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Dual inhibition of topoisomerase I and tubulin polymerization by BPR0Y007, a novel cytotoxic agent

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Abstract

Through the screening of DNA topoisomerase I (Top I) inhibitors, a new cytotoxic agent, BPR0Y007 [2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone], was identified. BPR0Y007 was less potent than camptothecin (CPT) in the inhibition of Top I *in vitro*. Also, *in vitro* data showed that BPR0Y007 induces DNA cleavage in the presence of Top I at micromolar concentrations, with a cleavage specificity similar to that of CPT. High concentrations of BPR0Y007 did not produce detectable DNA unwinding, suggesting that BPR0Y007 is not a DNA intercalator. However, BPR0Y007 displaced Hoechst 33342 dye, suggesting that BPR0Y007 binds to DNA at the Hoechst 33342 binding site. Furthermore, BPR0Y007 generated protein-linked DNA breaks in a cell-based study. Cell cycle analysis demonstrated that the cell cycle effect of BPR0Y007 differs from that of CPT. Cells accumulated in the S-phase when treated with high concentrations of CPT, whereas cells accumulated gradually in the G_2/M phase when treated with increasing concentrations of BPR0Y007. Further studies showed that BPR0Y007 inhibits tubulin polymerization *in vivo* and *in vitro*, and induces apoptosis in a concentration-dependent manner. No cross-resistance with BPR0Y007 was observed in CPT-, VP-16-, or vincristine-resistant cell lines. The IC_{50} of BPR0Y007 for various human cancer cell lines ranged from 1 to 8 μM. Taken together, these results suggest that BPR0Y007 acts on both Top I and tubulin. Given its unique biochemical mechanisms of action, BPR0Y007 warrants exploration as an antitumor compound.

Keywords: Topoisomerase I; Tubulin; Dual inhibitor

1. Introduction

Eukaryotic DNA Top I is an essential enzyme that regulates the topological changes of DNA that accompany DNA replication, transcription, recombination, and chromosome segregation during mitosis [1–3]. Top I introduces transient single-strand DNA breaks in one of the phosphodiester backbones of the duplex DNA, resulting in a reversible Top I/DNA covalent complex [4,5]. Top I interactive agents, consisting primarily of CPT analogues, interact with the Top I/DNA complex. This interaction prevents the resealing of the Top I-mediated DNA single-strand breaks, causing a collision of the replication forks when replication occurs and, subsequently, cell death [6,7]. CPT is an S-phase-specific DNA-damaging agent [8]. However, high concentrations of CPT can kill both S-phase and non-S-phase cells [9]. The mechanism of cytotoxicity of CPT in non-S-phase tumor cells may involve apoptosis [10]. Although several CPT derivatives, including irinotecan and topotecan, have been introduced for the clinical treatment of colon and ovarian cancers, the response rates remain low, and the overall survival rate has not improved substantially [11,12]. Thus, interest remains in finding new Top I inhibitors.

Recently, as part of a program to identify clinically effective antitumor agents with antiproliferative properties, we identified a cytotoxic agent, BPR0Y007 [2,5-bis-(4-hydroxy-3-methoxybenzylidene)cyclopentanone]. The

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Abbreviations: Top I, topoisomerase I; CPT, camptothecin; PLDBs, protein-linked DNA breaks.

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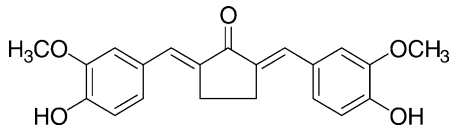

Fig. 1. Chemical structure of BPR0Y007.

structure of BPR0Y007 is shown in Fig. 1. In this study, we determined the mechanisms of action of BPR0Y007 and found that it could inhibit both Top I and tubulin polymerization. Furthermore, no cross-resistance with BPR0Y007 was observed in CPT-, vincristine-, or VP-16-resistant cells.

2. Materials and methods

2.1. Synthesis of BPR0Y007

BPR0Y007 was synthesized according to the procedure described by Sardjiman *et al.* [13]. Vanillin and cyclopentanone were heated in a water bath (45–50°) until a clear solution was obtained; then concentrated hydrochloric acid was added followed by stirring for 2 hr. After standing overnight, the mixture was treated with cold AcOH/water (1:1) and filtered. The solid materials were washed first with cold ethanol, then with hot water, and dried under vacuum. The yellow substance was recrystallized from ethanol. The structure of BPR0Y007 was confirmed by ^{1}H-NMR and liquid chromatography-mass spectrometry. BPR0Y007 was stable for more than 12 months when stored in the solid phase.

2.2. Drugs, enzymes, and chemicals

CPT, adriamycin, etoposide (VP-16), paclitaxel, and netropsin were purchased from the Sigma Chemical Co. Stock solutions of these drugs, except for those that were water-soluble, were prepared in DMSO at 20 mM, stored at −20°, and diluted in water immediately before use. Calf thymus Top I was purchased from TopGene. Microtubule-associated protein (MAP)-rich bovine brain tubulin was purchased from Cytoskeleton, and [α-^{32}P]dATP and [^{14}C]thymidine (specific activity, 50.5 mCi/mmol) were obtained from the Amersham Corp. All chemicals were standard analytical grade or higher.

2.3. Cell lines

All cell lines were maintained in RPMI 1640 medium containing 5% fetal bovine serum. The CPT-resistant cell lines, KB 100 and CPT 30, were maintained in growth medium supplemented with 100 or 30 nM CPT, respectively. The VP-16-resistant cell line, KB 7D, and the vincristine-resistant cell line, KB vin10, were maintained in the medium supplemented with a 7 μM or a 10 nM concentration of drug, respectively. KB 7D cells displayed a decrease in cellular Top II content and overexpression of multidrug-resistant-associated protein (MRP) [14]. KB 100 cells displayed a decrease in Top I and unidentified post-protein-linked DNA break (PLDB) resistance to Top I poisons [15]. KB vin10 cells overexpressed P-glycoprotein-170 (P-gp) (unpublished data). The cell doubling times for KB, KB 7D, KB 100, and KB vin10 cells were 20, 22, 29, and 22 hr, respectively. CPT 30 cells, a CPT-resistant cell line derived from a human nasopharyngeal carcinoma cell line, HONE-1, displayed a decrease in Top I level and a mutation in the *Top I* gene [16]. The cell doubling times for HONE-1 and CPT 30 cells were similar, on the order of 21 hr.

