BPR0L075, a Novel Synthetic Indole Compound with Antimitotic Activity in Human Cancer Cells, Exerts Effective Antitumoral Activity in Vivo

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ABSTRACT

BPR0L075 is a novel synthetic compound discovered through research to identify new microtubule inhibitors. BPR0L075 inhibits tubulin polymerization through binding to the colchicine-binding site of tubulin. Cytotoxic activity of BPR0L075 in a variety of human tumor cell lines has been ascertained, with IC50 values in single-digit nanomolar ranges. As determined by flow cytometry, human cervical carcinoma KB cells are arrested in G2-M phases in a time-dependent manner before cell death occurs. Terminal deoxynucleotidyl transferase-mediated nick end labeling assay indicates that cell death proceeds through an apoptotic pathway. Additionally, it is shown here that the effect of BPR0L075 on cell cycle arrest is associated with an increase in cyclin B1 levels and a mobility shift of Cdc2 and Cdc25C. The changes in Cdc2 and Cdc25C coincide with the appearance of phosphorytopes recognized by a marker of mitosis, MPF. Furthermore, phosphorylated forms of Bcl-2, perturbed mitochondrial membrane potential, and activation of the caspase-3 cascade may be involved in BPR0L075-induced apoptosis. Notably, several KB-derived multidrug-resistant cell lines overexpressing P-gp170/MDR and MRP are resistant to vincristine, paclitaxel, and colchicine but not to BPR0L075. Moreover, BPR0L075 shows potent activity against the growth of xenograft tumors of the gastric carcinoma MKN-45, human cervical carcinoma KB, and KB-derived P-gp170/MDR-overexpressing KB-VIN10 cells at i.v. doses of 50 mg/kg in nude mice. These findings indicate BPR0L075 as a novel antitumor compound with antimitotic activity that has potential for management of various malignancies, particularly for patients with drug resistance.

INTRODUCTION

Microtubules are hollow tubes consisting of α- and β-tubulin heterodimers that polymerize parallel to a cylindrical axis (1). Microtubules are critical elements in a variety of fundamental cell functions, including sustained shape, transportation of vesicles and protein complexes, and the regulation of motility and cell division (2). During mitosis, microtubules are at their highest dynamic instability during spindle formation and separation of chromosomes. Because microtubules play crucial roles in the regulation of the mitotic apparatus, disruption of microtubules can induce cell cycle arrest in M phase, formation of abnormal mitotic spindles, and final triggering of signals for apoptosis (3).

The discovery that the cytotoxic activity of various compounds is through interference with the mitotic spindle apparatus has attracted much attention within the past 2 decades, and microtubules have become an attractive pharmacologic target for anticancer drug discovery (4, 5). Microtubule inhibitors interfere with the dynamics of tubulin polymerization and depolymerization, which results in the inhibition of chromosome segregation in mitosis and consequently the inhibition of cell division (4). The well-characterized clinically used microtubule inhibitors are the taxanes and the Vinca alkaloids. Taxanes, such as paclitaxel and docetaxel, are newer antimitotic agents that stabilize microtubules and induce a net polymerization. The established class of Vinca alkaloids, such as vincristine, vinblastine, and vinorelbine, bind to the tubulin dimer, block the formation of new microtubules, and lead to the depolymerization of existing microtubules (4).

Although the taxanes and Vinca alkaloids are effective for the management of different malignancies, their potential is limited by the development of multidrug resistance (MDR) (6). MDR is multifactorial, with one pathway leading to resistance mediated by overexpression of transmembrane efflux pumps, namely, the M170,000 P-glycoprotein (P-gp170/MDR) and the multidrug resistance protein (MRP; Ref. 7). These efflux pumps are able to reduce the intracellular concentrations of taxanes and Vinca alkaloids to a nontoxic level. Therefore, there has been great interest in identifying novel microtubule inhibitors that overcome various modes of resistance and have improved pharmacologic profiles.

