Flow Cytometry Analysis of Cancer Cell Death Induced by the Extract of Thai Plant *Ellipeiopsis cherrevensis*

Ryoko Yumoto, Saki Kakizoe, Junya Nagai, Denpong Patanasethanont, Bung-orn Sripanidkulchai, and Mikihisa Takano

Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand

Center for Research and Development of Herbal Health Products, Khon Kaen University, Khon Kaen, Thailand

Full text of this paper is available at [http://www.jstage.jst.go.jp/browse/dmpk](http://www.jstage.jst.go.jp/browse/dmpk)

Summary: The mechanism of cancer cell death induced by KP018, an ethanol extract of the Thai plant *Ellipeiopsis cherrevensis*, was examined in paclitaxel-resistant HepG2 (PR-HepG2) and colon-26 cells using flow cytometry. In PR-HepG2 cells, KP018 induced necrosis in a concentration-dependent manner. Necrosis of PR-HepG2 cells induced by KP018 as well as by hydrogen peroxide was suppressed by co-treatment of the cells with N-acetylcysteine. KP018 decreased the viability of colon-26 cells with an IC$_{50}$ value of 15.1 µg/mL, which was estimated by XTT assay. As observed in PR-HepG2 cells, KP018 induced necrosis and the necrosis was suppressed by N-acetylcysteine in colon-26 cells. In addition, using colon-26 solid tumor-bearing mice, KP018 was found to suppress tumor growth without apparent toxicities under in vivo conditions. These results indicate that KP018 induces necrosis rather than apoptosis in these cancer cells, and reactive oxygen species such as hydrogen peroxide would be involved in KP018-induced necrosis. KP018 may be a useful source to search for a new anticancer drug that can be used for the chemotherapy of multidrug-resistant tumors.

Keywords: Thai plant; *Ellipeiopsis cherrevensis*; paclitaxel-resistant HepG2 cells; colon-26 cells; necrosis; apoptosis; reactive oxygen species

Introduction

Resistance of tumor cells to anticancer drugs is a serious problem in cancer chemotherapy. One of the main mechanisms involved in multidrug resistance of cancer cells is the active efflux of anticancer drug by the ATP-binding cassette (ABC) superfamily of drug transporters. P-glycoprotein (P-gp; MDR1/ABCB1) is a member of the ABC transporters and is well known to confer multidrug resistance to various cancer cells. One of the strategies to overcome P-gp-mediated multidrug resistance of cancer cells is to employ a potent and safe P-gp modulator that can be co-administered with an anticancer drug. Another strategy is to employ an anticancer drug that is not affected by P-gp function.

As a source for new P-gp modulators and anticancer drugs that are not affected by P-gp function, we have been studying the extracts and purified compounds from various Thai plants. Among various extracts, we have recently found that KP018, an ethanol extract from stems of the Thai plant *Ellipeiopsis cherrevensis*, has a potent cytotoxic effect, in addition to its P-gp modulating effect. Importantly, KP018 showed similar cytotoxic potential toward wild-type HepG2 cells (human hepatoma cell line) and PR-HepG2 cells (paclitaxel-resistant HepG2 cell with enhanced P-gp expression), and the cytotoxic effect of KP018 was not affected by verapamil, an inhibitor of P-gp. These findings suggest that KP018 would be a useful source to search for a new anticancer drug that can be used for the chemotherapy of multidrug-resistant tumors. Some other researchers also have been interested in the anticancer effect of the constituents of *Ellipeiopsis cherrevensis*. So far, however, little is known about the mechanism of cytotoxicity induced by the extract of *Ellipeiopsis cherrevensis*. Therefore, in the present study, we have analyzed the mechanism underlying KP018-induced cancer cell death in PR-HepG2 as well as in another cancer cell line, colon-26, using flow cytometry analysis.
Materials and Methods

Materials: KP018, an ethanol extract from the stem of *Ellipeiopsis cherrevensis*, was kindly supplied from Khon Kaen University (Thailand), and was stored at −20°C until use. Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Annexin V-FITC and propidium iodine (PI) (100 µg/mL) were purchased from Medical & Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan), N-Acetylcysteine (NAC) and hydrogen peroxide (H₂O₂) were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, inner salt (XTT) and N-methyl dibernopyrazine methyl sulfate (PMS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for the experiments were of the highest purity commercially available.

