# Evaluation of the Flavonoid Oroxylin A as an Inhibitor of P-Glycoprotein-Mediated Cellular Efflux

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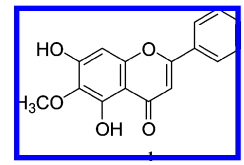
*Received May 18, 2009*

Oroxylin A (**1**), a flavonoid from the roots of *Scutellaria baicalensis*, increased the cellular accumulation of calcein AM in a concentration-dependent manner in NCI/ADR-RES cells overexpressing P-glycoprotein over the concentration range 0−40 $\mu$M. In addition, **1** significantly ($p < 0.05$) increased the cellular accumulation of paclitaxel in NCI/ADR-RES cells while it did not alter the cellular accumulation of paclitaxel in cells lacking P-glycoprotein expression. Accordingly, the concentrations that yielded 50% cytotoxicity of vinblastine and paclitaxel were reduced by approximately 5-fold in the presence of **1**. This indicated that cancer cells became more susceptible to the cytotoxicity of vinblastine and paclitaxel in the presence of **1**. The concomitant use of **1** (30 mg·kg$^{-1}$) significantly ($p < 0.05$) enhanced the oral exposure of paclitaxel (15 mg·kg$^{-1}$) in rats. The $C_{max}$ and AUC values of paclitaxel increased by 2.1−2.6-fold in the presence of **1** with no significant change in $T_{max}$. In conclusion, **1** was effective in inhibiting P-glycoprotein-mediated drug efflux both in vitro and in vivo, suggesting that it may be useful to improve the cellular availability of P-glycoprotein substrates such as anticancer drugs.

Multidrug resistance (MDR), one of the major causes of failure in cancer chemotherapy, is associated with excessively high expression of P-glycoprotein (P-gp) in cancer cells.[1−3] In addition, P-gp plays an important role in absorption, distribution, and elimination of various xenobiotics.[1−4] Considering that P-gp has a very broad spectrum of substrates including cancer chemotherapeutic agents, cardiovascular drugs, HIV protease inhibitors, immunosuppressants, antibiotics, steroids, and cytokines,[1−4] the modulation of P-gp activity may provide significant therapeutic benefits in the optimization of drug therapy for various therapeutic indications. Therefore, in the past several decades, there has been an enormous effort to identify and develop effective modulators of P-gp activity.[1] However, many synthetic compounds have only limited clinical applicability because of toxicity issues or pharmacokinetic issues.[1] Accordingly, in a continuing effort to identify safer P-gp inhibitors, there have been extensive investigations on the interaction characteristics of P-gp with natural compounds, particularly dietary flavonoids such as genistein, biochanin A, morin, and phloretin as well as the green tea catechins.[5−10] Among various phytochemicals, flavonoids are present widely in fruits, vegetables, and beverages derived from plants and also in many botanical dietary supplements or herbal medicines. Owing to their various health promoting effects and favorable safety profiles, dietary flavonoids have gained great attention in terms of the discovery of effective P-gp inhibitors. Indeed, some of these appear to be quite promising to inhibit P-gp-mediated drug efflux, indicating their potential utility to enhance the cellular availability of anticancer drugs.[7,10−13] However, many natural products have poor bioavailability and show limited efficacy in vivo.[14] Therefore, there are still continuing efforts ongoing to discover promising lead compounds for P-gp inhibition among flavonoids.

Oroxylin A (**1**), 5,7-dihydroxy-6-methoxyflavone, is a constituent of the roots of *Scutellaria baicalensis* Georgi (Lamiaceae), which is used widely in traditional Chinese medicine.[15] Hu et al.[16] have revealed that **1** induced programmed cell death effectively and suggested that it could be a promising antitumor drug. Also, **1** has potential anti-inflammatory activity by inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via the suppression of NF-$\kappa$B activation.[17] Compound **1** shows structural features similar to baicalein, a P-gp inhibitor.[18] In a structural comparison of **1** with baicalein, both have a planar structure but **1** has a 6-methoxy group instead of 6-hydroxy group, which confers more hydrophobicity to **1**. Given that hydrophobicity and planar structures are important for inhibitory potency on P-gp-mediated drug efflux,[19] compound **1** could possibly exhibit a strong hydrophobic interaction with P-gp like certain other flavones.[18−20] However, **1** has not been studied in detail yet for its potential to overcome the multidrug resistance resulting from the P-gp-mediated active efflux of anticancer drugs. Therefore, the present study aimed to investigate the interaction characteristics of **1** with P-gp in multidrug resistant cancer cells as well as in a rat model.