Combretastatin A-4 (CA-4), a naturally occurring stilbene derived from the South African tree Combretum caffrum, inhibits tubulin polymerization by binding to tubulin at the colchicine-binding site (8). It shows potent cytotoxicity against a broad spectrum of human cancer cell lines, including those of MDR-positive lines (9). CA-4P (disodium combretastatin-A-4–3-O-phosphate), a water-soluble prodrug, is a novel antitumor vascular targeting agent and is the first combretastatin analog to enter clinical trials (10, 11). Our previous work showed that 2-aminoazobenzophenone types of CA-4 analogs exhibited strong cytotoxic activity against a wide variety of human cancer cells, including MDR-positive cancer cells (12).

Our current work continues efforts to design and synthesize a series of 3-aryloylindole compounds as novel heterocyclic CA-4 analogs and to evaluate their biological activities for further drug development (13). Among hundreds of 3-aryloylindole synthetic compounds with various substituents on the indole ring, 6-methoxy-3-(3′,4′,5′-trimethoxy-benzoyl)-1H-indole (BPR0L075; Fig. 1) was identified as a potential lead based on extremely potent cytotoxicity with good pharmacologic properties. Herein, we describe the detailed molecular mechanism of action of BPR0L075 and examine whether its efficacy is affected by MDR status in selected cancer cell lines. Moreover, in vivo antitumor activities against human xenografts have been evaluated in murine preclinical models.

MATERIALS AND METHODS

BPR0L075. The compound BPR0L075 was synthesized at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taipei, Taiwan, ROC. BPR0L075 as a white solid was obtained in 72% yield from 6-methoxyindole and 3,4,5-trimethoxybenzoyl chloride. The detailed synthetic method will be published elsewhere.

Reagents. Colchicine, vincristine, and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibody to Cdc25C, monoclonal antibodies to Bcl-2, Cdc2, cyclin B1, and horseradish peroxidase-
conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific monoclonal antibody for MPM-2 was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody for α-tubulin was purchased from Sigma Chemical. FITC-conjugated secondary antibody was purchased from Auncell Corporation (Bayport, MN).

Cell culture reagents were obtained from Life Technologies, Inc. (Rockville, MD). Microtubule-associated protein-rich tubulin and biotin-labeled tubulin were purchased from Cytoskeleton, Inc. (Denver, CO). [3H]colchicine (specific activity, 60–87 Ci/mmol) and Western blot chemiluminescent reagent were purchased from Perkin-Elmer Life Sciences (Boston, MA). [3H]paclitaxel (specific activity, 4.6 Ci/mmol) and streptavidin-labeled poly(vinyl toluene) scintillation proximity assay (SPA) beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Human cervical carcinoma KB cells (this cell line was originally believed to be derived from an epidermal carcinoma of the mouth but has now been shown with HeLa characteristics), nasopharyngeal carcinoma HONE-1 cells, colorectal carcinoma HT29 cells, gastric MKN-45 carcinoma cells, and glioblastoma multiforme DBTRG cells were maintained in RPMI 1640 medium supplied with 5% fetal bovine serum. Human breast carcinoma MCF-7 cells, stomach carcinoma TSGH cells, and hepatocellular carcinoma Hep G2 cells were maintained in MEM supplied with 5% fetal bovine serum. Human leukemia CEM cells were maintained in RPMI 1640 medium supplied with 20% fetal bovine serum. Human Detoit 551 fibroblasts were maintained in MEM containing 10% fetal bovine serum. KB-derived MDR-positive cell lines (e.g., KB-VIN10, KB-TAX50, and KB-7D) were maintained in growth medium supplemented with 10 μM vinristine, 50 μM paclitaxel, and 7 μM VP-16, respectively. KB-VIN10 and KB-TAX50 cells were generated by vincristine- and paclitaxel-driven selection, respectively, and displayed overexpression of P-gp/MDR. KB-7D cells were generated by VP-16-driven selection, which displayed down-regulation of Top II and overexpression of MRP (14, 15).