Cell culture: A PR-HepG2 cell line was established from wild-type HepG2 cells as described previously. A colon-26 cell line derived from mouse adenocarcinoma, was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. PR-HepG2 and colon-26 cells were cultured in DMEM medium containing 3 nM paclitaxel and in RPMI 1640 medium, trypsin-EDTA and penicillin-streptomycin were purchased from Medical & Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan), N-Acetylcysteine (NAC) and hydrogen peroxide (H₂O₂) were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. In each medium, 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin were added. Each cell line was subcultured every 6–7 days (PR-HepG2), or every 3–4 days (colon-26 cells) using 0.25% trypsin and 1 mM EDTA. For the comparison, human renal proximal tubular cell line HK-2 derived from normal kidney (American Type Culture Collection, Manassas, VA, USA) was also employed to test the cytotoxicity of KP018 in normal cells.

Cytotoxicity evaluated by flow cytometry analysis: PR-HepG2 cells were seeded at a density of 10 × 10⁴/well in a 35-mm culture plate or a density of 5 × 10⁴/well in a 12-well flat-bottomed plate, and were cultured for 24 h. Colon-26 cells were seeded at a density of 12 × 10⁴/well in a 35-mm culture plate, and were cultured for 24 h. Then, each medium was replaced with serum-free medium containing KP018 (10, 50, 100 µg/mL) and 0.5% DMSO, and the cells were incubated at 37°C for 48 h. Following KP018 treatment, the medium was transferred to a 15 mL centrifuged tube and collect the detached cells. Then, PR-HepG2 and colon-26 cells on the culture plate were washed twice with PBS(−) containing 0.5% BSA and were incubated for 5 min at 37°C, respectively. After incubation, 0.5 mL of DMEM and 1 mL of RPMI medium [FBS(+)]) were added to each plate, and mixed well by pipetting. The cell suspension was transferred to the 15 mL centrifuged tube described above, and was centrifuged at 160 × g for 5 min. After the supernatant was removed, the cells (2 × 10⁵ cells) were resuspended with PBS(−) containing 0.5% BSA and were filtered through 30 µm mesh. After the cells were centrifuged at 160 × g for 5 min again, the cells were resuspended in 85 µL of binding buffer [10 mM HepES/NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂]. Ten µL of annexin V-FITC and 5 µL of PI were added to the cell suspension and incubated at room temperature for 15 min in the dark to stain the cells. Then, 400 µL of binding buffer was added and the cell sample was measured by EPICS XL flow cytometer (Beckman Coulter, Tokyo, Japan). FITC and PI fluorescence at 525 nm and 620 nm, respectively, were detected and subjected to quadrant analysis of fluorescence intensity using EXPO™32 MultiCOMP Software (Beckman Coulter, Tokyo, Japan).

To evaluate the effect of NAC, PR-HepG2 and colon-26 cells were pretreated for 1 h with or without 10 mM NAC, and then incubated for 48 h in combination with various concentrations of KP018 or 1 mM hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS).

Cytotoxicity evaluated by XTT assay: XTT assay was performed as described previously. Briefly, colon-26 cells were seeded at a density of 3 × 10⁵/well in a 24-well flat-bottomed plate, and were incubated for 24 h. The medium was replaced with serum-free RPMI 1640 medium containing various concentrations of KP018 and 0.5% DMSO, and the cells were incubated at 37°C for 48 h. Then, the cells were washed twice with PBS buffer containing 5 mM D-glucose (PBS-G buffer), and 0.2 mL of 250 µM XTT in PBS-G buffer containing 10 µM PMS was added to each well. The cells were incubated for 30 min at 37°C. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was quantitated using a VERSA max™ microplate reader (Molecular Devices, Toronto, Canada).

Cytotoxicity evaluated by protein assay: Colon-26 cells were seeded and treated with KP018 as described above. After the treatment, the cells were washed twice with PBS(−) buffer and were lysed in 300 µL of 0.1 M NaOH. The lysate was transferred to a 1.5 mL centrifuged tube and incubated at room temperature for 30 min. After centrifugation at 10,000 rpm for 5 min, protein concentration in the supernatant was measured by the method of Lowry with bovine serum albumin as a standard.

Animals and preparation of tumor-bearing mice model: Male balb/c mice (3 weeks) were purchased from Japan SLC (Hamamatsu, Japan). Animals were maintained at 20–26°C and 40–60% humidity, allowed free access to standard chow and water. To prepare tumor bearing mice, 10⁶ cells of colon-26 were subcutaneously inoculated into the groin of the mice. Experiments with animals were performed in accordance with the “Guide for Animal Experimentation” of Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University.

In vivo anti-tumor effect of KP018: When the tumor volume reached about 100 mm³ after inoculation of colon-26 cells, KP018 at a dose of 300 µg/mouse or DMSO for control was intraperitoneally administered every other day for 6 times. The experiments were ended when the tumor volume reached about 2,500 mm³ in control mice. Body weight and the size of tumor were measured every day with a caliper in two dimensions, and the tumor volume was calculated using the following equation: Tumor volume (mm³) = longer diameter (mm) × [shorter one (mm)]² × 0.52. After the mice were anesthetized, each tumor was removed and weighed on the 12th day.