## Results and Discussion

**Inhibitory Effect of Oroxylin A (1) on P-Glycoprotein-Mediated Drug Efflux.** Western blot analysis from several previous studies indicated that MCF-7 cells have no detectable level of P-gp, however, NCI/ADR-RES cells highly express P-gp.[21,22] Therefore, in the present study, MCF-7 and NCI/ADR-RES cells were used as the negative and the positive cells for P-gp-mediated efflux, respectively, although they have different origins.[23] The effect of **1** on the cellular accumulation of P-gp substrates, such as calcein AM and paclitaxel, was investigated in NCI/ADR-RES cells as well as MCF-7 cells. As illustrated in Figure 1, compound **1** increased the cellular accumulation of calcein AM in a concentration-dependent manner in NCI/ADR-RES cells over the concentration range 0−40 $\mu$M, implying that **1** might effectively inhibit the P-gp mediated cellular efflux of calcein AM. The $IC_{50}$ values were not determined due to the solubility limit of **1**. Calcein AM is also a substrate of multidrug resistance protein **1** (MRP1) in addition to P-gp.[24] However, the involvement of MRP1 in calcein AM accumulation in NCI/ADR-RES cells should be negligible because MRP1 is scarcely found in NCI/ADR-RES cells.[10,22] Similarly, the

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10.1021/np9003036 CCC: $40.75 
Published on Web 09/09/2009

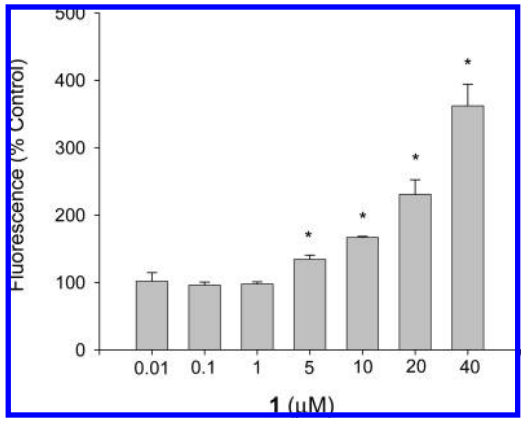


**Figure 1.** Effect of oroxylin A (**1**) on the cellular accumulation of calcein AM in NCI/ADR-RES cells (means ± SD, $n = 4$). *: $p < 0.05$ compared to the control group.

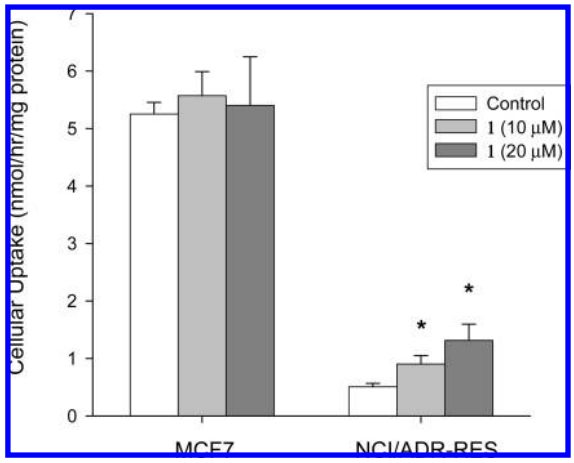


**Figure 2.** Effect of oroxylin A (**1**) on the paclitaxel accumulation in MCF-7 cells and NCI/ADR-RES cells (means ± SD, $n = 5$). *: $p < 0.05$ compared to the control group.

