM. *: p<0.05 vs. vehicle control. #: p<0.05 vs. cisplatin (5 mg/kg) in panel (C); vs. cisplatin (7.5 mg/kg), and BPR0L075 (50 mg/kg) in panel (D).*

BPR0L075 potentiates cisplatin activities prolonging the lifespans of leukemia mice. The murine leukemia P388 cells-inoculated DBA/2 inbred mice were treated with either BPR0L075 (25 mg/kg/day, *i.v.,* days 1-5 and 8-12) or CA-4P (25 mg/kg/day, *i.v.,* days 1-5 and 8-12). As shown in Figure 7 and the quantitative data summarized in Table I, none of the two agents showed a significant lifespan-prolonging activity. Cisplatin (5 mg/kg, *i.v.,* once on day 1) alone increased the lifespan of the leukemic animals by approximately 70% of the vehicle-treated leukemic mice. Cisplatin in combination with CA-4P (25 mg/kg/day, *i.v.,* on days 1-5 and 8-12) showed limited and not significant activity, compared to that of cisplatin alone, in prolonging the lifespans of the leukemic mice. On the other hand, BPR0L075 (25 mg/kg/day, *i.v.,* on days 1-5 and 8-12) synergistically potentiated the effects of cisplatin and resulted in an increase of lifespan of the leukemia mice from 170% (cisplatin alone) to 311% (cisplatin combined with BPR0L075) of the vehicle-treated control mice.

Discussion

BPR0L075 has been identified as an inhibiting factor for tubulin polymerization promoting tumor cell apoptosis, and therefore as an active agent *in vitro* against human leukemia, glioblastoma, oral, nasopharyngeal, breast, gastric, colorectal, and liver cancer cells (24). BPR0L075 is also active *in vivo* suppressing the growth of the xenografted human gastric MKN-45 and cervical KB tumors and P-gp-overexpressed

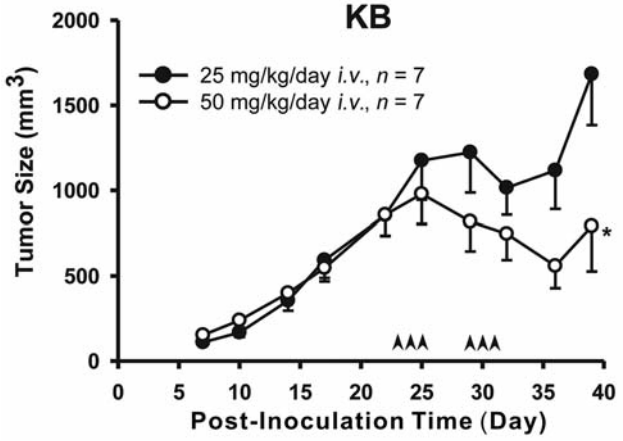

Figure 5. *BPR0L075 reduced the size of large growing human cervical KB tumors subcutaneously xenografted in nude mice. BPR0L075 treatments (once daily on days 23-25 and 29-31 as arrowheads indicated) suppressed the growth of large (>800 mm³) KB tumors. Data of tumor sizes are means±SEM, n=7. *: p<0.05 between groups.*