Statistical analysis: Data are expressed as mean ± SE. Statistical analysis was performed by Student’s t-test or by one-way ANOVA followed by Tukey’s test for multiple comparisons. The level of significance was set at *p < 0.05 or **p < 0.01.

Results and Discussion

Analysis of KP018-induced PR-HepG2 cell death: Before studying the effect of KP018 on PR-HepG2 cells, the effect of camptothecin (CPT) was examined using flow cytometry. CPT is a plant alkaloid derived from the tree *Camptotheca acuminata*, is a
potent inhibitor of topoisomerase I, and is known to induce apoptosis in various cancer cells. As expected, the percentage of apoptotic cells was increased by CPT in a concentration-dependent manner in PR-HepG2 cells (control, 7.3 ± 1.3%; 0.5 µM CPT, 26.3 ± 3.0%; 10 µM CPT, 63.1 ± 5.9%; mean ± SE of three determinations).

**Figure 1A** shows the typical results of quadrant analysis of fluorescence intensity of gated PR-HepG2 cells in annexin V-FITC and PI channels after treatment of the cells with various concentrations of KP018. In each figure, the lower left quadrant represents viable (normal) cells, the lower right quadrant represents apoptotic (early apoptotic) cells, and the upper right quadrant represents necrotic (late apoptotic) cells. In contrast to CPT that induced apoptosis in PR-HepG2 cells, KP018 induced necrosis in a concentration-dependent manner. Percentages of viable, apoptotic, and necrotic cells are summarized in **Figure 1B**.

**Involvement of reactive oxygen species in KP018-induced necrosis in PR-HepG2 cells:** In order to understand the mechanism underlying KP018-induced necrosis, the role of reactive oxygen species was examined. First, H₂O₂-induced cell death and the effect of NAC on its cytotoxicity was examined as a positive control. Hydrogen peroxide is one of the reactive oxygen species, and induces apoptosis and necrosis in various cancer cells. NAC is known to provide a sulphydryl group to synthesize reduced glutathione (GSH) and enhance GSH level, or attenuate the reduction of GSH level by cytotoxic compounds. In addition, NAC itself is an antioxidant that reacts with reactive oxygen species such as hydroxyl radical and hydrogen peroxide, resulting in protection from the cell death induced by oxidative stresses.

Under the present experimental conditions, hydrogen peroxide predominantly induced necrosis in PR-HepG2 cells (**Fig. 2A**). Necrosis induced by hydrogen peroxide was almost completely suppressed by the co-treatment of the cells with NAC. **Figure 2B** shows the effect of NAC on KP018-induced necrosis. NAC significantly suppressed KP018-induced necrosis. These results suggest the involvement of reactive oxygen species such as hydrogen peroxide in KP018-induced necrosis in PR-HepG2 cells. However, the suppression of KP018-induced necrosis by NAC was not so efficient compared with that observed in H₂O₂-induced necrosis in PR-HepG2 cells. Therefore, factors other than reactive oxygen species may also be involved in KP018-induced cytotoxicity in PR-HepG2 cells.

**Effect of KP018 on the viability of colon-26 cells:** In order to examine whether the cytotoxic effect of KP018 can be observed in other cancer cell lines, the effect of KP018 on the viability of colon-26 cells was examined. Colon-26 is a cell line that was initially developed from an *in vivo* transplantable C-26 adenocarcinoma tumor mass.

First, the effect of KP018 on the viability of colon-26 cells was examined using XTT assay. As shown in **Figure 3A**, KP018 decreased the viability of colon-26 cells in a concentration-dependent manner, and the half maximal inhibitory concentration (IC₅₀)
determined by the Hill equation was 15.1 µg/mL. A similar cytotoxic effect of KP018 on colon-26 cells was observed when evaluated by measuring the amount of cellular protein after treatment, and the IC50 value was estimated to be 22.9 µg/mL (Fig. 3B).

We previously reported that IC50 values of KP018 on HepG2 and PR-HepG2 cells estimated by XTT assay were 4.3 and 4.7 µg/mL, respectively.3) In addition, Prayong et al.16) reported that ethanol extract from the leaves of *Ellipeiopsis cherrevensis* had cytotoxic activity on HepG2 and Vero cells with IC50 values of 104 µg/mL and 86 µg/mL, respectively. Thus, though the IC50 values may differ among various cancer cells and experimental conditions, extracts from *Ellipeiopsis cherrevensis* would have potent cytotoxic activity on various cancer cells. In this study, we also examined the cytotoxicity of KP018 on the human renal proximal tubular cell line HK-2 derived from the normal kidney of an adult male. The IC50 value on HK-2 cells estimated by XTT assay was 186.4 µg/mL, and was much higher than those on PR-HepG2 and colon-26 cancer cells. Thus, though further studies are needed to clarify the cell selectivity of KP018, KP018 may induce more potent cytotoxicity in cancer cells than in normal cells.