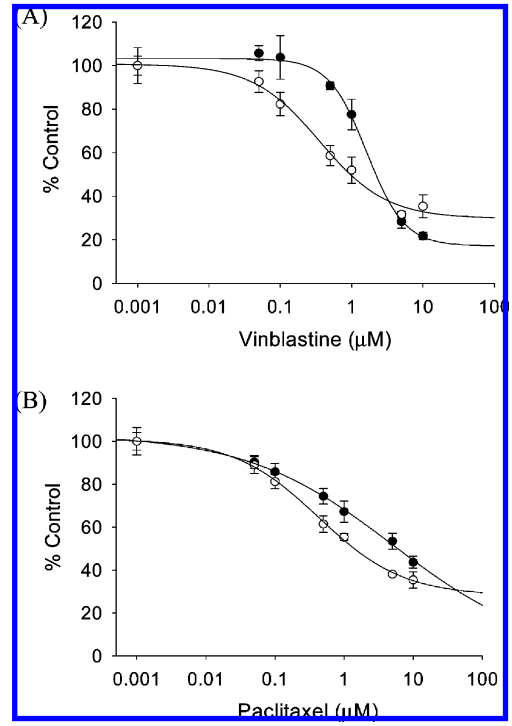


**Figure 3.** Cytotoxicity of vinblastine (A) and paclitaxel (B) in NCI/ADR-RES cells (means ± SD, $n = 10$). (○) with 10 μM of **1**; (●) without **1**.

**Table 1.** Pharmacokinetic Parameters of Paclitaxel after Oral Administration (15 mg·kg$^{-1}$) to Rats in the Presence and Absence of **1** (30 mg·kg$^{-1}$) (means ± SD, $n = 6$)

| parameter | paclitaxel only | paclitaxel + **1** |
|---|---|---|
| $C_{max}$ (ng·mL$^{-1}$) | 17.9 ± 6.51 | 37.7 ± 9.55[a] |
| $T_{max}$ (h) | 0.5 ± 0.1 | 0.5 |
| $AUC_{last}$ (ng·h·mL$^{-1}$) | 135 ± 41.4 | 352 ± 96.5[a] |
| $F_{rel}$ (%) | 100 | 261 |

[a] $p < 0.05$ compared to the control group given paclitaxel alone.

cellular accumulation of paclitaxel was also increased significantly ($p < 0.05$) by the concomitant use of **1** in NCI/ADR-RES cells, while there was no change in MCF-7 cells lacking P-gp[21,22] (Figure 2). These results suggest that **1** is effective in reducing P-gp-mediated drug efflux and may be useful to overcome the P-gp mediated multidrug resistance of cancer cells.

The interaction mechanisms of flavonoids with P-gp have not yet been clearly defined. So far, several potential mechanisms have been proposed as follows:[10,25,26] (i) flavonoids modulate P-gp by interacting with the vicinal ATP-binding site and the steroid binding site, (ii) flavonoids may interact with P-gp directly either by competitive binding to the substrate-binding site or by binding to other drug-binding sites and changing protein conformation, and (iii) flavonoids may bind to the allosteric site or other binding sites. The elucidation of the exact interaction mechanisms of flavonoids with P-gp is difficult due to the complexity of interaction characteristics between P-gp and its modulators and also because different flavonoids may interact with P-gp differently. So far, the underlying mechanism of P-gp interaction with **1** is not clear and should be further investigated in a future study.

**Effect of Oroxylin A (1) on the Cytotoxicity of Vinblastine and Paclitaxel.** The cytotoxicity of anticancer drugs such as paclitaxel and vinblastine was determined in the presence and the absence of **1** in NCI/ADR-RES cells. Compound **1** alone was not cytotoxic over the tested concentrations. As shown in Figure 3, the 50% cytotoxic concentration ($CC_{50}$) was reduced from 1.63 to 0.36 μM for vinblastine and from 5.11 to 1.02 μM for paclitaxel, in the presence of **1**. Those results suggest that the net influxes of vinblastine and paclitaxel were enhanced significantly by coincubation with **1**, and consequently cells became more susceptible to the cytotoxicity of vinblastine and paclitaxel in the presence of **1**.

**Effect of Oroxylin A (1) on the Pharmacokinetics of Paclitaxel in Rats.** The pharmacokinetic profiles of paclitaxel after the oral administration of paclitaxel (15 mg·kg$^{-1}$) in the presence and the absence of **1** (30 mg·kg$^{-1}$) were characterized in rats, and the mean pharmacokinetic parameters are summarized in Table 1. As shown in Table 1, combined use of **1** with paclitaxel altered the oral pharmacokinetics of paclitaxel significantly when compared to the control group given paclitaxel alone. The $C_{max}$ and AUC values of oral paclitaxel were increased by 2.1−2.6-fold in the presence of **1**, while there was no significant change in $T_{max}$. Consequently, the relative bioavailability of paclitaxel was improved significantly ($p < 0.05$) in rats as a result of the combined use of **1**.

