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

Jang-Yang Chang[a,*], Hsing-Pang Hsieh[b], Wen-Yu Pan[a], Jing-Ping Liou[b], Shian-Jy Bey[b], Li-Tzong Chen[a,c], Jin-Fen Liu[a], Jen-Shin Song[b]

[a]*Division of Cancer Research, National Health Research Institutes, 7, Chung-Shan South Road, Taipei, Taiwan, ROC*
[b]*Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taipei, Taiwan, ROC*
[c]*Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, ROC*

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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

* Corresponding author. Tel.: +886-2-2356-2879; fax: +886-2-2389-2737.
E-mail address: jychang@nhri.org.tw (J.-Y. Chang).
Abbreviations: Top I, topoisomerase I; CPT, camptothecin; PLDBs, protein-linked DNA breaks.

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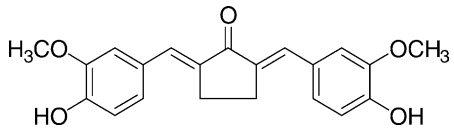

Fig. 1. Chemical structure of BPR0Y007.

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.