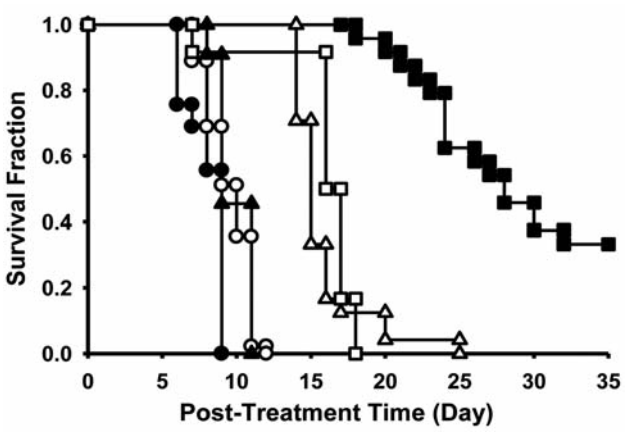

Figure 7. *Comparative activities of BPR0L075 and CA-4P combined with cisplatin on prolonging the lifespan of leukemia mice. Combination treatments of cisplatin with BPR0L075, not CA-4P, synergistically increased the lifespan of the P388-inoculated leukemia mice. ●: vehicle; ○: BPR0L075 (25 mg/kg/day, daily), Δ: cisplatin (5 mg/kg, once), ▲: CA-4P (25 mg/kg/day, daily), ❑: cisplatin (5 mg/kg, once)+CA-4P (25 mg/kg/day, daily), ■: cisplatin (5 mg/kg, once)+BPR0L075 (25 mg/kg/day, days 1-5, 8-12).*

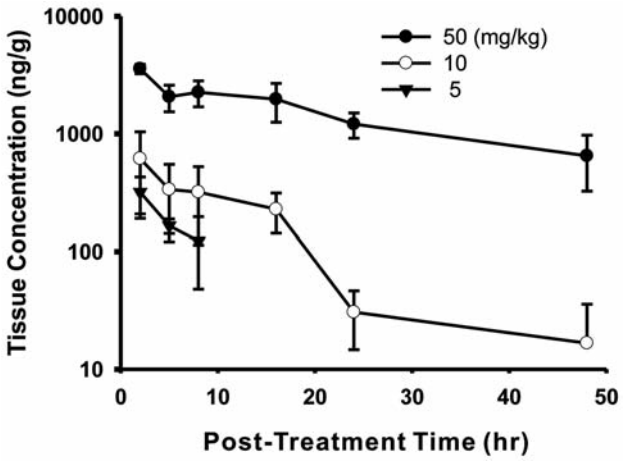

Figure 6. *Tissue levels of BPR0L075 in subcutaneously xenografted human tumors in mice. Human gastric MKN-45 tumors subcutaneously growing in the nude mice intravenously administered with BPR0L075 were harvested at the indicated time points after the dosing treatment and the tumor tissue levels of BPR0L075 were measured using LC-MS/MS analysis. Data are means±SEM, n=3.*

multidrug-resistant KB tumors in nude mice. In the present study, we further demonstrated BPR0L075's antiangiogenic activities in *in vitro*/*ex vivo* capillary tube formations of the endothelial cells and *in vivo* VEGF-mediated angiogenesis in Matrigel™ plug in mice. In addition to the previously reported mechanisms of anticancer activity, BPR0L075 concentration-dependently reduces the colony-forming abilities of human cancer cells, such as the cervical KB and colorectal Colo205 cells in 3-dimensional cultures. The *in vivo* antitumor spectrum of BPR0L075 extends to the human lung H1299 and colorectal HCT-116 tumors subcutaneously growing in nude mice. Most importantly, combinational treatments of cisplatin plus BPR0L075 display better anticancer activity against the growth of human tumor xenografts in nude mice and a potentiated cisplatin activity by BPR0L075, not CA-4P, on prolonging the lifespan of the murine leukemia P388 cells-inoculated mice.

As previously reported, the pharmacodynamic relationship: $C^n \times T = K$ was proposed to compare the anticancer drug exposures in different experimental conditions and a minimum (C×T) was used to estimate the therapeutic end point efficacies (34, 35). This pharmacodynamic approach on estimation of the efficacy was herein adopted. We found that the IC_{50}s (2.3-109 nM) of BPR0L075 against HUVECs growth and migration, and capillary tube formations of the endothelial cells were approximate to the IC_{50}s (1-23 nM) in cytotoxicity assays against a panel of human cancer cells (24) and to 14-15 nM in the clonogenic growths of human cancer cells (this study). These results indicate that BPR0L075 exerts dual action mechanisms against tumor growths through direct cytotoxic and indirect anti-angiogenic effects. Furthermore, after a single intravenous dose at 10 or 50 mg/kg, the tissue levels of BPR0L075 in the xenografted tumors were maintained for 48 h at concentrations higher than 50 nM, which is within the range of the IC50s (*i.e.*, 2.3-109 nM) against the human cancer cells and endothelial