A previous study has demonstrated that about 54% of a paclitaxel oral dose is extruded to the gut lumen by P-gp.[27] Moreover, studies using *mdr 1a*(−/−) mice have demonstrated directly that P-gp strictly limits the uptake from the intestinal tract of paclitaxel administered orally.[28] Therefore, given that the major factor leading to the low bioavailability of orally administered paclitaxel is considered to be the active efflux by P-gp within the intestinal tract, the enhanced oral exposure of paclitaxel in the presence of oroxylin A (**1**) could be due mainly to the increased intestinal absorption

via the inhibition of P-gp by **1**. This pharmacokinetic observation is consistent with our in vitro observation in multidrug-resistant cancer cells and also comparable to previous reports.[11,13]

Collectively, the in vitro and in vivo data obtained from the present study strongly suggest that **1** may be effective in reducing P-gp-mediated drug efflux. Given that after oral administration, a drug concentration is higher in the intestinal lumen than in the plasma, particularly for poorly absorbable or highly metabolized drugs, the drug concentration needed for P-gp inhibition may be more readily achievable in the intestine than in the plasma. Therefore, orally dosed **1** may have a greater impact on the intestinal absorption of P-gp substrates, as is supported also by the present pharmacokinetic study in rats. However, the plasma concentration of **1** after an oral administration would not be high enough to interact with P-gp in cancer cells. In that case, intravenous administration of **1** with anticancer drugs may be an alternative way to overcome P-gp-mediated multidrug resistance.

## Experimental Section

**General Experimental Procedures.** Calcein-AM, vinblastine, paclitaxel, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and BCA protein assay kit were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), cell culture media, antibiotics, and all other reagents used in cell culture studies were purchased from Seolin Science Company (Seoul, Korea). Oroxylin A (**1**) was provided by Dr. Kun Ho Son (Andong National University, Korea), and its purity was 99.9%. NCI/ADR-RES cells were obtained from National Cancer Institute (Frederick, MD). All other chemicals were of reagent grade, and all solvents were of HPLC grade. Cells were routinely maintained in RPMI 1640 culture medium containing 10% FBS and penicillin (50 IU·mL$^{-1}$)/streptomycin (50 $\mu$g·mL$^{-1}$). All cells were maintained in an atmosphere of 5% $CO_2$ and 90% relative humidity at 37 °C.

**Effect of Oroxylin A (1) on P-Glycoprotein-Mediated Drug Efflux.** Cells were seeded into 24-well plates at a density of $10^5$ cells·cm$^{-2}$ and incubated at 37 °C for two days. After removing the medium, cells were washed twice with 1 mL of uptake buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 5 mM D-glucose, 1 mM $NaH_2PO_4$, 10 mM MES, pH 6.5) and then incubated with drug solutions [calcein-AM (2.5 $\mu$M) or paclitaxel (5 $\mu$M)] in the presence and the absence of **1** (0−40 $\mu$M). After 30 min incubation at 37 °C, each solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline (PBS). In the case of calcein-AM, the cells were lysed with 1% Triton X-100 and supernatants were transferred into a 96-well fluorescence plate. Fluorescence intensity of each sample was determined using a spectrophotometer (excitation 496 nm and emission 516 nm). In the case of paclitaxel, the cellular concentration was examined by HPLC. After the cell lysis, cells were harvested and sonicated for 1−2 min. Acetonitrile was added to the cell lysate, vortexed rigorously, and centrifuged for 5 min at 3000 rpm. After the filtration of the supernatant through a membrane filter (0.45 $\mu$m), the drug concentration of each sample was determined by HPLC. The protein amount of each sample was determined using a BCA protein assay kit following the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO).

**Effect of Oroxylin A (1) on the Cytotoxicity of Vinblastine and Paclitaxel.** The resistance of NCI/ADR-RES cells to vinblastine and paclitaxel cytotoxicity was determined in the presence and the absence of **1**. Cells were seeded into 96-well plates at a density of $5 \times 10^3$ cells·well$^{-1}$. After 24 h incubation at 37 °C, various concentrations of vinblastine and paclitaxel with or without **1** (10 $\mu$M) were added to the cells and the plates were incubated for three days. At the end of incubation, cell viability was determined by a modified colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).[29] Briefly, medium with the drug was removed and replaced by fresh medium (200 $\mu$L·well$^{-1}$) containing 0.1 mg·mL$^{-1}$ MTT. After 4 h incubation at 37 °C, the medium was aspirated and the cells were extracted with 150 $\mu$L·well$^{-1}$ of DMSO. The concentration of the extracted formazan metabolite was determined by the measurement of absorbance at 560 nm in a 96-well plate reader. The 50% cytotoxic concentration ($CC_{50}$) was determined from the nonlinear regression of a dose−response curve using Sigma Plot 9.0 (Systat Software Inc., Point Richmond, CA).

