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ORIGINAL ARTICLE

Wogonin potentiates the antitumor action of etoposide and ameliorates its adverse effects

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Abstract Wogonin, a flavone in the roots of Scutellaria baicalensis, reduced etoposide-induced apoptotic cell death in normal cells, such as bone marrow cells and thymocytes. On the other hand, wogonin potentiated the proapoptotic or cytotoxic action of etoposide in tumor cells, such as Jurkat, HL-60, A549, and NCI-H226. These contradictory actions of wogonin on apoptosis are distinguished by normal or cancer cell types. Wogonin had no effect on apoptosis induced by other anticancer agents in the tumor cells. Thus, the potentiation effect of wogonin was observed only in etoposide-induced apoptosis in tumor cells. In a functional assay for P-glycoprotein (P-gp), wogonin suppressed excretion of calcein, a substrate for P-gp, in these tumor cells. Moreover, wogonin decreased the excretion of radiolabeled etoposide and accordingly increased intracellular content of this agent in the cells. P-gp inhibitors showed a similar potentiation effect on etoposide-induced apoptosis in these tumor cells. Thus, wogonin is likely to potentiate the anticancer action of etoposide due to P-gp inhibition and accumulation of this agent. These findings suggest that wogonin may be a useful chemotherapeutic adjuvant to potentiate the pharmacological action of etoposide and ameliorate its adverse effects.

Keywords Wogonin · Etoposide · Apoptosis · Antitumor action · Chemotherapeutic adjuvant · P-glycoprotein

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Introduction

Flavonoids are one of the most common active ingredients of medicinal herbs. Flavonoids possess a wide spectrum of pharmacological properties, such as anti-inflammatory [1, 2], antioxidant [3], and antitumor [4–9] activities. Some flavonoids are thought to be sources of new anti-cancer drugs or chemotherapeutic adjuvants [10]. However, the molecular mechanisms remain poorly understood and warrant further investigation. Wogonin is one of the active ingredients extracted from the roots of Scutellaria baicalensis Georgi and has been reported to induce apoptosis in tumor cells and suppress tumor growth [4, 5, 11–14]. Although the mechanism of the antitumor action of wogonin has been discussed in these reports, investigations are insufficient and the details are unclear.

We recently reported that wogonin shows not only a proapoptotic effect but also a potentiation effect on etoposide-induced apoptosis in HL-60 and Jurkat cells [15]. We also found that wogonin acts as an anti-apoptotic agent in thymocytes. Fas et al. [16] reported that wogonin sensitizes TNFα-resistant leukemia cells to TNFα-induced apoptosis. They indicated that wogonin does not affect the viability of normal T-cells in the report. Since the pharmacological properties of wogonin may be distinguished by the cell type, it may enhance the pharmacologic actions of antitumor agents and ameliorate its severe adverse effects.