Growth Inhibition Assay. Cells in logarithmic growth phase were cultured at a density of 5000 cells/ml/well in a 24-well plate. Drug-resistant cells were cultured in drug-free medium for 3 days before use. The cells were exposed to various concentrations of the test drugs for 72 h. The methylene blue dye assay was used to evaluate the effect of test drugs on cell growth, as has been described previously (16). The IC50 value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with the effect of various antimitotic agents on tubulin polymerization in vivo as described by Blagosklomny et al. (21). In brief, KB and KB-VIN10 cells at a density of 1×10^6/100-mm² dishes were treated with indicated concentrations of test agents for the selected treatment duration. Cells then were washed with PBS three times before adding lysis buffer containing 20 mM Tris-HCl (pH 6.8), 1 mM MgCl₂, 2 mM EGTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM propylthiophenylmethylsulfon fluoride, 1 mM orthovanadate, and 0.5% Nonidet. Supernatants were collected after centrifugation at 15,000×g for 10 min at 4°C. The pellets were dissolved in an SDS-PAGE loading buffer and heated at 95°C for 10 min to dissolve the pellets; the resulting material was subjected to electrophoresis on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% skim milk/PBS-Tween 20 overnight at 4°C. The relative amounts of tubulins were detected by anti-α-tubulin monoclonal antibody and horseradish peroxidase-conjugated secondary antibody. Detection of immunoreactive signal was accomplished with Western blot chemiluminescent reagent.

Immunocytochemistry. KB and KB-VIN10 cells plated on coverslips were treated with indicated concentration of test agents for the selected treatment duration. After treatment, cells were placed in the fixation solution (methanol:acetone = 1:1, v/v) for 1 h at −20°C and washed with PBS. Cells then were blocked with 5% skim milk in PBS for 1 h before further incubation with 5% skim milk in PBS containing anti-α-tubulin monoclonal antibody for 2 h at room temperature. After being washed with PBS, cells were reincubated with FITC-conjugated secondary antibody in the dark room for 1 h. Cellular microtubules were observed with an Olympus BX50 fluorescence microscope (Tokyo, Japan).

Analysis of Cell Cycle Distribution. Cells were initially seeded at 1×10^5 cells in 100-mm² dishes and then incubated in various concentrations of BPR0L75 for the indicated time. Adherent and floating cells after pooling were treated with indicated concentration of test agents for the selected treatment duration. After treatment, cells were placed in the fixation solution (methanol:acetone = 1:1, v/v) for 1 h at −20°C and washed with PBS. Cells then were blocked with 5% skim milk in PBS for 1 h before further incubation with 5% skim milk in PBS containing anti-α-tubulin monoclonal antibody for 2 h at room temperature. After being washed with PBS, cells were reincubated with FITC-conjugated secondary antibody in the dark room for 1 h. Cellular microtubules were observed with an Olympus BX50 fluorescence microscope (Tokyo, Japan).

Western Blot Analysis for Bel-2 and G2-M Regulatory Proteins. Cells were initially seeded at a density of 1×10^6 in 100-mm² dishes. After treatment for the indicated time with various concentrations of BPR0L75, adherent cells were washed twice with PBS, gently scraped from the dishes, centrifuged, lysed in ice-cold lysis buffer [50 mM Tris (pH 7.4), 0.8 mM NaCl, 5 mM MgCl₂, 0.5% NP40, 1 mM propylthiophenylmethylsulfon fluoride, 1 μg/ml pepstatin, and 50 μg/ml leupeptin], and cleared by microcentrifugation. Total cell lysates (50 μg/well) were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk/PBS-Tween 20 overnight at 4°C and probed with appropriate dilutions (as recommended by the manufacturer) of primary antibody for 1 h at room temperature. Membranes then were washed three times in PBS-Tween 20 and subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing,
immunoreactive proteins were visualized using Western blot chemiluminescent reagent.