**Effect of Oroxylin A (1) on the Pharmacokinetics of Paclitaxel in Rats.** Pharmacokinetic profiles of paclitaxel were determined after the oral administration of paclitaxel (15 mg·kg$^{-1}$) to rats in the presence and the absence of **1** (30 mg·kg$^{-1}$). All animal studies were performed in accordance with the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA). Male Sprague−Dawley rats (280−300 g) were purchased from Samtako Bio Company (Osan, Korea) and had free access to normal standard chow diet (Superfeed Company, Wonju, Korea) and tap water. Rats were fasted for 24 h prior to the beginning of experiments. Rats ($n = 6$ per each treatment) were divided into two groups: group 1 (15 mg·kg$^{-1}$ paclitaxel, po) and group 2 (15 mg·kg$^{-1}$ paclitaxel + 30 mg·kg$^{-1}$ compound **1**, po). Dosing suspensions of paclitaxel and **1** were prepared using 10% Tween 80 in 0.5% methylcellulose solution, and the dosing volume was 1 mL per each animal. Blood samples were collected from the femoral artery at 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, and 24 h postdose. Blood samples were centrifuged at 13000 rpm for 5 min, and the plasma obtained was stored at −40 °C until being analyzed.

**HPLC Analysis.** The chromatographic system consisted of a pump (LC-10AD), an automatic injector (SIL-10A), and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Tokyo, Japan). Cellular and plasma concentrations of paclitaxel were determined by a HPLC assay described below. Atorvastatin was used as an internal standard for the assay. For cellular concentrations, an octadecylsilane column (Gemini $C_{18}$, 4.6 mm × 250 mm, 5 $\mu$m; Phenomenex, Torrance, CA) was eluted with a mobile phase [0.05 M phosphate buffer−acetonitrile−methanol (48:42.6:9.4)] and the column was maintained at 40 °C. The flow rate was 1.0 mL·min$^{-1}$ with the detection wavelength set at 229 nm. For plasma concentrations of paclitaxel, 4 mL of *tert*-butyl methyl ether was added into each sample. After rigorous vortexing, each sample was centrifuged at 13000 rpm for 15 min and the supernatant was evaporated under a vacuum. The residue was reconstituted with mobile phase, and then a 50 $\mu$L aliquot was injected into the HPLC system. An octadecylsilane column (Gemini $C_{18}$, 4.6 mm × 150 mm, 5 $\mu$m; Phenomenex) was eluted with a mobile phase [0.05 M phosphate buffer−acetonitrile−methanol (60:41:9)], and the column was maintained at 40 °C. The flow rate was 1.2 mL·min$^{-1}$, with the detection wavelength set at 229 nm.

**Pharmacokinetic Analysis.** Noncompartmental analysis was performed by using WinNonlin software version 5.2.1 (Pharsight Co., Mountain View, CA). The area under the plasma concentration−time curve (AUC) was calculated using the linear trapezoidal method. The peak plasma concentration ($C_{max}$) and the time to reach the peak plasma concentration ($T_{max}$) were observed values from the experimental data. The relative bioavailability ($F_{rel}$) of paclitaxel was estimated by AUC(paclitaxel+**1**)/AUC(paclitaxel only) × 100.

**Statistical Analysis.** All the means obtained are presented with their standard deviation. The statistical significance of the difference in the parameters was determined using ANOVA followed by a Dunnett's post hoc test or by a Student's *t* test. A $p$ value <0.05 was considered statistically significant.

**Acknowledgment.** The authors thank Dr. Kun Ho Son (Andong National University, Korea) for providing oroxylin A. This work was supported by the Pioneer Research Program for Converging Technology of the Ministry of Education, Science and Technology, Republic of Korea (grant no. M1071118001-08M1118-00110).