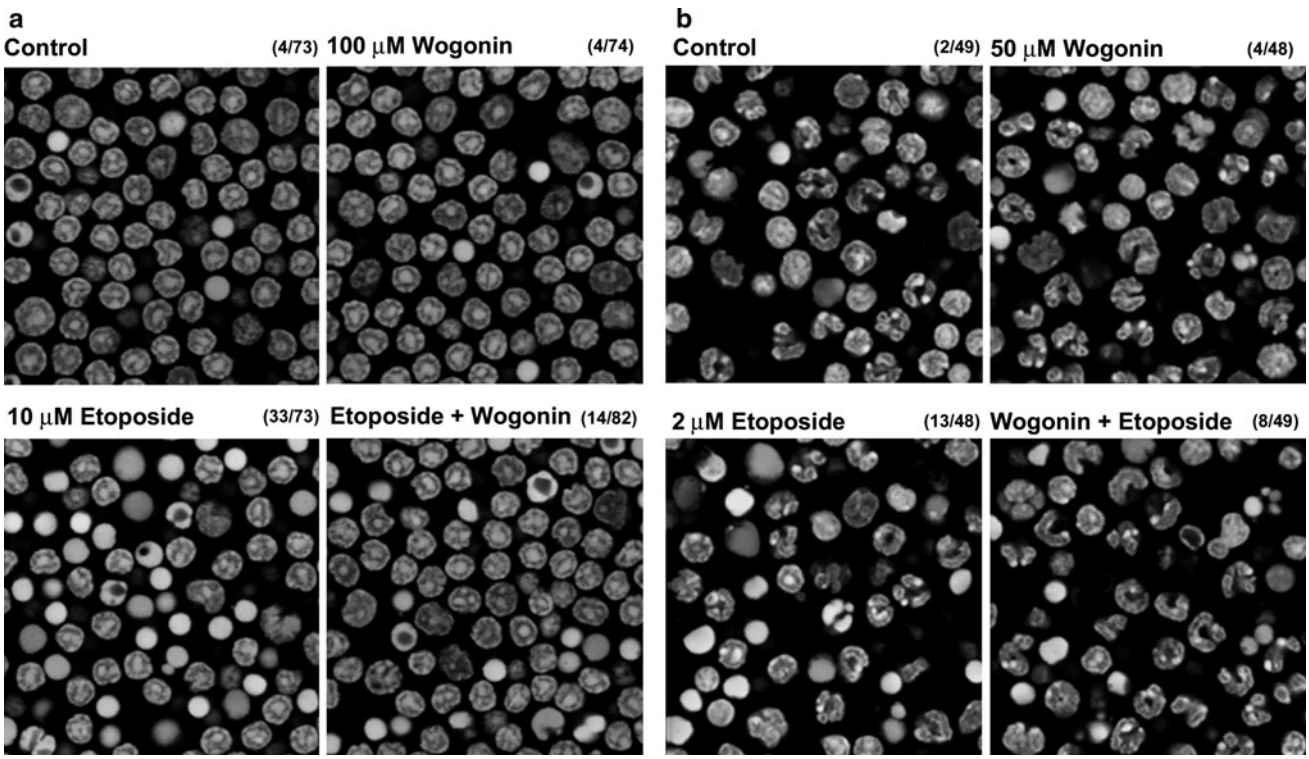


Fig. 1 Wogonin inhibits etoposide-induced apoptosis in normal cells. Thymocytes (**a**) and bone marrow cells (**b**) were treated with 100 μM wogonin in the presence or absence of 10 μM etoposide for 4 h. Nuclei were stained by Hoechst 33342, and nuclear structure was observed with a laser scanning microscope. The percentage of condensed nuclei is shown in the *upper right* corner of each image (condensed/total). Typical data of three times of independent experiments were shown in the figure. **c**, *upper* Thymocytes were treated with various concentrations of wogonin in the presence or absence of 10 μM etoposide for 6 h. **c**, *lower* Cells were treated with various concentrations of etoposide in the presence or absence of 100 μM wogonin for 6 h. Cells were collected and DNA fragmentation determined. Values are mean ± SEM of four separate experiments. **d** Cells were treated with various concentrations of wogonin in the presence or absence of 10 μM etoposide for 4 h. Cells were collected and PS translocation analyzed using FACS. Values are mean ± SEM of three separate experiments. **e** Cells were treated with 10 μM etoposide in the presence or absence of 100 μM wogonin for the indicated time. Caspase preparation from the cells was used to determine DEVDase activity. Values are mean ± SEM of four separate experiments

In this study, the potential of wogonin as a chemotherapeutic adjuvant is evaluated using tumor and normal cells and its mechanism of action is also discussed.

Materials and methods

Extraction and isolation of wogonin

Wogonin was extracted from commercial Scutellariae Radix and isolated by column chromatography and preparative TLC. The isolated compound was single peak in the HPLC analysis. Wogonin was identified by NMR analysis and mass spectrometry. Details of 1H-NMR, 13C-NMR, and mass spectral data are given below: 1H-NMR (DMSO-d6): 3.87 (3H, s, 8-OMe), 6.32 (1H, s, 6-H), 7.00 (1H, s, 3-H), 7.61 (3H, m, 2′,4′,6′-H), 8.08 (2H, m, 3′,5′-H), 12.50 (2H, s, 7,5-OH). 13C-NMR (DMSO-d6): 61.0 (q, 8-OMe), 99.1 (d, C-6), 103.7 (s, C-4a), 105.0 (d, C-3), 126.3 (d, C-3′,5′), 128.3 (s, C-8), 129.2 (d, C-2′,6′), 130.8 (s, C-2), 132.1(d, C-4′), 149.6 (s, C-8a), 156.2 (s, C-7), 157.4 (s, C-5) 163.0 (s, C-1′), 182.0 (s, C-4). EI-MS m/z: 284 (M^+).

Animals

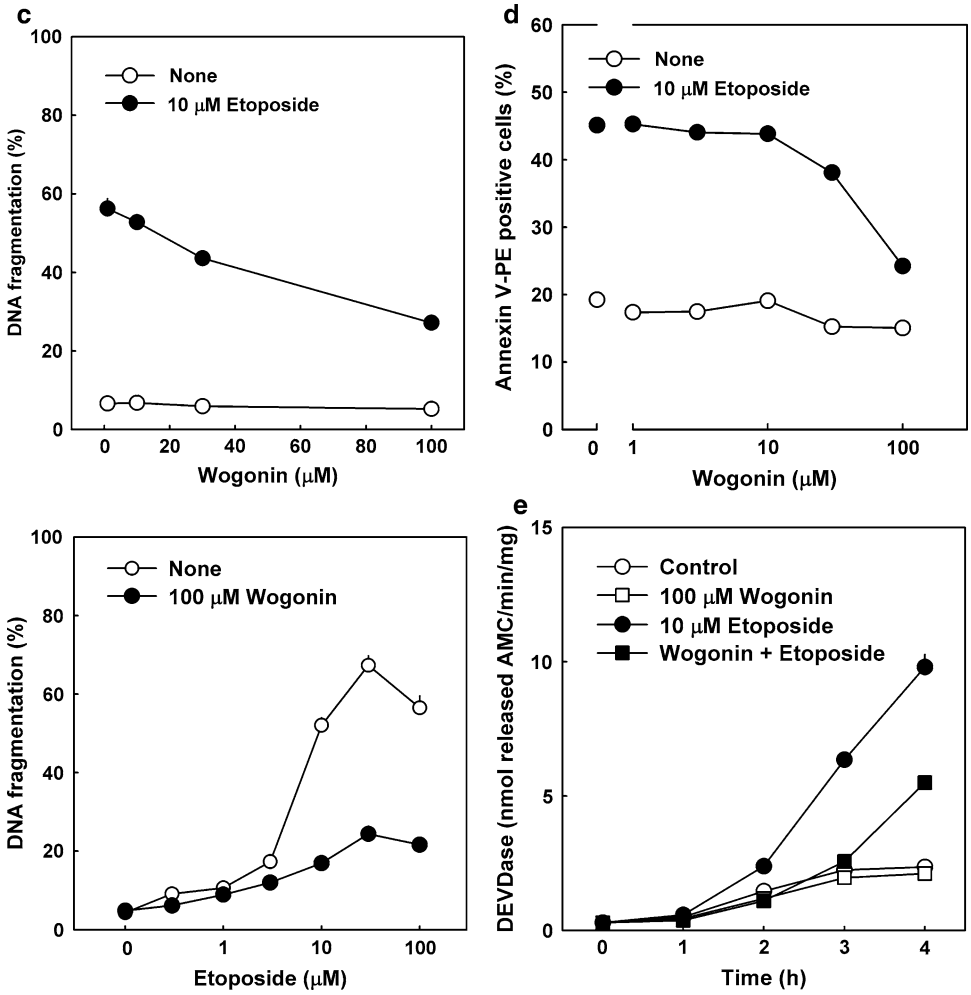
All procedures conformed to the "guiding principles for the care and use of laboratory animals" approved by the Japanese Pharmacological Society, and all efforts were made to minimize animal suffering and the number of animals used.