cells. The dose-dependent tumor tissue concentrations of BPR0L075, therefore, demonstrate that the once daily intravenous dosing regimen for BPR0L075 provides effective BPR0L075 concentrations in the xenografted tumors and the *in vivo* antitumor activities observed. Overall, our findings on the BPR0L075 tumor tissue levels provide a pharmacokinetic basis for the observed *in vivo* antitumor efficacies of BPR0L075.

An *in vivo* growing tumor needs a constant supply of nutrients *via* the newly formed blood vessels recruited from the adjacent existing vasculatures (3). Tumors of large sizes are associated with drug resistance (36, 37). It has also been reported that the cytotoxic activity of a thymidylate synthase-inhibiting antimetabolite agent 5-fluorouracil is less active against large colon tumors, compared to that against small tumors in mice (38). BPR0L075 demonstrates *in vitro* and *in vivo* antiangiogenic activities. It is active in reducing the size of large solid tumors in nude mice, which may be, in part, due to the inhibition of angiogenesis in the targeting tumors. This indicates that BPR0L075 may act directly on the tumor-surrounding blood vessels, disrupting the nutrients supplying machineries without being distributed or acting directly onto the targeting tumor cells. The observed effective antiangiogenic activity of BPR0L075 at low concentrations, however, does not exclude the possibility of the cytotoxicity-enhanced tumor tissue penetration of BPR0L075. This mechanism of penetration of a cytotoxic drug caused by drug-induced cancer cell death has been reported (39). Nevertheless, the tissue distribution kinetics of BPR0L075 into the tumors was found to be dose-dependent, which indicates that BPR0L075 can readily penetrate tumor tissues. Overall, these results suggest that BPR0L075 exerts both cytotoxic and antiangiogenic activities simultaneously against the targeting tumors for its *in vivo* antitumor activities. Whether this observation can be translated further for treating cancer patients with a large tumor as perioperative treatments in combination with surgery and/or other chemotherapeutics remains to be explored in preclinical and clinical studies.

Cisplatin has been widely used as an anticancer drug alone and more frequently in combination. Combinational chemotherapies of cisplatin plus drugs including methotrexate (40), gemcitabine (41), and vindesine (42) synergistically enhance the antitumor activities of individual drugs. Inclusion of cisplatin in a combinational chemotherapy with irinotecan has been suggested for S-1- and paclitaxel-refractory metastatic gastric cancer in patients (43). Here, we report that the combination of cisplatin plus BPR0L075 increases antitumor activities against the human cervical and gastric xenograft tumors in nude mice, which is in agreement with the additive effects previously observed in the combination of cisplatin and a microtubule targeting paclitaxel (44, 45). There are mechanistically proven evidences showing that RAD001 enhances cisplatin-induced apoptosis by inhibition of the phosphoinositide-3-kinase-AKT signaling pathway (46). Bortezomib enhances the delivery of cisplatin to the targeted tumors (47), and gemcitabine decreases the intracellular glutathione that detoxifies cisplatin activity by conjugation (48). Whether BPR0L075 exhibits any of these effects attributed to the mechanisms of the potentiated cisplatin activities remains to be further explored. In addition to the antimitotic activities arresting cancer cell cycle leading to tumor cell apoptosis, BPR0L075 also shows potent anti-angiogenic activity which is likely to contribute to the observed antitumor growth activities and synergistic efficacies in the combination uses with cisplatin. Agreeably, AC-7700 a vascular-disrupting agent augments the cisplatin efficacy by inhibiting the excretion of cisplatin distributed to the tumors *via* decreasing the tumor blood flow (49). To investigate if BPR0L075 reduces the excretion and thus increases the accumulation of cisplatin in the targeting tumors, further studies are needed.