2.4. Screening the topoisomerase inhibitor

A two-step combinational screening strategy was used to identify novel inhibitors for human topoisomerases in the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes. A Beckman Coulter high-throughput cell screening system was used to screen the compounds for antiproliferative activity. The growth inhibition assay using a human gastric cancer cell line, NUGC3, was performed as described by Goodwin *et al.* [17]. If the growth inhibitory activity against NUGC3 cells was greater than 50% at a concentration of 10 μM, biological screening using an *in vitro* topoisomerase-mediated DNA relaxation assay [18] was performed.

2.5. Inhibition of the catalytic activity of Top I

Top I catalytic activity was determined by the ATP-independent relaxation of pBR322 supercoiled DNA, as described previously [18]. Each reaction mixture contained 0.25 μg supercoiled DNA, 50 mM Tris–HCl (pH 7.5), 60 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 50 μg/mL of BSA, one unit of enzyme, and various concentrations of drugs. The reaction was terminated by adding 1% SDS, and separated in a 1% agarose gel. Photographs of the resulting ethidium bromide-stained agarose gel were taken under UV light.

2.6. Top I-induced DNA cleavage

Evaluation of Top I-induced single-strand DNA breaks was performed as described previously [7]. Linear pBR322 DNA was 3′-end labeled, as described previously [19]. The reaction was carried out at 37° for 15 min, stopped by the addition of 5 μL of 5% SDS and 0.75 mg/mL of proteinase K, and incubated for 30 min at 50°. Prior to loading onto a 1% agarose gel in 1× TBE (0.09 M Tris-borate and 0.002 M EDTA) with 0.1% SDS, 10-μL samples were denatured with 0.45 N NaOH, 30 mM EDTA, 15% (v/w) sucrose, and 0.1% bromophenol blue. After electrophoresis, the gel was dried and autoradiographed for 24 hr.

2.7. Measurement of PLDBs in vivo

Cells in log phase growth were labeled with [^{14}C]thymidine for 24 hr. After labeling, the cells were trypsinized, resuspended in fresh medium at a density of 5×10^5/mL, and shaken gently in a 37° water bath for 30 min in suspension. Various concentrations of drugs were added, and incubation was continued for an additional 0.5 hr. The cells were collected and analyzed for protein-linked DNA breaks by a potassium-SDS (K-SDS) co-precipitation method, as described previously [20].

2.8. DNA unwinding measurements

Drug-induced DNA unwinding was assayed as described previously [21]. The DNA circle-ligation assay, using nicked DNA as a substrate, was performed using the method of Montecucco *et al*. [22].

2.9. Measurement of fluorescence of DNA-bound Hoechst 33342

The minor groove binding effect of BPR0Y007 was assayed as described previously [23]. Briefly, salmon sperm DNA and 0.8 μg Hoechst 33342 were mixed in 200 μL of PBS and preincubated for 10 min at room temperature in a 96-well plate. Netropsin, a known minor groove binder, and VP-16 were included as positive and negative controls, respectively. Then BPR0Y007 was added at various concentrations. After another 10-min incubation, the fluorescence derived from Hoechst 33342 bound to DNA was measured with a fluorometer (excitation wavelength, 355 nm; and emission wavelength, 460 nm).

2.10. Cell cycle analysis

Flow cytometric analysis of propidium iodide-stained cells was performed with a FACS IV flow cytometer (Becton Dickinson). Cell cycle analysis was performed according to the mathematical model of Jett [24].

2.11. In vitro tubulin polymerization assay

Microtubule polymerization was conducted in a 96-well UV microtiter plate with MAP-rich tubulin and various concentrations of BPR0Y007 in a buffer containing 100 mM PIPES (pH 6.9), 2 mM $MgCl_2$, 1 mM GTP, and 4% (v/v) DMSO. The increase in absorbance was measured at 350 nm in a PowerWave X (Biotek) microplate reader at 37° and recorded every 30 s for 30 min. The area under the curve (AUC) was used to determine the concentration that inhibited tubulin polymerization by 50% (IC_{50}). The AUC of the untreated control was set to 100% polymerization, and the IC_{50} was calculated using nonlinear regression.

2.12. In vivo tubulin polymerization assay

Cells at a density of 1×10^6/dish were treated with various concentrations of test agents. Then the cells were washed with PBS three times before adding a lysis buffer containing 20 mM Tris–HCl (pH 8.6), 1 mM $MgCl_2$, 2 mM EGTA, 20 μg/mL of aprotinin, 20 μg/mL of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 0.5% Nonidet P-40. Supernatants were collected after centrifugation at 9168 *g* for 10 min at 4°. The pellets were dissolved in an SDS–PAGE sampling buffer and subjected to electrophoresis on a 10% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with α-tubulin monoclonal antibody (Sigma). Detection of immunoreactive signal was accomplished with Western Blot Chemilluminescent Reagent Plus (Perkin Elmer Life Sciences).

2.13. Colchicine and tubulin binding assays

Tubulin was incubated with [^{3}H]colchicine in the presence of different concentrations of either unlabeled colchicine or BPR0Y007 in a buffer containing 0.05 M PIPES (pH 6.9), 1 mM $MgCl_2$, and 1 mM GTP. The reaction mixtures were incubated at 37° for 1 hr. The samples were loaded onto a Sephadex G-50 column (Amersham Pharmacia) previously equilibrated with the buffer solution. The columns were placed into 1.5-mL tubes, spun at a speed of 750 *g* for 2 min at room temperature, and radioactivity in the flow-through was analyzed by a scintillation counter.