Male Sprague–Dawley rats and BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan) were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle. The animals had free access to food and water. They were euthanized with diethyl ether and then, thymocytes and bone marrow cells were isolated.

Cell culture

Thymocytes were isolated from the thymus of male Sprague–Dawley rats (5 weeks old). Bone marrow cells were

Fig. 1 continued



isolated from the femur of male BALB/c mice (5 weeks old). Both cell types were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) at a density of 10×10^6 cells/ml. HL-60 and Jurkat cells (Health Science Research Resource Bank, Osaka, Japan) were maintained in RPMI 1640 medium containing 10% FCS. NCI-H226 cells (ATCC, Manassas, VA) were maintained in the same medium. A549 cells (Health Science Research Resource Bank, Osaka, Japan) were maintained in Eagle's MEM containing 10% FCS.

Drug treatment

All drugs, including wogonin, were dissolved in dimethyl sulfoxide (DMSO) and then diluted 1,000-fold in the culture medium. DMSO concentration (0.2%) did not affect cell viability. Thymocytes and bone marrow cells at a density of 10×10^6 cells/ml were treated with drugs in the culture medium containing 10% FCS. HL-60 and Jurkat cells at a density of 4×10^6 and 3×10^6 cells/ml, respectively, were treated with drugs in all experiments except for the WST-8 assay. A549 and NCI-H226 cells were treated with drugs in a subconfluent 35-mm dish.

Measurement of apoptotic markers

Cells were treated with etoposide with or without wogonin and then tested for the following apoptotic markers. For nuclear condensation, cell nuclei were fixed with formaldehyde solution and nuclei stained by Hoechst 33342 (Invitrogen Co., Carlsbad, CA) [17]. Nuclei were then observed using a laser scanning microscope (GB200, Olympus Optical Co., Ltd., Tokyo, Japan). Condensed and normal nuclei in each image were counted and percentages of condensed nuclei calculated. DNA fragmentation was determined by the diphenylamine method. Briefly, the cells were lysed in lysis buffer, and intact and fragmented DNAs were separated by centrifugation. DNA contents were respectively measured, and the percentage of fragmented DNA in total DNA (intact DNA + fragmented DNA) was calculated [18]. Externalization of phosphatidylserine (PS) was analyzed using BD PharmingenTM Annexin V-PE Apoptosis