Evaluation of the Mitochondrial Transmembrane Potential. The amphotropic cationic fluorescent probe DiOC₆ was used to monitor drug-induced changes in the mitochondrial transmembrane potential (ΔΨ_m; Ref. 24). Briefly, cells were initially seeded at a density of 1 × 10⁶ in 100-mm² dishes and then treated with various concentrations of BPR0L075 for 24 h. After drug treatment, cells were loaded with the probe DiOC₆ (40 nm) for 30 min at 37°C before cytometric analysis. The supernatant was removed, and the cells were harvested and resuspended in PBS. Measurement of the retained DiOC₆ was examined twice a week after implantation, and the volume of tumor mass was determined by ANOVA (P < 0.05).

Antitumoral Activity. The implantation of cancer cells was carried out similarly to previous reports with modification (25). Male Ncr and CD1 nude mice (5–6-weeks-old) were purchased from Taconic (Germantown, NY) and Charles River Laboratories (Wilmington, MA), respectively. The animals were housed and the experiments were carried out at an International Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of the Developmental Center for Biotechnology, Taipei, Taiwan. The Institutional Animal Care and Use Committees for Biotechnology and the National Health Research Institutes approved uses of animals in these studies.

The animals were s.c. implanted with 5 × 10⁵ MKN-4, 1 × 10⁶ KB cells, or 1 × 10⁶ KB-VIN10 mixed with equal volume of Matrigel (Becton Dickinson) in 0.1 ml at one flank per mouse via a 22-gauge needle. Tumor growth was examined twice a week after implantation, and the volume of tumor mass was measured with an electronic caliper and calculated as 1/2 × length × width² in mm³.

Drug Treatments and Monitoring of in Vivo Antitumoral Activity. BPR0L075 was dissolved completely in a vehicle mixture of DMSO/cremophor/saline (1:4:15). In MKN-45 and KB xenograft studies, when the size of a growing tumor reached ≥100 mm³, the xenograft tumor-bearing nude mice were treated with BPR0L075 i.v. via the tail veins at 10, 25, or 50 mg/kg for 5 days/week for 2 consecutive weeks or 50 mg/kg for 5 days/week for the entire period of observation. In KB-derived P-gp170/MDR-overexpressing KB-VIN10 xenograft study, mice were treated with BPR0L075 at 50 mg/kg for 5 days/week for 2 consecutive weeks. Vincristine was included for comparisons and was administered at a dose regimen close to its maximal tolerated dose of 1 mg/kg at 1 dose/week for 2 weeks in mice. The control group was treated with vehicle mixture only. Tumor size and animal body weight were measured twice a week after drug treatment. At the end of the experiments, animals were euthanized with carbon dioxide inhalation, followed by cervical dislocation. Data were statistically analyzed, and significant differences between testing groups were determined by ANOVA (P < 0.05).

RESULTS

Growth Inhibition of BPR0L075 against Various Human Tumor Cells. Initial experiments were conducted for evaluation of growth inhibition by BPR0L075 against various human cancer cell lines. The results showed that BPR0L075 inhibited the growth of several human cancer cell lines from different tissues and organs, including cervical carcinoma KB cells, nasopharyngeal carcinoma HONE-1 cells, colorectal carcinoma HT29 cells, glioblastoma multimforme DBTRG cells, breast carcinoma MCF-7 cells, gastric carcinoma TSGH and MKN-45 cells, hepatoma Hep G2 cells, and leukemia CEM cells, with IC_{50} values in single-digit nanomolar ranges (data not shown). It also displayed similar or greater growth inhibitory
activities than that of CA-4 toward several human cancer cells. However, the IC$_{50}$ of BPR0L075 toward cultured fibroblast Detroit 551 was >1000 nM. This result demonstrated that BPR0L075 possessed great selectivity between normal and cancer cells.