In agreement with previous reports (9), we observed a marginal activity (26% increased lifespan) of CA-4P structurally analogous to BPR0L075 in prolonging the lifespan of the leukemia mice. Unlike CA-4P, BPR0L075 showed significant activity enhancing the cisplatin efficacy in prolonging the lifespan of the leukemia mice. Furthermore, the combination uses of cisplatin plus either vascular targeting CA-4P or 5,6-dimethyl-xanthenone-4 acetic acid were reported with a greater tumor cell killing effect (50). In the present study, we also observed an enhanced antitumor activity in the combination use of cisplatin with BPR0L075, than either one acting alone against the growth of solid tumors. Nevertheless, not all cisplatin-based chemotherapies are superior to the drug alone. Negative cisplatin effects-counteracting results have been reported when combining cisplatin with another anticancer agent such as paclitaxel in pre-clinical (51) and in clinical (52) studies, whereas positive augmenting cisplatin-based combinational activities with paclitaxel have also been reported (53, 54). Therefore, controversial clinical findings on the combination chemotherapies using cisplatin exist and an optimal regimen for the combination uses of cisplatin plus BPR0L075 may be further explored in preclinical and clinical studies.

In conclusion, we report here, in addition to the antimitotic tubulin binding and cell cycle arresting activities of BPR0L075 against human cancer cells and cancer cells of multidrug resistant phenotypes, that anti-angiogenesis contributes to the antitumor effects of BPR0L075. A broadened *in vivo* antitumor activity spectrum of BPR0L075 and an enhanced cisplatin activity following a combinational treatment of cisplatin plus BPR0L075 have been demonstrated. Whether BPR0L075 can be used as a single agent or in combination with a cytotoxic agent (*e.g.*, cisplatin) against chemotherapy naïve and/or multidrug resistant tumors remains to be further investigated.