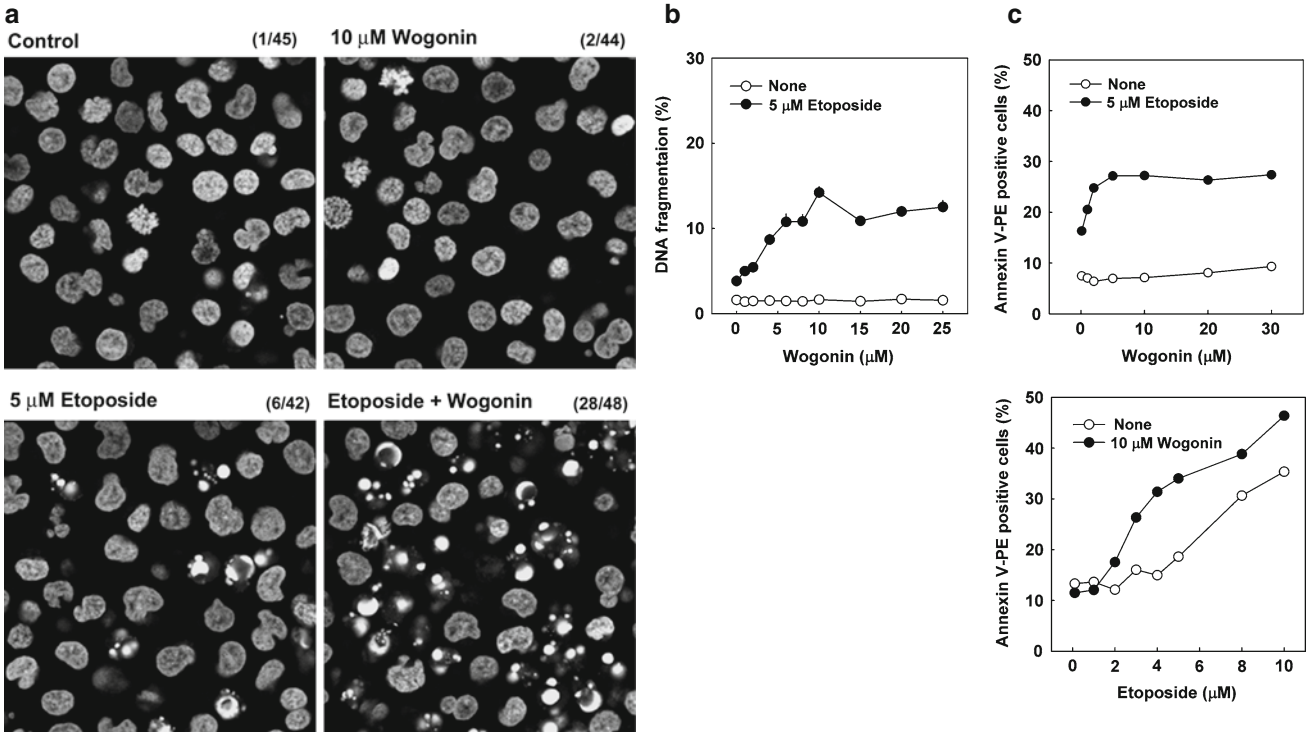


Fig. 2 Wogonin enhances etoposide-induced apoptosis in tumor cells. **a** Jurkat cells were treated with various concentrations of wogonin in the presence or absence of 5 μM etoposide for 6 h. Nuclei were stained by Hoechst 33342 and the nuclear structure observed using a laser scanning microscope. The percentage of condensed nuclei is shown in the *upper right* corner of each image (condensed/total). **b** Jurkat cells were treated with various concentrations of wogonin in the presence or absence of 5 μM etoposide for 6 h. Cells were collected and DNA fragmentation determined. Values are mean ± SEM of four separate experiments. **c**, *upper* Cells were treated with various concentrations of wogonin in the presence or absence of 5 μM etoposide for 4 h. **c**, *lower* Cells were treated with various concentrations of etoposide in the presence or absence of 10 μM wogonin for 4 h. Cells were collected and PS translocation analyzed using FACS. Values are mean ± SEM of three separate experiments

Detection Kit I (BD Biosciences Pharmingen, San Diego, CA). Cells were incubated with various compounds for 6 h and stained according to manufacturer's instructions. Annexin V-PE-positive cells were analyzed using FACSCanto (BD Biosciences, Franklin Lakes, NJ) with FCS Express (De Novo Software, Los Angeles, CA). Caspase-3-like activity was measured using the fluorescence substrate acetyl-Asp-Glu-Val-Asp α-(4-methyl-cumaryl-7-amide). Cell extract (caspase preparation) was prepared by repeated freezing and thawing of cells after treatment. The fluorescence of aminomethylcoumarin was determined by ARVOsx (PerkinElmer, Inc., Waltham, MA) [19].

Cell viability

Assay of cell viability was performed at a density of 1×10^4 cells/well in a 96-well plate. Cell viability was determined by WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Functional assay for P-glycoprotein (P-gp)

For transport assay, cells were incubated with 50 or 100 nM calcein-AM (Invitrogen Co., Carlsbad, CA) for 30 min. Then the cells were washed and resuspended in fresh culture medium with or without wogonin for 30 and/or 120 min. The fluorescence of residual calcein was analyzed using FACScanto.

Etoposide accumulation

Etoposide accumulation was measured using a radiolabeled compound. Cells were incubated with 4 μM [3H]etoposide (500 mCi/mmol; Moravek Biochemicals, Inc., Brea, CA,) in the presence or absence of various compounds for 1 h. After incubation, the cells were collected and washed with ice-cold PBS. The final cell suspension was applied to Ready Cap® with Xtalscint® (Beckman Coulter, Inc., Fullerton, CA).