**BPR0L075 binds to the Colchicine-Binding Site of Tubulin and Inhibits the Polymerization of Microtubules.** Because BPR0L075 is a novel heterocyclic CA-4 analog, we wanted to determine whether BPR0L075 interacts directly with tubulin through binding of the colchicine-binding site. According to tubulin competition-binding SPA, we found that, for a concentration range of 0.016–100 μM, BPR0L075 competed with [3H]colchicine binding to tubulin. The binding capacity of BPR0L075 to the colchicine-binding site of tubulin is stronger than that of colchicine. The K$_i$ values for colchicine and BPR0L075 are 1.24 and 0.023 μM, respectively. However, it did not compete with either [3H]paclitaxel or [3H]vinblastine binding to tubulin (Fig. 2A).

To test the effect of BPR0L075 on microtubule assembly in vitro, we incubated purified, unpolymerized, microtubule-associated protein-rich tubulin with various concentrations of BPR0L075 and measured polymerization. In the control sample without the addition of any test agent, absorbance at 350 nm increases with time. In the presence of 5 μM colchicine, tubulin polymerization is inhibited ~54% compared with that of a control sample after 10-min incubation. Furthermore, in the presence of BPR0L075, tubulin polymerization is inhibited in a concentration-dependent manner. The inhibitory concentration that reduces polymerized tubulin by 50% is 3.3 ± 0.5 μM ($n = 3$; Fig. 2B). In Fig. 2C, the effect of BPR0L075 on microtubule assembly is compared with that of paclitaxel, vincristine, and colchicine in an in vivo assay. After KB cells are treated with various antimitotic agents for 6 h in the presence of 800 nM colchicine and 80 nM vincristine, inhibition of microtubule assembly is observed. In contrast, 800 nM paclitaxel promotes tubulin polymerization. Similar to the effect of colchicine and vincristine, BPR0L075 inhibits tubulin polymerization in a concentration-dependent manner (Fig. 2C).

We further examined the effect of BPR0L075 on cellular microtubule networks by using immunofluorescence techniques. As shown in Fig. 3A, the microtubule network exhibits normal arrangement and organization in KB cells in the absence of drug treatment. However, after 6-h drug treatment, 500 nM of colchicine significantly causes cellular microtubule depolymerization; we noted that most cells had short microtubule fragments scattered throughout the cytoplasm. In contrast, 500 nM of paclitaxel dramatically promotes microtubule polymerization with an increase in the density of cellular microtubules and formation of long thick microtubule bundles. Furthermore, BPR0L075 treatment results in findings similar to those of colchicine-induced microtubule change. We observed an almost complete loss of microtubules with only a diffuse stain visible throughout the cytoplasm (Fig. 3A). In addition, KB cells treated with low concentration of BPR0L075 ranging from 5–80 nM for 24 h also significantly induce microtubule disassembly in a concentration-dependent manner (Fig. 3B).

**BPR0L075 is cytotoxic toward P-gp170/MDR and MRP Tumor Cell Lines via Prevention of Tubulin Polymerization.** One major mechanism of acquired drug resistance is the overexpression of efflux pumps, namely, P-gp170/MDR and MRP (7). Cell lines selected for expression of drug efflux pumps by long-term drug exposure.
sure also were used to compare the antitumoral activity of BPR0L075 with that of other microtubule inhibitors. As summarized in Table 1, KB-VIN10 cells have profound resistance to paclitaxel (4024-fold), vincristine (150-fold), and colchicine (10-fold) compared with parental cells. KB-TAX50 and KB-7D cells are moderately resistant to paclitaxel, vincristine, and colchicine (2–33-fold). In contrast, BPR0L075 is equally potent toward the parental KB cells and those of KB-derived MDR-positive cell lines. The IC50 of BPR0L075 for a variety of KB-derived MDR-positive cells ranged from 2–4 nM.

Furthermore, we examined the microtubule polymerization status in KB-VIN10 cells compared with their parental KB cell line after 6-h treatment with colchicine, vincristine, paclitaxel, or BPR0L075. We observed an effect on microtubules after colchicine, vincristine, or paclitaxel treatment in parental KB cells but not in the KB-VIN10 cells that overexpress P-gp170/MDR (Fig. 2C). However, BPR0L075 still can inhibit microtubule assembly in a concentration-dependent manner in KB-VIN10 cells (Fig. 2C). Results from immunocytochemistry studies also demonstrate that changes on the microtubule network are not observed after colchicine and paclitaxel treatment in KB-VIN10 cells. However, BPR0L075 treatment induces similar changes in cellular microtubule networks between KB and KB-VIN10 cells (Fig. 3A).