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References

1 Folkman J: Angiogenesis. Annu Rev Med *57*: 1-18, 2006.
2 Cao Y: Angiogenesis: What can it offer for future medicine? Exp Cell Res *316*: 1304-1308, 2010.
3 Eichhorn ME, Kleespies A, Angele MK, Jauch KW and Bruns CJ: Angiogenesis in cancer: molecular mechanisms, clinical impact. Langenbecks Arch Surg *392*: 371-379, 2007.
4 Kessenbrock K, Plaks V and Werb Z: Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. Cell *141*: 52-67, 2010.
5 Hung H: Bevacizumab plus 5-fluorouracil induce growth suppression in the CWR-22 and CWR-22R prostate cancer xenografts. Mol Cancer Ther *6*: 2149-2157, 2007.
6 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. New Engl J Med *350*: 2335-2342, 2004.
7 Shimizu K and Oku N: Cancer anti-angiogenic therapy. Biol Pharm Bull *27*: 599-605, 2004.
8 Chaplin DJ, Pettit GR and Hill SA: Anti-vascular approaches to solid tumour therapy: evaluation of combretastatin A4 phosphate. Anticancer Res *19*: 189-195, 1999.
9 Siemann DW, Chaplin DJ and Walicke PA: A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). Expert Opin Investig Drugs *18*: 189-197, 2009.
10 Pettit GR, Singh SB, Boyd MR, Hamel E, Pettit RK, Schmidt JM and Hogan F: Antineoplastic agents. 291. Isolation and synthesis of combretastatins A-4, A-5, and A-6(1a). J Med Chem *38*: 1666-1672, 1995.
11 Tron G, Pirali T, Sorba G, Pagliai F, Busacca S and Genazzani A: Medicinal chemistry of combretastatin A4: present and future directions. J Med Chem *49*: 3033-3044, 2006.
12 Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, Stirling JJ, Sena L, Padhani AR and Rustin GJ: Combretastatin A4 phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. J Clin Oncol *21*: 2831-2842, 2003.
13 Koh DM, Blackledge M, Collins DJ, Padhani AR, Wallace T, Wilton B, Taylor NJ, Stirling JJ, Sinha R, Walicke P, Leach MO, Judson I and Nathan P: Reproducibility and changes in the apparent diffusion coefficients of solid tumours treated with combretastatin A4 phosphate and bevacizumab in a two-centre phase I clinical trial. Eur Radiol *19*: 2728-2738, 2009.
14 Siemann DW, Chaplin DJ and Walicke PA: A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). Expert Opin Investig Drugs *18*: 189-197, 2009.
15 Peterman EJ and Scholey JM: Mitotic microtubule crosslinkers: insights from mechanistic studies. Curr Biol *19*: R1089-1094, 2009.
16 Decordier I, Cundari E and Kirsch-Volders M: Survival of aneuploid, micronucleated and/or polyploid cells: crosstalk between ploidy control and apoptosis. Mutat Res *651*: 30-39, 2008.
17 Rossi A, Gridelli C, Gebbia V, Rosati G, Tortoriello A, Maione P, Borsellino N, Rossi N, Pisano A, Colantuoni G, Iaffaioli RV, Castaldo V and Manzione L: Single agent vinorelbine as first-line chemotherapy in elderly patients with advanced breast cancer. Anticancer Res *23*: 1657-1664, 2003.
18 Kamath K and Jordan MA: Suppression of microtubule dynamics by epothilone B is associated with mitotic arrest. Cancer Res *63*: 6026-6031, 2003.
19 Katsumata N: Docetaxel: an alternative taxane in ovarian cancer. Br J Cancer *89(Suppl 3)*: S9-S15, 2003.
20 Dumontet C, Isaac S, Souquet P, Bejui-Thivolet F, Pacheco Y, Peloux N, Frankfurter A, Luduena R and Perol M: Expression of class III beta tubulin in non-small cell lung cancer is correlated with resistance to taxane chemotherapy. Bull Cancer *92*: E25-30, 2005.
21 Kavallaris M, Tait AS, Walsh BJ, He L, Horwitz SB, Norris MD and Haber M: Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells. Cancer Res *61*: 5803-5809, 2001.
22 Carlson RO: New tubulin targeting agents currently in clinical development. Expert Opin Investig Drugs *17*: 707-722, 2008.
23 Zhou J and Giannakakou P: Targeting microtubules for cancer chemotherapy. Curr Med Chem Anticancer Agents *5*: 65-71, 2005.
24 Kuo CC, Hsieh HP, Pan WY, Chen CP, Liou JP, Lee SJ, Chang YL, Chen LT, Chen CT and Chang JY: BPR0L075, a novel synthetic indole compound with antimitotic activity in human cancer cells, exerts effective antitumoral activity in vivo. Cancer Res *64*: 4621-4628, 2004.
25 Hung JJ, Chow KC, Wang HW and Wang LS: Expression of dihydrodiol dehydrogenase and resistance to chemotherapy and radiotherapy in adenocarcinoma cells of lung. Anticancer Res *26*: 2949-2955, 2006.
26 Ali N, Yoshizumi M, Fujita Y, Izawa Y, Kanematsu Y, Ishizawa K, Tsuchiya K, Yano S, Sone S and Tamaki T: A novel Src kinase inhibitor, M475271, inhibits VEGF-induced human umbilical vein endothelial cell proliferation and migration. J Pharmacol Sci *98*: 130-141, 2005.
27 Ho PY, Liang YC, Ho YS, Chen CT and Lee WS: Inhibition of human vascular endothelial cells proliferation by terbinafine. Int J Cancer *111*: 51-59, 2004.
28 Huang YC, Chen CT, Chen SC, Lai PH, Liang HC, Chang Y, Yu LC and Sung HW: A natural compound (ginsenoside Re) isolated from Panax ginseng as a novel angiogenic agent for tissue regeneration. Pharm Res *22*: 636-646, 2005.
29 Wang HS, Hwang LL, Sue HF, Lee KM and Chen CT: A simple quantitative method for evaluation of angiogenesis activity. Assay Drug Dev Technol *2*: 31-38, 2004.
30 Kimura Y, Taniguchi M and Baba K: Antitumor and antimetastatic effects on liver of triterpenoid fractions of Ganoderma lucidum: mechanism of action and isolation of an active substance. Anticancer Res *22*: 3309-3318, 2002.
31 Yeh TK, Li CM, Chen CP, Chuu JJ, Huang CL, Wang HS, Shen CC, Lee TY, Chang CY, Chang CM, Chao YS, Lin CT, Chang JY and Chen CT: Antitumor activities and pharmacokinetics of silatecans DB-67 and DB-91. Pharmacol Res *61*: 108-115, 2010.