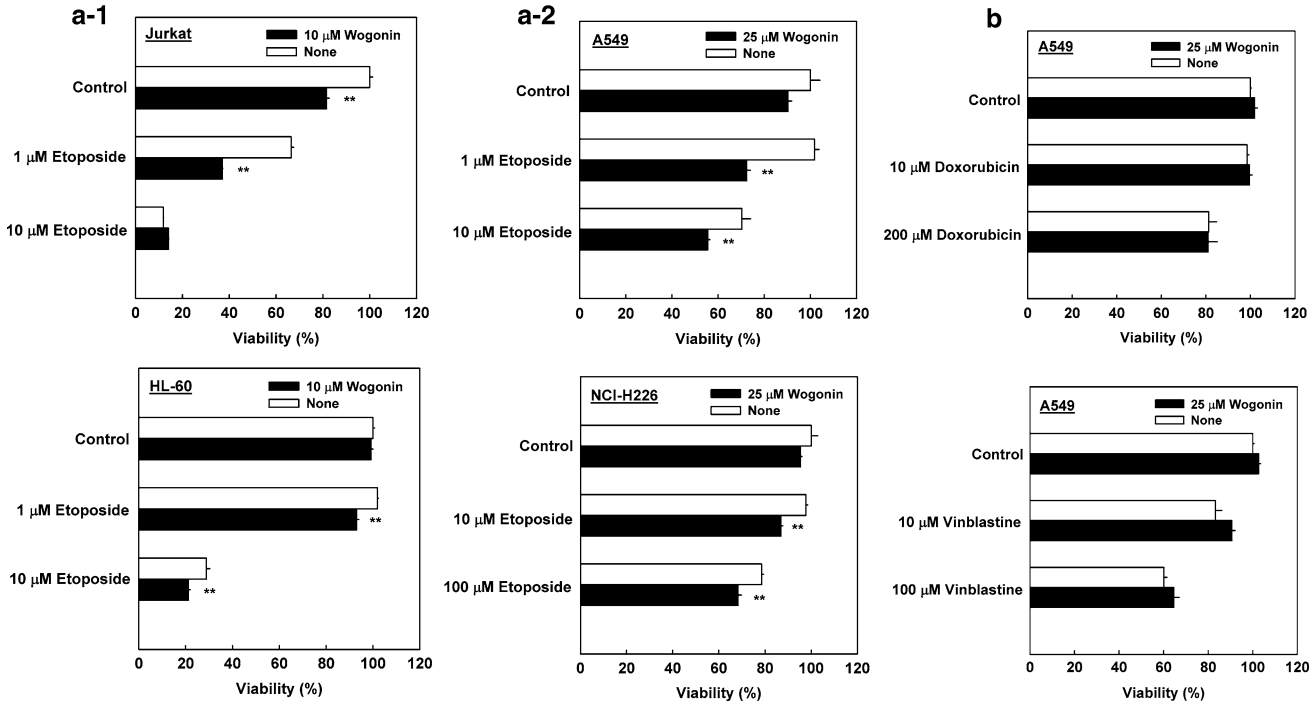


Fig. 3 Wogonin enhances the antitumor action of etoposide in various tumor cells. **a** Jurkat, HL-60, NCI-H226, and A549 cells were treated with various concentrations of etoposide in the presence or absence of wogonin for 24 h. **b** A549 cells were treated with various concentrations of antitumor drugs (doxorubicin and vinblastine) in the presence or absence of wogonin for 24 h. After incubation, cell viability was determined using the Cell Counting Kit-8. Values are mean ± SEM. of four separate experiments. $^{**}P < 0.01$ (Student's *t* test)

Results

Effect of wogonin on etoposide-induced apoptosis in normal cells

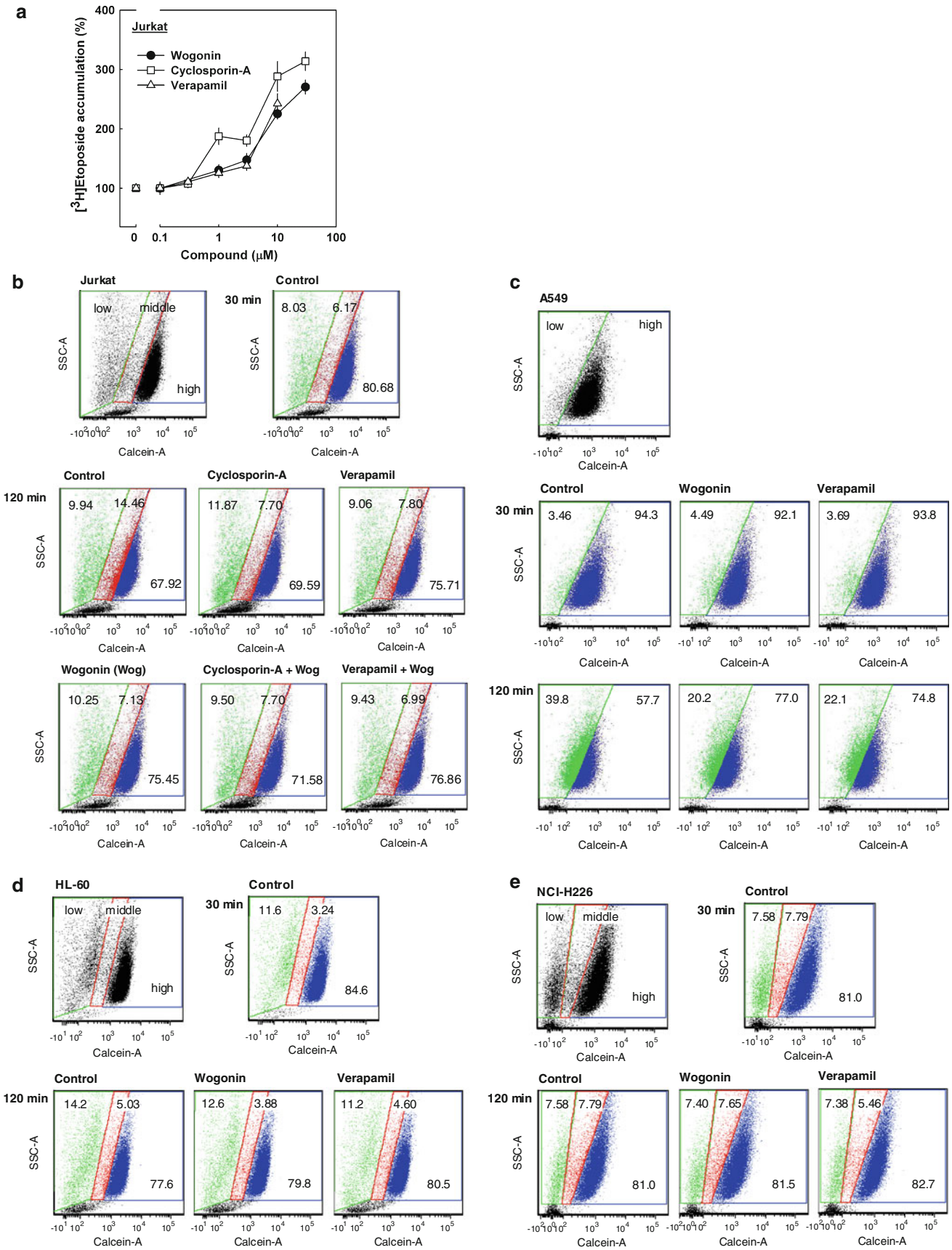
Figure 1 shows the effect of wogonin on etoposide-induced apoptosis in rat thymocytes (a) and mouse bone marrow cells (b). Wogonin reduced nuclear condensation induced by etoposide, a morphological hallmark of apoptosis, in thymocytes (45–17%) and bone marrow cells (27–17%). Wogonin also reduced etoposide-dependent biological changes of apoptosis, such as DNA fragmentation (Fig. 1c), PS translocation (Fig. 1d), and caspase activation (Fig. 1e) in thymocytes. The inhibitory effect of wogonin was observed at a concentration greater than 10 μM and the effect was concentration dependent. On the other hand, wogonin at a concentration up to 100 μM had no effect on these morphological (Fig. 1a, b) and biological (Fig. 1c–e) apoptotic changes in cells. Direct addition of wogonin to the assay medium for caspase had no effect on activity (data not shown).