**BPR0L075 Induces G2-M Phase Arrest and Change in Expressed and Phosphorylated Status of G2-M Regulators in KB Cells.** The effect of BPR0L075 on cell cycle progression of KB cells was examined by flow cytometry. BPR0L075 treatment results in a time-dependent accumulation of KB cells in the G2-M phase with concomitant losses from G0-G1 phase. No change of S-phase was observed (Fig. 4A). BPR0L075 treatment results in accumulation of cells in G2-M phase starting with an 8-h exposure, with a maximum accumulation observed by 16 h. However, a characteristic hypodiploid DNA content peak (sub-G1), indicated as apoptotic cells, can be detected with treatment duration of ≥24 h. The value of sub-G1 phase reaches a peak at 48 h (~50%; Fig. 4A). These results indicate that BPR0L075-induced cells are arrested in G2-M phase before cell death occurs.

Furthermore, we investigated the association between BPR0L075-induced G2-M phase arrest and alteration in G2-M regulatory protein expression. As shown in Fig. 4B, BPR0L075 causes a concentration-dependent increase in cyclin B1. In addition, slower migrating forms of Cdc25C protein are present in cells after 24-h treatment with BPR0L075 in a concentration-dependent manner, indicative of changes in the phosphorylation state of Cdc25C (Fig. 4B). In contrast, there is a shift to the faster migrating form of the cyclin-dependent kinase Cdc2, consistent with the presence of the hypophosphorylated active form of the protein (Fig. 4B). We further examined the status of phosphorylated polypeptides found only in mitotic cells using MPM-2 antibody. After 24-h treatment with BPR0L075, significant elevation in the levels of MPM-2 phosphoepitopes at a concentration of 20 nM is observed (Fig. 4B).

**BPR0L075 Initiates Bcl-2 Phosphorylation, Followed by Damage to Mitochondria and Induced Apoptosis.** The Bcl-2 protein located on the outer mitochondrial membrane is important for suppression of mitochondrial manifestations of apoptosis (26), and its phosphorylation is a common characteristic of antimicrotubotics (27). We examined whether BPR0L075-induced G2-M phase arrest and apoptosis are associated with Bcl-2 phosphorylation. As shown in Fig. 5A, the appearance of the characteristically slower migrating form of

![Fig. 4.](image-url)
Bcl-2, consistent with phosphorylation, is detected after treatment with up to 20 nM BPR0L075 for 24 h. Because Bcl-2 prevents the initiation of the cellular program by stabilizing mitochondrial permeability and preventing subsequent release of cytochrome c to prevent caspase activation, we used the ampholytic cationic fluorochrome DiOC6 to monitor changes in \( \Delta \psi_m \) induced by BPR0L075 (28). As presented in Fig. 5B, cells treated with BPR0L075 exhibit a significant reduction in cellular uptake of the fluorochrome DiOC6, indicating a loss of \( \Delta \psi_m \) in a concentration-dependent manner. The percentage of cells with reduced DiOC6 fluorescence reaches 17.7% and 35.5% after treatment with 10 and 20 nM BPR0L075, respectively, for 24 h. Caspase-1 and caspase-3 are key executioners of apoptosis, and their involvement in apoptosis also was investigated in KB cells treated with BPR0L075 (29). As shown in Fig. 5C, incubation of KB cells with BPR0L075 results in activation of caspase-3 in a time-dependent manner, starting with a 6-h exposure (3.13-fold compared with control group) and reaching a maximum of 41.5-fold induction by 30 h of BPR0L075 treatment. In contrast, no cleavage of caspase-1 substrate was detected after BPR0L075 treatment (data not shown). To further confirm that BPR0L075 leads to apoptosis, the TUNEL assay was used to characterize DNA strand breaks in situ (30). As shown in Fig. 5D, control cells containing intact genomic DNA appear in TUNEL-positive cells at nearly undetectable levels (0.3%), whereas ~4.9%, 50.6%, and 98.6% of TUNEL-positive cells are detected within the population of cells treated with 20 nM BPR0L075 for 24, 48, or 72 h, respectively.