2.14. TUNEL assay

To determine whether BPR0Y007 could induce apoptosis, a TUNEL assay using an *In Situ* Cell Death Detection Kit (Roche Molecular Biochemicals) was performed. Cells treated with various concentrations of BPR0Y007 for 24 hr at 37° were subjected to a TUNEL assay according to the instructions of the manufacturer.

2.15. Caspase-3 activation assay

After treatment with different BPR0Y007 concentrations for 24 hr, and 12 μM BPR0Y007 for different durations, in triplicate at 37° in 96-well microtiter plates, caspase-3 activation by KB ATCC cells was measured by the cleavage of the fluorometric substrate Ac-DEVD-AMC (Promega Corp.) according to the procedure of the manufacturer.

2.16. Growth inhibition assay

Cells in logarithmic phase were cultured at a density of 5000 cells/mL/well in a 24-well plate. The resistant cells were cultured in drug-free medium for 3 days prior to use. The cells were exposed to various concentrations of

the test compounds for 72 hr. The methylene blue dye assay was used to evaluate the effects of the test compounds on cell growth, as described previously [25], and to determine the concentration of drug that inhibited 50% of cell growth (IC_{50}).

3. Results

3.1. Top I-mediated DNA relaxation inhibited by BPR0Y007

Using a high-throughput cell-based growth inhibition assay to screen part of a chemical library of 26,000 compounds, we identified 260 compounds with significant inhibitory activity against human gastric cancer NUGC3 cells. Through the screening of the Top I and II inhibitors using a DNA relaxation assay, we determined that one compound, BPR0Y007 (Fig. 1), could inhibit Top I catalytic activity, but not Top II activity (data not shown). The HPLC profile and the ^{1}H-NMR spectrum of the synthetic compound were identical with those produced by a reference sample on the same spectrometer at the same time. As shown in Fig. 2A, BPR0Y007 inhibited Top I-mediated DNA relaxation, but was less potent than CPT.

3.2. Top I-mediated DNA cleavage induced by BPR0Y007

Induction of DNA cleavage in the presence of Top I was tested in a linearized pBR322 DNA. As shown in Fig. 2B, Top I-mediated cleavage in the presence of BPR0Y007 was detectable at 5 μM and reached a plateau at 15 μM. A number of cleavage sites detected in the presence of BPR0Y007 were also induced by CPT. The relative intensity of similar sites varied between BPR0Y007 and CPT.

3.3. Induction of protein-linked DNA breaks

Drug-induced PLDBs were evaluated in HONE-1 cells and their CPT-resistant variant (CPT 30) cells by K-SDS assay. BPR0Y007 induced PLDBs in HONE-1 cells in a concentration-dependent manner, which reached a plateau at a concentration of 10 μM. The maximal level of PLDBs generated by BPR0Y007 was one-fifth of that of CPT (Fig. 3). Moreover, in CPT 30 cells, which displayed a reduced level of Top I and a mutation in the *Top I* gene [16], the number of PLDBs induced by CPT was much lower than that in its parental cells, HONE-1. There were almost no detectable PLDBs in CPT 30 cells in the presence of 1–25 μM BPR0Y007.

3.4. Interaction of BPR0Y007 with DNA

Most of the non-CPT Top I inhibitors are DNA intercalators and/or minor groove binders. To investigate whether BPR0Y007 intercalates into DNA, an unwinding assay was performed with linearized DNA and T4 ligase. In this assay, adriamycin, a strong intercalator, and CPT, a non-intercalator, were included as positive and negative controls, respectively. As seen in Fig. 4A, adriamycin produced a concentration-dependent DNA shift, indicating a change in the DNA linking number. In the presence of adriamycin at concentrations greater than 2.5 μM, substrate linear DNA remained unchanged, indicating that the strong intercalating activity of adriamycin causes inhibition of T4 ligase, as reported previously [22]. Similar to the effect of CPT, BPR0Y007 produced no DNA shift at concentrations up to 50 μM (Fig. 4A). To determine whether BPR0Y007 binds to the DNA, a Hoechst 33342 dye displacement assay was performed. Hoechst 33342 is a fluorochrome that binds to the DNA minor groove and generates specific fluorescence [26,27]. In a cell-free system, netropsin, another DNA minor groove binder [28], and VP-16 were included as positive and negative controls, respectively. As shown in Fig. 4B, similar to the effect of netropsin, the fluorescence of DNA-bound Hoechst 33342 was quenched after BPR0Y007 was added in a concentration-dependent manner. This result indicated that BPR0Y007 displaced the Hoechst 33342 bound to the DNA.

3.5. Effect of BPR0Y007 on the cell cycle

The impact of different concentrations of BPR0Y007 and CPT on the cell cycle progression of HONE-1 cells was studied after one generation of drug exposure (Fig. 5). Consistent with previous reports [15], cells treated with CPT accumulated in S-phase with concomitant losses from the G_0/G_1 and G_2/M phases, especially at higher drug concentrations. However, with increasing BPR0Y007 concentrations, cells accumulated gradually in the G_2/M phase with concomitant losses from the G_0/G_1 phase. No change of S-phase was observed.

3.6. Microtubule assembly studies

The effect of BPR0Y007 on microtubule polymerization was examined *in vitro*. Figure 6A shows the result of microtubule assembly *in vitro* using MAP-rich tubulin. In control samples (without the addition of any test agents), absorbance at 350 nm ($A_{350\ nm}$) increased with time. The initial rate of increase was slow but accelerated within ~3 min after initiation of the reaction. The increase in $A_{350\ nm}$ reached a plateau in ~12 min. In the presence of 10 μM colchicine, tubulin polymerization was inhibited completely throughout the reaction (data not shown). Furthermore, in the presence of different concentrations of BPR0Y007, tubulin polymerization was inhibited in a concentration-dependent manner. The inhibitory concentration that reduced the amount of polymerized tubulin by 50% was 1.5 ± 0.2 μM ($N = 2$).