32 Chang YW, Chen WC, Lin KY, Chang L, Yao HT, Hsieh HP, Lan SL, Chen CT, Chao YS and Yeh TK: Development and validation of a liquid chromatography-tandem mass spectrometry for the determination of BPR0L075, a novel antimicrotuble agent, in rat plasma: application to a pharmacokinetic study, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci *846*: 162-168, 2007.

33 Li WT, Hwang DR, Chen CP, Shen CW, Huang CL, Chen TW, Lin CH, Chang YL, Chang YY, Lo YK, Tseng HY, Lin CC, Song JS, Chen HC, Chen SJ, Wu SH and Chen CT: Synthesis and biological evaluation of N-heterocyclic indolyl glyoxylamides as orally active anticancer agents. J Med Chem *46*: 1706-1715, 2003.

34 Adams DJ, Watkins PJ, Knick VC, Tuttle RL and Bair KW: Evaluation of arylmethylaminopropanediols by a novel *in vitro* pharmacodynamic assay: correlation with antitumor activity *in vivo*. Cancer Res *50*: 3663-3669, 1990.

35 Schmittgen TD, Wientjes MG, Badalament RA and Au JLS: Pharmacodynamics of mitomycin C in cultured human bladder tumors. Cancer Res *51*: 3849-3856, 1991.

36 De Vita VT: The relationship between tumor mass and resistance to chemotherapy. Cancer *51*: 1209-1220, 1983.

37 Jain RC: Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst *81*: 570-576, 1989.

38 Van Laar JAM, Rustum YM, Van der Wilt CL, Smid K, Kuiper CM, Pinedo HM and Peters GJ: Tumor size and origin determine the antitumor activity of cisplatin or 5-fluorouracil and its modulation by leucovorin in murine colon carcinomas, Cancer Chemother Pharmacol *39*: 79-89, 1996.

39 Zheng JH, Chen CT, Au JLS and Wientjes MG: Time- and Concentration-dependent penetration of doxorubicin in prostate tumors. AAPS PharmSci *3*: article 15, DOI: 10.1208/ps030215, 2001.

40 Mead GM, Russell M, Clark P, Harland SJ, Harper PG, Cowan R, Roberts JT, Uscinska BM, Griffiths GO and Parmar MK: A randomized trial comparing methotrexate and vinblastine (MV) with cisplatin, methotrexate and vinblastine (CMV) in advanced transitional cell carcinoma: results and a report on prognostic factors in a Medical Research Council study. MRC Advanced Bladder Cancer Working Party. Br J Cancer *78*: 1067-1075, 1998.

41 Kaufman D, Raghavan D, Carducci M, Levine EG, Murphy B, Aisner J, Kuzel T, Nicol S, Oh W and Stadler W: Phase II trial of gemcitabine plus cisplatin in patients with metastatic urothelial cancer. J Clin Oncol *18*: 1921-1927, 2000.