**Efficacy of BPR0L075 in Xenograft Experiments in Vivo.** The potential antitumoral effect of BPR0L075 in vivo was assessed in human tumor xenografts in mice. KB cells were grown as s.c. tumors in nude mice. Nine days afterward, when well-established KB xenografts were palpable with tumor size of \( \sim 100 \text{ mm}^3 \), mice were randomized into vehicle control and treatment groups of seven animals each. The treated mice received 50 mg/kg of BPR0L075 i.v. for the entire period of observation (days 9–30 after cancer cell inoculation), or they received 10, 25, or 50 mg/kg of BPR0L075 for 5 days/week for 2 consecutive weeks (days 9–13 and 16–20 after inoculation). There was a dose-dependent decrease in tumor volume in mice treated with BPR0L075 5 days/week for 2 consecutive weeks. Moreover, there was an ~82% decrease in tumor volume on day 30 in the animals daily treated with 50 mg/kg/day BPR0L075 for the...
BPR0L075, a novel antimitotic agent

**DISCUSSION**

BPR0L075 is a novel synthetic 3-arylidindole compound that exerts a broad spectrum of activity against human leukemia, glioblastoma, oral, nasopharyngeal, breast, gastric, colorectal, and liver cancer cells *in vitro* and strongly inhibits tubulin polymerization through binding of tubulin’s colchicine-binding site (Fig. 2). Consistent with these biochemical effects, BPR0L075 disrupts intracellular microtubule network in intact cells as demonstrated in the immunocytochemistry studies (Fig. 3).

As with other microtubule-interacting agents, BPR0L075 arrests the growth of cancer cells at the G2-M phase (Fig. 4A) and then induces apoptotic cell death (Fig. 5). Different classes of cyclins and their cyclin-dependent kinases control cell cycle progression. In eukaryotic cells, cyclin B and Cdc2 kinase regulate the onset of M phase. Extensive studies have shown that activation of Cdc2 kinase at the G2-M transition requires accumulation of cyclin B and dephosphorylation of Cdc2 (31). The conversion of Cdc2 from inactive to active form is accomplished by the phosphatase Cdc25C (32, 33). Phosphorylation of Cdc25C directly stimulates its phosphatase activity, and this is necessary to activate Cdc2/cyclin B1 kinase on entry into mitosis (34). Many antimitotic drugs that interfere with normal formation of mitotic spindles either by increasing microtubule stability or by depolymerization can cause cells to arrest at the prometaphase/metaphase-to-anaphase transition known as the mitotic checkpoint (35). In this study, we demonstrate that in addition to directly disrupting microtubules, BPR0L075 treatment leads to inappropriate accumulation of cyclin B and initiates a phosphorylation cascade resulting in the engagement of active Cdc2 kinase and phosphorylation of Cdc25C (Fig. 4B). The changes in Cdc2 and cdc25C coincide with the appearance of phosphoepitopes recognized by a marker of mitosis, PMP2, which is an antibody that recognizes phosphorylated polypeptides found only in mitotic cells (Ref. 36; Fig. 4B). These changes in protein phosphorylation are consistent with cell cycle arrest in mitosis (37).