In Fig. 6B, the effect of BPR0Y007 on microtubule assembly was compared with those of paclitaxel, vincristine, and colchicine during an *in vivo* study. In the presence

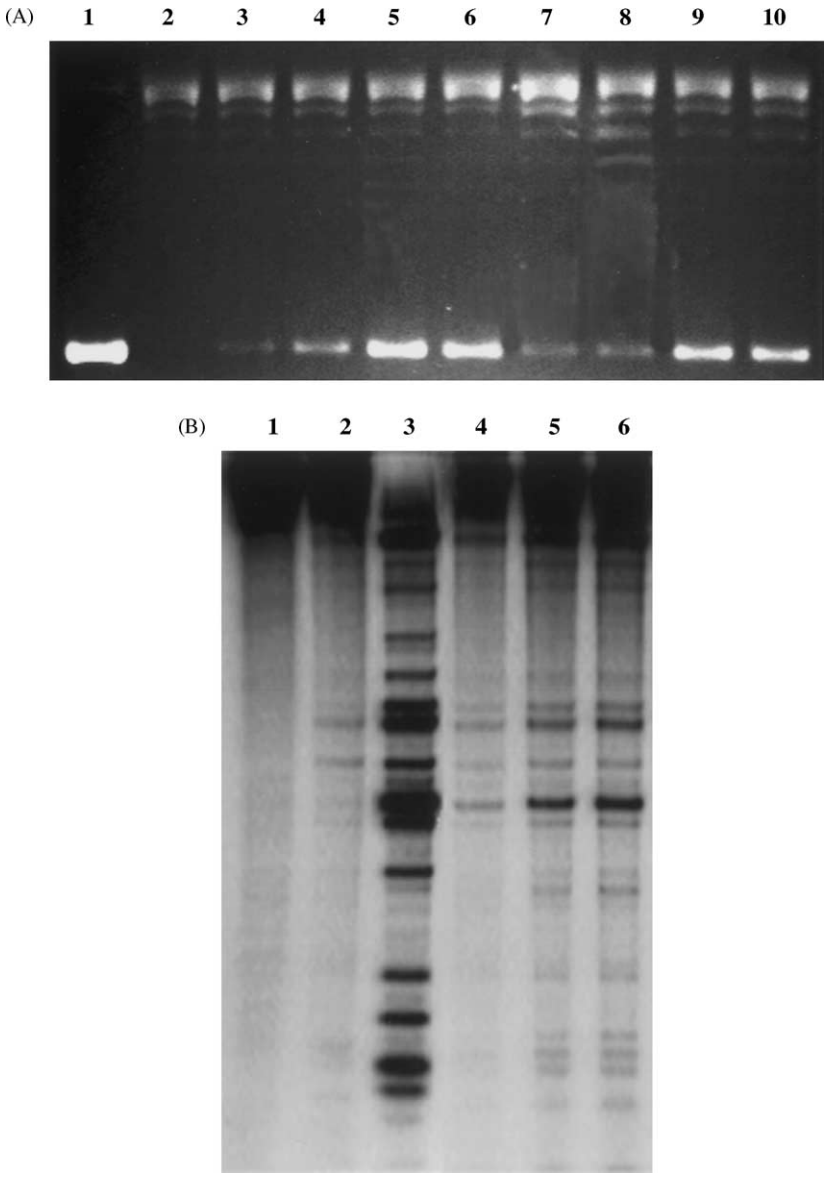

Fig. 2. (A) Effect of BPR0Y007 on Top I catalytic activity. Supercoiled pBR322 DNA was incubated with one unit of Top I in the presence of CPT and BPR0Y007 in a reaction mixture for 30 min at 37°. The DNA was analyzed by electrophoresis in a 1% agarose gel. Lane 1, control DNA; lane 2, no drug; lanes 3–6, CPT 5, 10, 25, and 50 μM, respectively; lanes 7–10, BPR0Y007 5, 10, 25, and 50 μM, respectively. (B) DNA cleavage pattern induced by CPT and BPR0Y007 in the presence of Top I. 3′-Ended-^{32}P-labeled pPR322 DNA was incubated with 20 units of purified Top I in the presence of CPT and BPR0Y007 in a reaction mixture for 15 min at 37°. The reactions were terminated by SDS-proteinase K treatment. Samples were analyzed by alkali gel electrophoresis. Lane 1, control DNA; lane 2, no drug; lane 3, CPT 10 μM; lanes 4–6, BPR0Y007 5, 15, and 50 μM, respectively.

of 100 nM colchicine and 10 nM vincristine, inhibition of microtubule assembly was observed, whereas 100 nM paclitaxel promoted tubulin polymerization. Similar to the effect of colchicine and vincristine, BPR0Y007 prevented tubulin polymerization and reached a plateau at a concentration of 5 μM. We further determined the nature of BPR0Y007 interactions with tubulin by binding colchicine-binding domains on tubulin using a spin column assay. As shown in Fig. 6C, tubulin incubation with [^{3}H]colchicine in the presence of different concentrations of unlabeled colchicine reduced the amount of [^{3}H]colchicine found in the flow-through in a concentration-dependent manner. At concentrations up to 10 μM, BPR0Y007 reduced the amount of [^{3}H]colchicine in the flow-through. No further reduction in radioactivity was observed at BPR0Y007 concentrations greater than 10 μM.