Potentiation of the antitumor effect of etoposide in various tumor cells by wogonin

Figure 2a shows that wogonin increased condensed or fragmented nuclei in etoposide-treated Jurkat cells. Wogonin also increased the biological hallmarks of apoptosis in these cells (Fig. 2b, c). The potentiation effect of wogonin was observed at a concentration greater than 1 μM, and the maximal effect was detected at 10 μM in these cells. Etoposide-induced cell death was markedly potentiated by wogonin not only in Jurkat cells but also in A549 cells (Fig. 3a). On the other hand, wogonin showed weak potentiation effect on etoposide-treated HL-60 and NCI-H226 cells (Fig. 3a). However, the flavone had no effect on doxorubicin and vinblastine (Fig. 3b) and other antitumor agents (cisplatin, 5-FU, carboquone, and camptotecin; data not shown) in A549 cells.

Drug accumulation and P-gp inhibition by wogonin

Typical inhibitors of P-gp, such as verapamil and cyclosporin-A increased the content of [3H]etoposide in Jurkat and HL-60 cells. A similar increase in [3H]etoposide content was observed in these cells treated with wogonin. Wogonin and P-gp inhibitors markedly increased the content of [3H]etoposide in Jurkat cells (Fig. 4a), whereas these compounds slightly increased the content in HL-60 cells (data not shown). Furthermore, wogonin inhibited excretion of calcein-AM, a substrate of P-gp, and maintained intracellular calcein, the fluorescent hydrolysis product of calcein-AM, in Jurkat cells. Similar inhibition of


a
Jurkat
Wogonin
Cyclosporin-A
Verapamil
[3H]Etoposide accumulation (%)
Compound (μM)
b
Jurkat
Control
30 min
low
middle
high
8.03
6.17
80.68
120 min
9.94
14.46
67.92
11.87
7.70
69.59
9.06
7.80
75.71
Wogonin (Wog)
Cyclosporin-A + Wog
Verapamil + Wog
10.25
7.13
75.45
9.50
7.70
71.58
9.43
6.99
76.86
c
A549
3.46
94.3
4.49
92.1
3.69
93.8
39.8
57.7
20.2
77.0
22.1
74.8
d
HL-60
11.6
3.24
84.6
14.2
5.03
77.6
12.6
3.88
79.8
11.2
4.60
80.5
e
NCI-H226
7.58
7.79
81.0
7.40
7.65
81.5
7.38
5.46
82.7
SSC-A
Calcein-A

◀ **Fig. 4** Wogonin increases [^{3}H]etoposide accumulation and inhibits excretion of calcein-AM via P-gp in tumor cells. **a** Jurkat cells were incubated with 4 μM [^{3}H]etoposide in the presence or absence of various concentrations of wogonin, cyclosporin-A, and verapamil for 1 h. Cells were then collected and washed. The final cell suspension was applied to Ready Cap and radioactivity determined using LSC. Values are mean ± SEM of four separate experiments. **b** Jurkat cells were incubated with 100 nM calcein-AM for 30 min. Cells were washed with fresh growth medium and then incubated in the same medium containing 10 μM of wogonin and/or P-gp inhibitors for 30 and 120 min. **c** and **d** A549 and NCI-H226 cells were incubated with 50 nM calcein-AM for 30 min. **e** HL-60 cells were incubated with 100 nM calcein-AM for 30 min. Cells were washed with fresh growth medium and then incubated with the same medium containing 10 μM of wogonin or P-gp inhibitor for 30 and/or 120 min. The fluorescence of accumulated calcein was analyzed using FACSCanto and indicated as 2-D *plot* (fluorescence/side scatter). Typical data of three times of independent experiments were shown in the figure. The percentage of the cells in each gate is shown in the figure (each gate/total)

calcein-AM excretion was observed in these cells treated with verapamil and cyclosporin-A. Combination of wogonin and these P-gp inhibitors did not increase the accumulation of calcein (Fig. 4b). The inhibition of calcein-AM excretion by wogonin treatment was also detected in A549 cells (Fig. 4c). On the other hand, wogonin had little effect on the excretion of calcein-AM in HL-60 and NCI-H226 cells (Fig. 4d, e). Increase in [3H]etoposide content (Fig. 5a) and calcein accumulation (Fig. 5b) was also observed in rat thymocytes.

Potentiation of etoposide-induced apoptosis in cancer cell lines by P-gp inhibitors

Cyclosporin-A and verapamil enhanced etoposide-induced apoptosis at a concentration greater than 1 μM in Jurkat cells (Fig. 6a). However, in contrast to wogonin, these P-gp inhibitors did not protect etoposide-induced apoptosis in thymocytes (Fig. 6b).

Discussion

Medicinal herbs have various active ingredients and have attracted interest as sources of therapeutic drugs. In particular, several authors have discussed the biological and pharmacological actions of flavonoids. Anti-inflammatory [1, 2], antioxidant [3], and antitumor actions [4–9] are the well-known actions of flavonoids. Some flavonoids are thought to be applicable to tumor chemotherapy [10].

In this study, we have shown that wogonin inhibits etoposide-induced apoptosis in normal cells and potentiates it in tumor cells. We also reported that it shows an anti-apoptotic effect on thymocyte apoptosis induced by various compounds [19]. On the other hand, its potentiation effect was selective on etoposide-induced apoptosis in tumor cells. It seems to act reversibly on target cells because this effect disappeared when cells were treated with etoposide after wogonin removal (data not shown).