Apoptosis induced by antimitotic agents has been associated with alterations in a variety of cellular signaling pathways. It is known that Bcl-2 is a guardian of microtubule integrity (27). Microtubule inhibitors such as vincristine, vinblastine, colchicine, paclitaxel, and doxetaxel induce growth arrest, followed by phosphorylation and inactivation of Bcl-2, and eventually leading to apoptotic cell death in the G2-M phase of the cell cycle (3, 18, 38–40). As with other microtubule-damaging agents, BPR0L075 also is able to induce Bcl-2 hyperphosphorylation in a concentration-dependent manner (Fig. 5A). The mechanism by which Bcl-2 opposes apoptosis may involve prevention of mitochondrial damage (*e.g.*, loss of ∆Ψm; Refs. 41, 42). The collapse of ∆Ψm results in an uncoupling of the respiratory chain and the efflux of small molecules (*e.g.*, cytochrome c and calcium) and

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**Fig. 6.** Inhibition of human xenografts growth *in vivo* by 6-methoxy-3-(3’,4’,5’-trimethoxy-benzoyl)-1H-indole (BPR0L075). Nude mice bearing (A) human cervical carcinoma KB and (B) human gastric carcinoma MKN-45 xenograft xenografts were treated with vehicle control (●), 10 mg/kg BPR0L075 for 5 days/week for 2 weeks (on days 9–13 and 16–20; ▲), 25 mg/kg BPR0L075 for 5 days/week for 2 weeks (on days 9–13 and 16–20; ▼), 50 mg/kg BPR0L075 for 5 days/week for 2 weeks (on days 9–13 and 16–20; ◻), or 50 mg/kg BPR0L075 daily for the entire period of observation (■). To evaluate the efficacy of BPR0L075 in vivo, MKN45 xenograft tumor to evaluate the efficacy of BPR0L075 against gastric tumor growth. Compared with tumor growth in control mice, the growth of MKN45 tumor was significantly inhibited in a dose-dependent manner in mice that received BPR0L075 treatment, with the greatest antitumoral efficacy in animals treated daily with BPR0L075 at 50 mg/kg for the entire period of observation (Fig. 6B). Notably, BPR0L075 also is effective toward MDR xenograft as it is in cultured MDR cells (Fig. 6C). BPR0L075 was well tolerated after dosing up to 50 mg/kg with no signs of toxicity in these three xenograft tumor models because loss of body weight after the treatment was <15% in all treatment groups (data not shown). Thus, BPR0L075 exerts potent antitumoral efficacy toward these three solid tumor xenografts.
certain proteins (including caspase-2 and caspase-9; Ref. 43), as well as the apoptosis-inducing factor that can, in turn, stimulate proteolytic activation of caspase-3 (44). We show here that there is a substantial loss of $\Delta W_{mt}$ (Fig. 5B) and activation of caspase-3 (Fig. 5C) after KB cells are treated with BPROL075. These results confirm that BPROL075-induced apoptosis is associated with phosphorylation of Bcl-2, loss of $\Delta W_{mt}$, and activation of caspase-3.

Drug resistance is a common problem in the management of neoplastic disease, and the effectiveness of many clinically useful drugs is limited by the fact that they are substrates for the efflux pumps P-gp170/MDR and MRP. Notably, BPROL075 in vitro was shown to be equally effective against three KB-derived MDR-positive cell lines (Table 1) via inhibition of tubulin polymerization despite P-gp170/MDR or MRP status (Fig. 2C and Fig. 3A); these findings suggest that BPROL075 is a poor substrate of P-gp170/MDR and MRP. This feature is distinct from those of paclitaxel, vincristine, and colchicine because three KB-derived MDR-positive cells are much more resistant to these chemotherapeutic agents than KB cells. More importantly, BPROL075 shows marked in vivo antitumoral activity in the KB, MKN-45, and P-gp170/MDR-overexpressing KB-VIN10 xenograft tumor models (Fig. 6C).

In conclusion, our data demonstrate that BPROL075, a novel synthetic indole compound designed from CA-4, has properties distinct from colchicine, vincristine, and paclitaxel and is efficacious in suppressing cell growth in a variety of solid tumor models despite P-gp170/MDR or MRP status in vitro (45, 46). Furthermore, it exhibits significant antitumoral efficacy in vivo. These findings indicate BPROL075 is a promising new tubulin-binding compound with potential for management of various malignancies, particularly for patients with demonstrated drug resistance.

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