3.7. Apoptosis induction by BPR0Y007

The effect of BPR0Y007 on the induction of apoptosis was also examined. Using the TUNEL assay (Fig. 7A), green fluorescein was detectable after treating cells with 10 μM BPR0Y007 and was increased with concentrations up to 50 μM. We also observed an increase in the ability of cell lysates to clear the caspase-3 substrate, Ac-DEVD-AMC, in a concentration-dependent (Fig. 7B) and

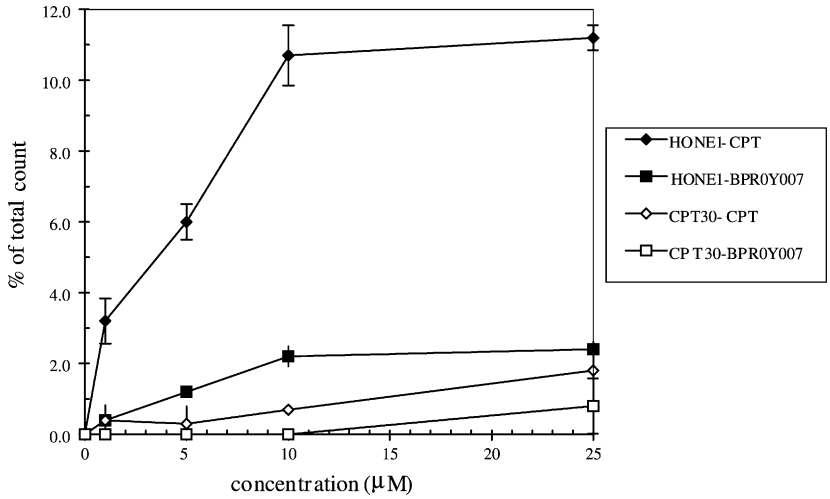

Fig. 3. Measurement of PLDBs *in vivo* by a K-SDS co-precipitation assay. HONE-1 and CPT 30 cells were treated with 1, 5, 10, and 25 μM CPT and BPR0Y007 for 30 min at 37°, respectively. The experimental procedures were described in Section 2. Data represent means ± SD from three independent experiments performed in duplicate.

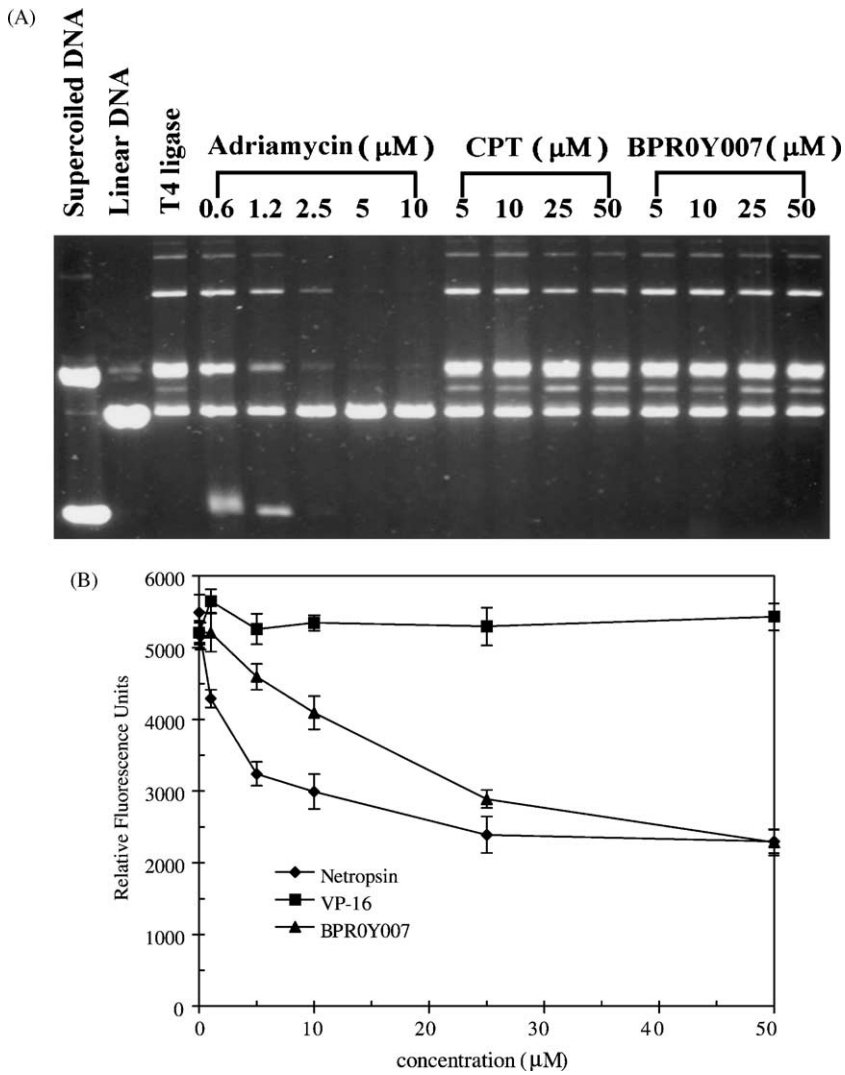

Fig. 4. (A) DNA unwinding assay. Linear DNA and an excessive amount of T4 ligase were incubated with different concentrations of adriamycin, CPT, and BPR0Y007, as indicated, at 15° for 60 min. Unwinding measurements were performed as described in Section 2. (B) Quenching of the fluorescence of DNA-bound Hoechst 33342 in a cell-free system by BPR0Y007. Netropsin and VP-16 were used as a positive and negative control, respectively. The concentrations were 1, 5, 10, 25, and 50 μM for each compound.