It is well known that etoposide is a typical inhibitor of topoisomerase-II, and that late S-phase cells and G2-M-phase cells are highly sensitive to it. Furthermore, it had been widely reported that flavonoids affect cell cycle [20–26]. However, wogonin had no effect on the cell cycle in Jurkat cells in our study (data not shown). The cell cycle therefore had no relation to potentiation of the antitumor action of etoposide by wogonin. We presumed that topoisomerase-II may be involved in the mechanism of action of wogonin because wogonin enhanced only the action of etoposide, and some flavonoids have been reported to act as topoisomerase-II inhibitors [27, 28]. However, wogonin did not enhance the antitumor action of doxorubicin, another inhibitor of topoisomerase-II. Doxorubicin has multiple actions, such as inhibition of topoisomerase-II, DNA polymerase, and RNA polymerase. In addition, it shows its antitumor action by DNA crosslinking, and then inhibition of the abovementioned enzymes, whereas etoposide shows its action by DNA cleavage and formation of the drug-topoisomerase-II complex [29, 30]. The difference in the inhibitory mechanisms of topoisomerase-II may be attributable to the difference in the effect of wogonin.

It is well known that the sensitivity of antitumor agents in tumor cells is involved with the expression of P-gp and multidrug resistance–associated protein (MRP). These proteins are members of the ATP-binding cassette transporter family and act as an ATP-dependent drug-efflux pump. Etoposide is a substrate for P-gp and some MRPs. We speculated that wogonin enhanced the antitumor action of etoposide due to the inhibition of efflux of etoposide from cells because some flavonoids were reported to inhibit P-gp or MRPs [31–37]. In this study, wogonin inhibited P-gp and accumulated etoposide in some tumor cells. Cotreatment with wogonin brought the effect equal to the treatment of two-fold the amount of etoposide in Jurkat cells. Moreover, typical inhibitors of P-gp exerted a similar effect on the action of etoposide. Thus, P-gp inhibition seems to be part of the mechanism of potentiation of the action of etoposide by wogonin. However, the flavone had no effect on the antitumor actions of doxorubicin and vinblastine although these agents are the substrates for P-gp as well as etoposide. Our results suggest that the potentiation effect of wogonin involves multiple mechanisms. It has been reported that wogonin sensitized TNFα- and TRAIL-induced apoptosis in malignant T-cells, but not in normal T-cells [16]. These results partly coincided with our findings. The authors accounted for its mechanism by production of reactive oxygen species (ROS) and the difference in