42 Rixe O, Gatineau M, Jauffret E, Spano JP, Orcel B, Vannetzel JM, Berille J, Derenne JP and Khayat D: Sequential administration of docetaxel followed by cisplatin-vindesine: a pilot study in patients with locally advanced or metastatic non-small cell lung cancer (NSCLC). Bull Cancer *92*: E1-6, 2005.

43 Yoshida T, Yoshikawa T, Tsuburaya A, Kobayashi O, Hasegawa S, Osaragi T and Sairenji M: Feasibility study of biweekly CPT-11 plus CDDP for S-1- and paclitaxel-refractory, metastatic gastric cancer. Anticancer Res *26*: 1595-1598, 2006.

44 Levasseur LM, Greco WR, Rustum YM and Slocum HK: Combined action of paclitaxel and cisplatin against wildtype and resistant human ovarian carcinoma cells. Cancer Chemother Pharmacol *40*: 495-505, 1997.

45 Engblom P, Rantanen V, Kulmala J, Helenius H and Grènman S: Additive and supra-additive cytotoxicity of cisplatin-taxane combinations in ovarian carcinoma cell lines. Br J Cancer *79*: 286-292, 1999.

46 Mabuchi S, Altomare DA, Cheung M, Zhang L, Poulikakos PI, Hensley HH, Schilder RJ, Ozols RF and Testa JR: RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. Clin Cancer Res *13*: 4261-4270, 2007.

47 Jandial DD, Farshchi-Heydari S, Larson CA, Elliott GI, Wrasidlo WJ and Howell SB: Enhanced delivery of cisplatin to intraperitoneal ovarian carcinomas mediated by the effects of bortezomib on the human copper transporter 1. Clin Cancer Res *15*: 553-560, 2009.

48 Smith JA, Gaikwad A, Ramondetta LM, Wolf JK and Brown J: Determination of the mechanism of gemcitabine modulation of cisplatin drug resistance in panel of human endometrial cancer cell lines. Gynecol Oncol *103*: 518-522, 2006.

49 Morinaga Y, Suga Y, Ehara S, Harada K, Nihei Y and Suzuki M: Combination effect of AC-7700, a novel combretastatin A-4 derivative, and cisplatin against murine and human tumors in vivo. Cancer Sci *94*: 200-204, 2003.

50 Siemann DW, Mercer E, Lepler S and Rojiani AM: Vascular targeting agents enhance chemotherapeutic agent activities in solid tumor therapy. Int J Cancer *99*: 1-6, 2002.

51 Villa D, Miloso M, Nicolini G, Rigolio R, Villa A, Cavaletti G and Tredici A: Low-dose cisplatin protects human neuroblastoma SH-SY5Y cells from paclitaxel-induced apoptosis. Mol Cancer Ther *4*: 1439-1447, 2005.

52 Gatzemeier U, von Pawel J, Gottfried M, ten Velde GP, Mattson K, DeMarinis F, Harper P, Salvati F, Robinet G, Lucenti A, Bogaerts J and Gallant G: Phase III comparative study of high-dose cisplatin versus a combination of paclitaxel and cisplatin in patients with advanced non-small-cell lung cancer. J Clin Oncol *18*: 3390-3399, 2000.

53 Nagata N, Kimura M, Hirabayashi N, Tuburaya A, Murata T, Kondo K, Fukuda Y, Kobayashi M, Miyashita Y, Nakao A and Sakamoto J: Phase II study of weekly paclitaxel and cisplatin combination therapy for advanced or recurrent gastric cancer. Hepatogastroenterology *55*: 1846-1850, 2008.

54 Zhang X, Shen L, Li J, Li Y and Jin M: A phase II trial of paclitaxel and cisplatin in patients with advanced squamous-cell carcinoma of the esophagus. Am J Clin Oncol *31*: 29-33, 2008.

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