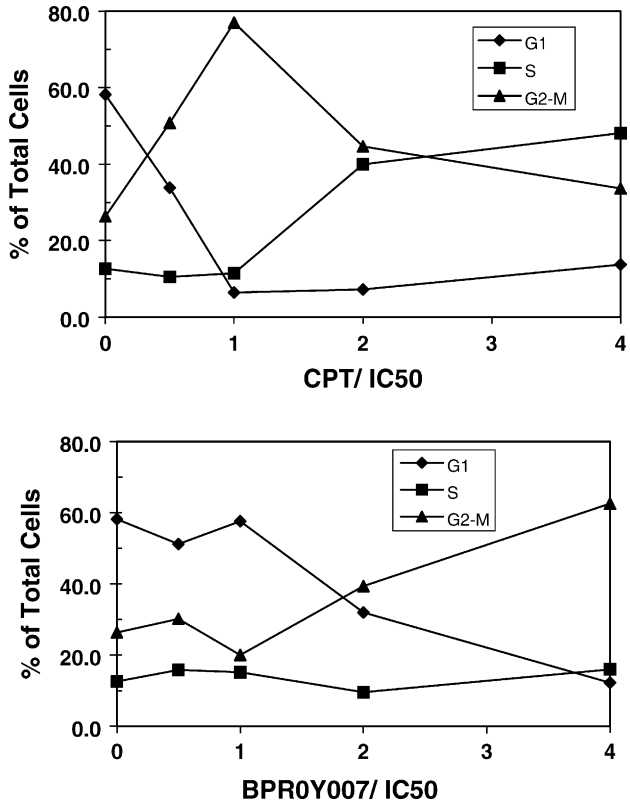

Fig. 5. Effects of CPT and BPR0Y007 on cell cycle distribution of KB cells. The cell cycle analysis was done as described in Section 2. Cells were treated with various concentrations of CPT (top panel) and BPR0Y007 (bottom panel) expressed as multiples of IC_{50} values [(CPT or BPR0Y007): IC_{50}] for 24 hr. The IC_{50} was based on the growth inhibition assay. This result is representative of three independent experiments.

time-dependent (Fig. 7C) manner, which was independent of caspase-1 (data not shown).

3.8. No cross-resistance to CPT-, vincristine- and VP-16-resistant cells

The cytotoxicity of BPR0Y007 toward various human cancer and drug-resistant cell lines was evaluated. As shown in Table 1, KB vin10 cells, which displayed overexpressed P-gp and down-regulated Top II, and KB 7D cells, which overexpressed MRP, were 3-fold more sensitive to BPR0Y007 than their parental KB cells. Furthermore, two CPT-resistant cell lines, KB 100 and CPT 30, which exhibited a decrease in Top I content and a mutation in the *Top I* gene, were as sensitive to BPR0Y007 as their parental cells. The IC_{50} of BPR0Y007 to various human cancer cell lines tested ranged from 1 to 8 μM (Table 2).

Table 2
Growth inhibition of BPR0Y007 against human tumor cell lines

Cell lines	Origin	IC_{50} (μM)
KB	Epidermoid carcinoma	3.5 ± 0.3
HONE-1	Nasopharyngeal carcinoma	1.9 ± 0.1
MCF-7	Breast carcinoma	7.7 ± 1.0
TSGH	Stomach carcinoma	4 ± 0.5
HT-29	Colon carcinoma	7.2 ± 0.4
H460	Non-small cell lung cancer	1.8 ± 0.3
DBTRG	Glioblastoma multiformi	4.6 ± 1.0

Values are averages ± SD of at least three independent experiments.

4. Discussion

By screening potential antitumor agents using a high-throughput cytotoxicity assay, we identified BPR0Y007 as an agent possessing antiproliferative properties. We further determined that BPR0Y007 inhibits Top I-mediated DNA relaxation with less potency than does CPT (Fig. 2A). Furthermore, BPR0Y007 induced Top I-mediated single-strand DNA breaks. The cleavage specificity was similar to that of CPT, whereas the relative cleavage intensity induced by BPR0Y007 was less than that of CPT. In a cellular-based study, BPR0Y007 generated protein-linked DNA breaks in a concentration-dependent manner. Moreover, the number of PLDBs generated by BPR0Y007 was lower than that generated by CPT, consistent with the *in vitro* findings that BPR0Y007 possessed lower inhibitory potency against Top I than did CPT. Similar to CPT, the number of PLDBs induced by BPR0Y007 in a CPT-resistant cell line was lower than that in parental HONE-1 cells, further indicating that BPR0Y007 targets Top I. Several lines of evidence have shown that non-CPT Top I inhibitors are DNA intercalators and/or minor groove binders (for review, see Ref. [29]). Our unwinding assay data suggest that BPR0Y007 does not intercalate into DNA (Fig. 4A). However, similar to the effect of netropsin,

Table 1
Growth inhibition of various compounds against different drug-resistant cell lines

	IC_{50}					
	KB	KB vin10	KB 100	KB 7D	HONE-1	CPT 30
BPR0Y007 (μM)	3.5 ± 0.3	1.2 ± 0.2	3 ± 0.5	1 ± 0.2	1.9 ± 0.2	1.3 ± 0.1
Vincristine (nM)	0.6 ± 0.2	65 ± 5	0.7 ± 0.3	1.2 ± 0.4	1.4 ± 0.3	1.6 ± 0.2
CPT (nM)	50 ± 10	420 ± 15	1286 ± 33	15 ± 1.5	27 ± 5	355 ± 30
VP-16 (μM)	0.9 ± 0.2	25 ± 2.3	3.1 ± 0.5	44 ± 3.5	0.5 ± 0.1	0.4 ± 0.1
Taxol (nM)	4 ± 1	16500 ± 707	7.9 ± 0.3	7.9 ± 0.5	1.2 ± 0.3	0.8 ± 0.1
Colchicine (nM)	10.5 ± 1	115 ± 7	33 ± 2.5	55 ± 7	31 ± 0.8	30 ± 1.2

Values are averages ± SD of at least three independent experiments.