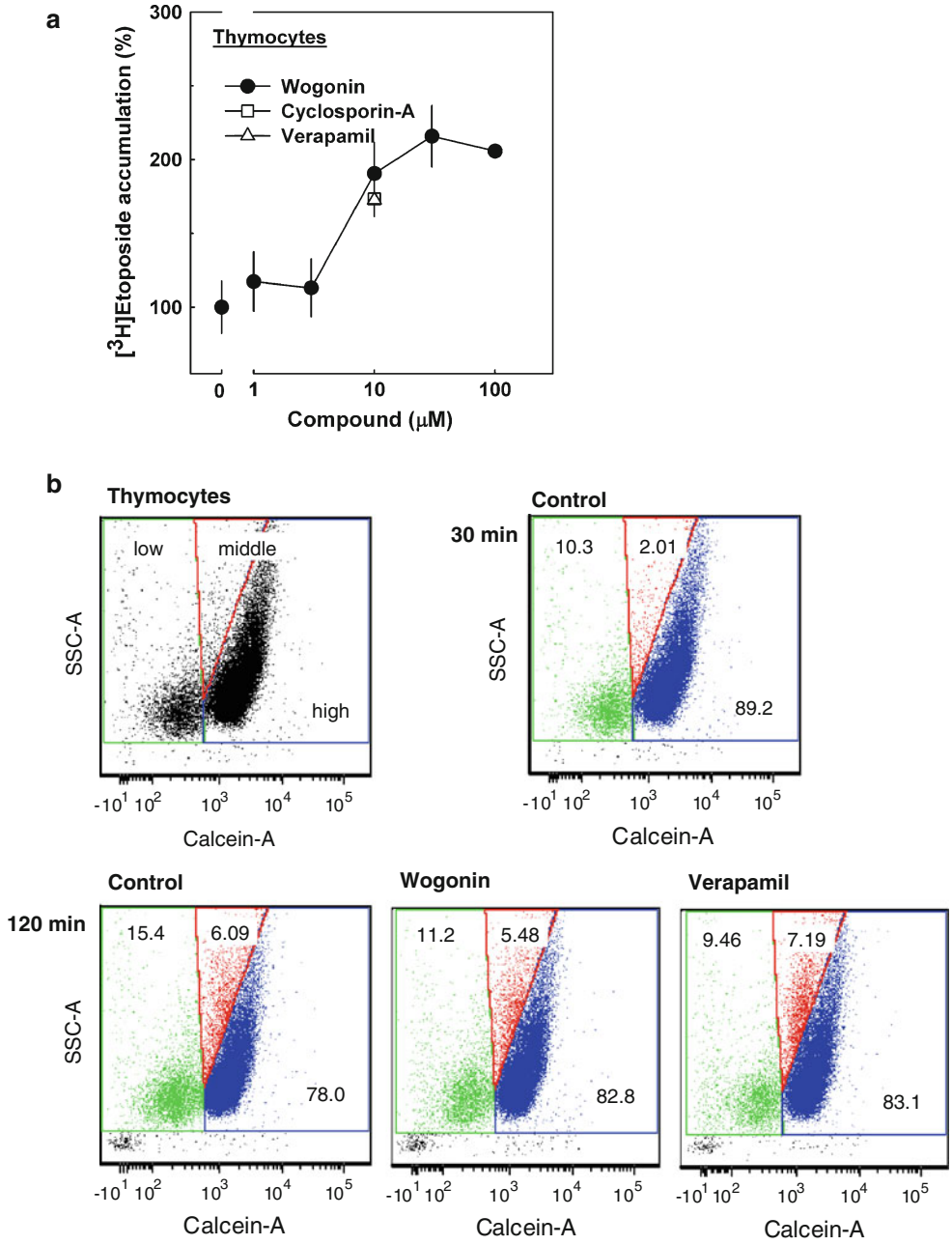


Fig. 5 Wogonin accumulates [^{3}H]etoposide and calcein in normal cells. **a** Thymocytes were incubated with 4 μM [^{3}H]etoposide in the presence or absence of various concentrations of wogonin, 10 μM cyclosporin-A, and 10 μM verapamil for 1 h. Cells were then collected and washed. The final cell suspension was applied to Ready Cap and radioactivity determined using LSC. Values are mean ± SEM of four separate experiments. **b** Thymocytes were incubated with 100 nM calcein-AM for 30 min. Cells were washed with fresh growth medium and then incubated with the same medium containing 10 μM of wogonin or P-gp inhibitor for 120 min. The fluorescence of accumulated calcein was analyzed using FACSCanto and indicated as 2-D *plot* (fluorescence/side scatter). Typical data of three times of independent experiments were shown in the figure. The percentage of the cells in each gate is shown in the figure (each gate/total)

the oxidation–reduction status of both cells. ROS accumulation may be partly involved in the potentiation effect of wogonin because etoposide is known to increase ROS generation.

Although wogonin also accumulated etoposide in thymocytes, the flavone reduced etoposide-induced apoptosis in the same cells. Moreover, we found that the anti-apoptotic effect disappeared when wogonin was added to cells before etoposide treatment (data not shown). These results suggest that wogonin acts on upstream processes of the common extrinsic pathway of apoptosis. We also confirmed that the anti-apoptotic effect disappeared when thymocytes

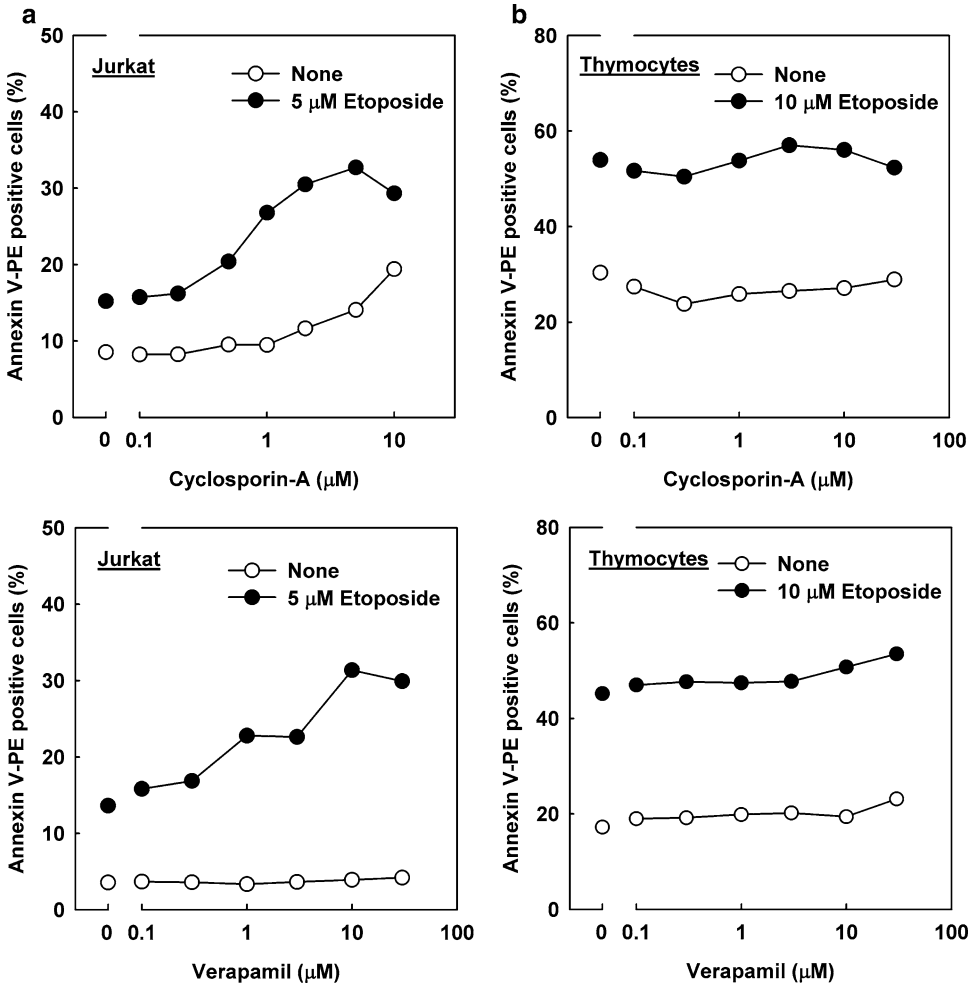


Fig. 6 Etoposide-induced PS translocation enhanced by MDR inhibitors only in tumor cells. **a** Jurkat cells were treated with various concentrations of P-gp inhibitors in the presence or absence of 5 μM etoposide for 4 h. **b** Thymocytes were treated with various concentrations of P-gp inhibitors in the presence or absence of 10 μM etoposide for 4 h. After incubation, cells were collected and PS translocation analyzed using FACS. Values are mean ± SEM of three separate experiments

were treated with etoposide after wogonin was washed out of cells (data not shown). Wogonin therefore seems to act reversibly on target cells.

These findings confirm that wogonin is a chemotherapeutic adjuvant that ameliorates severe adverse effects such as myelosuppression and increases the pharmacological action of etoposide.

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