The pathways of cell death induced by a toxic compound are not necessarily the same and vary depending on the cancer cell lines. For example, cisplatin generally induces apoptosis in various cancer cells including colon-26 cells.17,18) On the other hand, Lim et al.19) reported that cisplatin induced necrosis in HepG2 cells, though the necrosis could be switched to apoptosis by ursodeoxycholic acid. Therefore, the pathway of cell death induced by KP018 was examined in colon-26 cells, and was compared with that in PR-HepG2 cells. Figure 3C shows the flow cytometry analysis of KP018-induced cell death in the colon-26 cell line. As observed in PR-HepG2 cells, KP018 induced necrosis in colon-26 cells in a concentration-dependent manner.

**Involvement of reactive oxygen species in KP018-induced necrosis in colon-26 cells:** The effects of H2O2 and NAC were examined in colon-26 cells. As shown in Figure 4A, H2O2 induced necrosis in colon-26 cells, and the necrosis was completely suppressed by co-treatment of the cells with NAC. Figure 4B shows the effect of NAC on KP018-induced necrosis in colon-26 cells. NAC significantly suppressed KP018-induced necrosis. These results suggest that KP018 would induce necrosis, and reactive oxygen species such as hydrogen peroxide are involved in the necrosis in colon-26 cells, as well as in PR-HepG2 cells.

Mitochondria are an important source of reactive oxygen within most mammalian cells, and the production of reactive oxygen species would be enhanced by various mitochondrial dysfunctions such as high NADH/NAD+ ratio and low ATP production.20) Therefore, though the precise mechanism is not known, KP018 may have inhibitory effects on normal mitochondrial functions, resulting in the enhanced production of the reactive oxygen species in both PR-HepG2 and colon-26 cells.

**In vivo anti-tumor effect of KP018:** Finally, the anti-tumor effect and toxicity of KP018 was examined in vivo using colon-26...
Mechanism of Cancer Cell Death from Thai Plant Extract

Figure 5. In vivo anti-tumor effect of KP018 in colon-26-bearing mice
KP018 at a dose of 300 µg/mouse (closed symbols) or DMSO (control; open symbols) was intraperitoneally administered every other day (indicated by arrows). Circle shows tumor volume and triangle shows body weight. Each value represents the mean ± SE of 3–5 experiments.

solid tumor-bearing mice. As shown in Figure 5, KP018 injected intraperitoneally suppressed tumor growth. Tumor weights measured on the 12th day were 2,049.7 ± 182.5 mg in control mice and 1,137.6 ± 90.5 mg in KP018-treated mice (p < 0.01; mean ± SE of 3–5 experiments). On the other hand, KP018 did not significantly affect the body weight of mice, and other apparent toxicities such as diarrhea were not observed. These results indicate that KP018 could effectively suppress tumor growth without apparent toxicities under in vivo condition.

Various compounds were isolated and identified from Ellipeiopsis cherrevensis, such as polyoxygenated cyclohexenes (zeylenol, ferrudiol), their analogues (ellipeiopsols A, B and C), C-benzylated chalcone, and flavonoids (tilisioide, kaempferol 3-O-rutinoside). Among these compounds, C-benzylated chalcone was shown to have cytotoxic activity against small-cell lung cancer, epidermoid carcinoma, and breast cancer cell lines. In future, constituent(s) of KP018 having an anti-tumor effect should be isolated and identified. The constituent(s) would be useful for the chemotherapy of tumors, which may be used by injection or by oral administration depending on the physicochemical and pharmacokinetic properties of the constituent(s).

In conclusion, based on flow cytometry analysis, KP018 was found to induce necrosis in cancer cell lines, PR-HepG2 and colon-26. Reactive oxygen species such as hydrogen peroxide would be involved in the necrosis induced by KP018. The anti-tumor effect of KP018 was also confirmed under in vivo condition. Because the cytotoxic effect of KP018 was not affected by P-gp, KP018 would be a useful source to search for a new anticancer drug that can be used for the chemotherapy of P-gp-related multidrug-resistant tumors.

Acknowledgments: We thank the Research Facilities for Laboratory Animal Science and the Analysis Center of Life Science, National Science Center for Basic Research and Development, Hiroshima University.

References