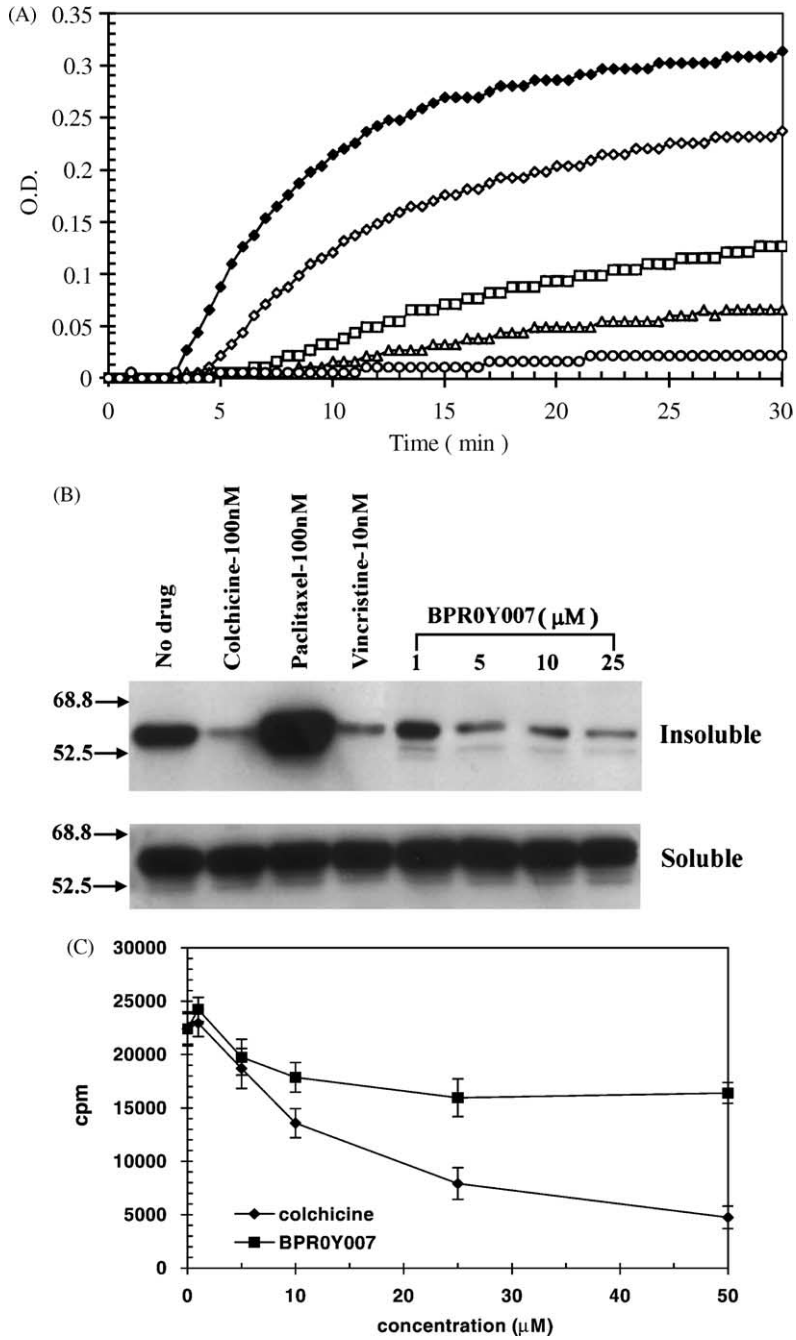

Fig. 6. (A) Effect of BPR0Y007 on microtubule assembly *in vitro*. MAP-rich tubulin in a reaction buffer was incubated at 37° in the presence of 4% (v/v) DMSO (◆), and 1 μM (◇), 2.5 μM (□), 5 μM (△) and 10 μM (○) BPR0Y007. This result is representative of two independent experiments. (B) Effect of tubulin polymerization *in vivo*. Cells were treated with colchicine (100 nM), paclitaxel (100 nM), vincristine (10 nM), and 1, 5, 10, and 25 μM BPR0Y007 for 24 hr at 37°. The *in vivo* tubulin polymerization assay was done as described in Section 2. (C) Binding of BPR0Y007 to the colchicine site of tubulin, as determined by a spin column assay, as described in Section 2. The concentrations for colchicine and BPR0Y007 were 1, 5, 10, 25, and 50 μM. Data represent means ± SD from three independent experiments performed in duplicate.

BPR0Y007 displaced Hoechst 33342 dye in a cell-free system (Fig. 4B). Both Hoechst 33342 and netropsin bind to AT-rich sites in the minor groove of DNA and cause widening of the minor grooves [28]. Therefore, these results suggest that BPR0Y007 binds to DNA, probably in a way similar to that of Hoechst 33342. Previous studies of non-CPT Top I inhibitors indicate that Top I-mediated DNA cleavage by these strong intercalators is suppressed at higher drug concentrations because the change of DNA conformation blocks Top I access to DNA [30,31]. In the case of BPR0Y007, however, Top I-mediated DNA cleavage reached a plateau at 15 μM, and no suppression of DNA cleavage occurred at concentrations up to 50 μM. Thus, BPR0Y007 can be categorized as a drug with low DNA binding affinity, which induces DNA cleavage without suppression at high concentrations.