## References and Notes

(1) Leonard, G. D.; Fojo, T.; Bates, S. E. *Oncologist* **2003**, *8*, 411–424.
(2) Dean, M.; Allikmets, R. *J. Bioenerg. Biomembr.* **2001**, *33*, 475–479.
(3) Thiebaut, F.; Tsuruo, T.; Hamada, H.; Gottesman, M. M.; Pastan, I.; Willingham, M. C. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7735–7738.
(4) Marchetti, S.; Mazzanti, R.; Beijnen, J. H.; Schellens, J. H. *Oncologist* **2007**, *12*, 927–941.
(5) Boumendjel, A.; Di Pietro, A.; Dumontet, C.; Barron, D. *Med. Res. Rev.* **2002**, *22*, 512–529.
(6) Deferme, S.; Augustijns, P. *J. Pharm. Pharmacol.* **2003**, *55*, 153–162.
(7) Morris, M. E.; Zhang, S. *Life Sci.* **2006**, *78*, 2116–2130.
(8) Scambia, G.; Ranelletti, F. O.; Panici, P. B.; De Vincenzo, R.; Bonanno, G.; Ferrandina, G.; Piantelli, M.; Bussa, S.; Rumi, C.; Cianfriglia, M.; Mancuso, S. *Cancer Chemother. Pharmacol.* **1994**, *34*, 459–464.
(9) Castro, A. F.; Altenberg, G. A. *Biochem. Pharmacol.* **1997**, *53*, 89–93.

(10) Zhang, S.; Morris, M. E. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1258–1267.
(11) Choi, B. C.; Choi, J. S.; Han, H. K. *Int. J. Pharm.* **2006**, *323*, 81–85.
(12) Dupuy, J.; Larrieu, G.; Sutra, J. F.; Lespine, A.; Alvinerie, M. *Vet. Parasitol.* **2003**, *112*, 337–347.
(13) Choi, J. S.; Choi, H. K.; Shin, S. C. *Int. J. Pharm.* **2004**, *275*, 165–170.
(14) Yang, C. S.; Sang, S.; Lambert, J. D.; Lee, M. J. *Mol. Nutr. Food Res.* **2008**, *52*, S139–151.
(15) Huen, M. S.; Leung, J. W.; Ng, W.; Lui, W. S.; Chan, M. N.; Wong, J. T.; Xue, H. *Biochem. Pharmacol.* **2003**, *66*, 125–132.
(16) Hu, Y.; Yang, Y.; You, Q. D.; Liu, W.; Gu, H. Y.; Zhao, L.; Zhang, K.; Wang, W.; Wang, X. T.; Guo, Q. L. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 521–527.
(17) Chen, Y.; Yang, L.; Lee, T. J. *Biochem. Pharmacol.* **2000**, *59*, 1445–1457.
(18) Lee, Y.; Yeo, H.; Liu, S. H.; Jiang, Z.; Savizky, R. M.; Austin, D. J.; Cheng, Y. C. *J. Med. Chem.* **2004**, *47*, 5555–5566.
(19) Kitagawa, S.; Nabekura, T.; Takahashi, T.; Nakamura, Y.; Sakamoto, H.; Tano, H.; Hirai, M.; Tsukahara, G. *Biol. Pharm. Bull.* **2005**, *28*, 2274–2278.
(20) Patanasethanont, D.; Nagai, J.; Yumoto, R.; Murakami, T.; Sutthanut, K.; Sripanidkulchai, B. O.; Yenjai, C.; Takano, M. *J. Pharm. Sci.* **2007**, *96*, 223–233.
(21) Han, C. Y.; Cho, K. B.; Choi, H. S.; Han, H. K.; Kang, K. W. *Carcinogenesis* **2008**, *29*, 1837–1844.
(22) Zhang, S.; Yang, X.; Morris, M. E. *Mol. Pharmacol.* **2004**, *65*, 1208–1216.
(23) Liscovitch, M.; Ravid, D. *Cancer Lett.* **2007**, *245*, 350–352.
(24) Essodaigui, M.; Broxterman, H. J.; Garnier-Suillerot, A. *Biochemistry* **1998**, *24*, 2243–2250.
(25) Conseil, G.; Baubichon-Cortay, H.; Dayan, G.; Jault, J. M.; Barron, D.; Di Pietro, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9831–9836.
(26) Shapiro, A. B.; Ling, V. *Eur. J. Biochem.* **1997**, *250*, 130–137.
(27) Woo, J. S.; Lee, C. H.; Shim, C. K.; Hwang, S. J. *Pharm. Res.* **2003**, *20*, 24–30.
(28) Sparreboom, A.; van Asperen, J.; Mayer, U.; Schinkel, A. H.; Smit, J. W.; Meijer, D. K.; Borst, P.; Nooijen, W. J.; Bejinen, J. W.; van Tellingen, O. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2031–2035.
(29) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 943–946.

NP9003036