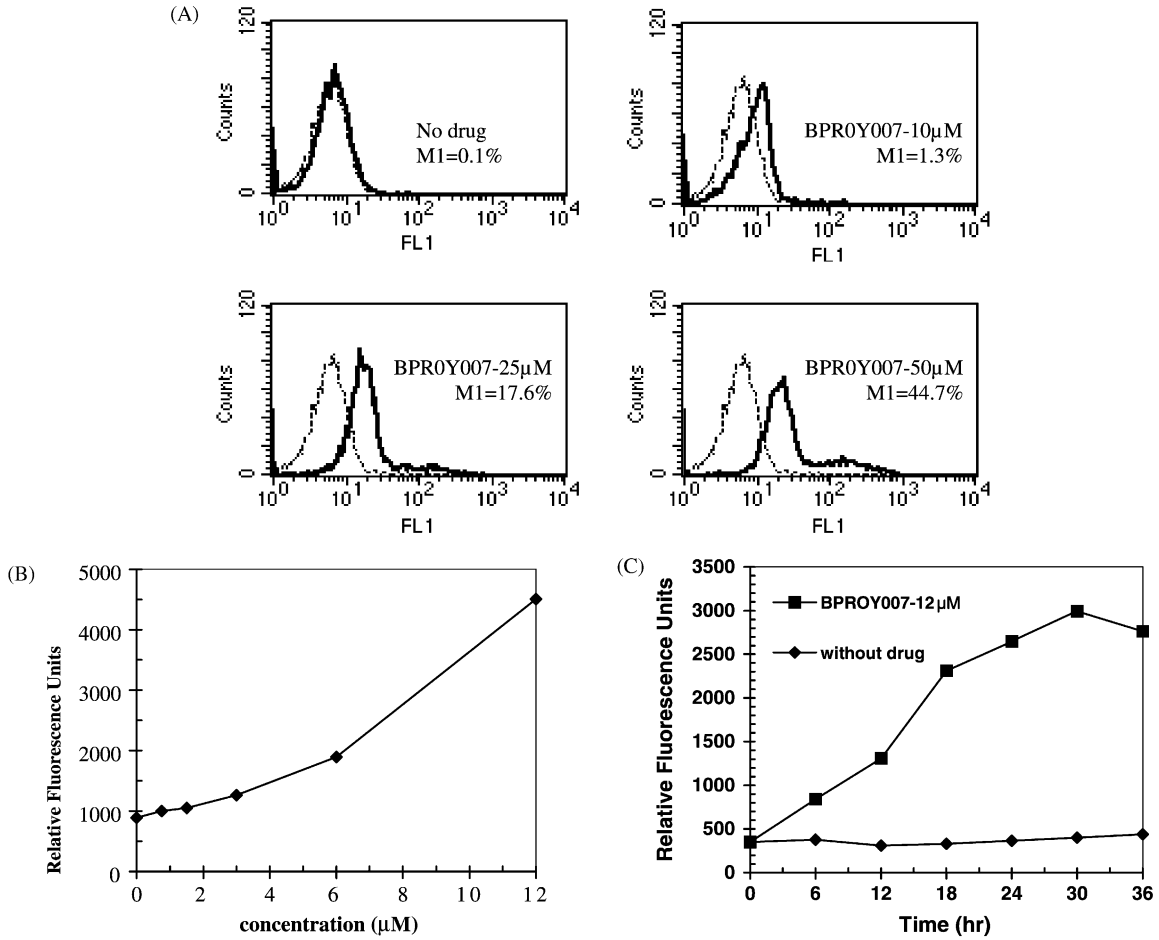

Fig. 7. (A) Effect of BPR0Y007 concentration on the induction of apoptosis. This effect was determined by a TUNEL test as described in Section 2. (B) Concentration-dependent increase in caspase-3 activation (Ac-DEVD-AMC) in KB cells after treatment for 24 hr. (C) Time-dependent increase in caspase-3 activation in KB cells after treatment with 12 μM BPR0Y007 for the indicated times. Results are representative of two independent experiments.

We were surprised to observe no cross-resistance with BPR0Y007 in two CPT-resistant cell lines (Table 1), CPT 30 and KB 100, despite the fact that BPR0Y007 generated fewer PLDBs in CPT 30 cells than in HONE-1 cells. Furthermore, it has been demonstrated that CPT is an S-phase-specific DNA-damaging agent [8]. In our cell cycle study, cells treated with CPT accumulated in S-phase with concomitant losses from the G_0/G_1 and G_2/M phases, especially at higher drug concentrations, whereas BPR0Y007 arrested cells in the G_2/M phase (Fig. 5). These results suggest that BPR0Y007 might act on other nuclear targets. Using *in vitro* and *in vivo* tubulin polymerization assays (Fig. 6A and B), we found that BPR0Y007 inhibits tubulin polymerization in a concentration-dependent manner. The mechanism of tubulin-interacting agents includes disruption of microtubule assembly (vinca alkaloids and colchicine) and stabilization of microtubules (paclitaxel and docetaxel). In contrast to paclitaxel-promoted tubulin polymerization, the pattern of inhibition of tubulin polymerization of BPR0Y007 was similar to those of colchicine and vincristine (Fig. 6B), which prevented tubulin polymerization. BPR0Y007 is thus classified as a "microtubule depolymerizing agent."

BPR0Y007 cytotoxicity exhibits features that are somewhat unusual, compared with those of other agents that inhibit Top I and tubulin. For example, a decrease in a Top I intracellular level and/or a mutation in the *Top I* gene, which are the common mechanisms of resistance to Top I inhibitors, does not inhibit cell sensitivity to BPR0Y007, as observed in two CPT-resistant cell lines, KB 100 and CPT 30. This indicates that BPR0Y007 cytotoxicity likely correlates with parameters other than anti-Top I activity, such as anti-tubulin activity. Furthermore, as previously reported, overexpression of P-gp or MRP best described the mechanism of resistance to tubulin-binding agents, including vincristine, colchicine, and paclitaxel [32,33]. In our study, different levels of resistance to vincristine, colchicine, and paclitaxel in a vincristine-resistant cell line (KB vin10) that overexpressed P-gp were observed, yet BPR0Y007 exhibited greater cytotoxicity toward KB vin10 cells than toward KB cells (Table 1). Similar findings were obtained from a VP-16-resistant cell line (KB 7D), which decreased cellular Top II content and overexpressed MRP. Therefore, BPR0Y007 exhibits a broad spectrum of activity and is cytotoxic to cells that are resistant to either Top I or Top II or tubulin inhibitors. It

will be interesting to determine whether Top I or tubulin is the principal target of BPR0Y007 in tumor cells. Investigating the cytotoxic effects on mammalian cells, which are resistant to BPR0Y007, may be able to supply the answer.

Bis-benzylidenecyclopentanone derivatives have been shown to possess antioxidative properties [13]. We demonstrated for the first time that this class of compounds possesses cytotoxicity, which acts through inhibition of Top I and tubulin. In addition, BPR0Y007 also induces apoptosis in solid tumor cells (Fig. 7).

Diferuloylmethane (curcumin), an antioxidant, induced apoptosis in a human leukemia cell line [34]. BCL-2 may play a role in the early stage of curcumin-triggered apoptotic cell death. Whether the BPR0Y007-induced apoptotic pathway is similar to curcumin is under investigation.

In summary, BPR0Y007 is a dual inhibitor of Top I and tubulin. The structure of this compound resembles neither the structure of CPTs nor the structure of known tubulin inhibitors. Moreover, no cross-resistance with BPR0Y007 in CPT-, vincristine-, and VP-16-resistant cells was observed. Given its unique biochemical properties, BPR0Y007 warrants further investigation as an antitumor agent.

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