The Novel Anticancer Drug KRN5500 Interacts with, but is Hardly Transported by, Human P-Glycoprotein

Kohji Takara,1 Yusuke Tanigawara,2 Fusao Komada,1, 4 Kohshi Nishiguchi,1 Toshiyuki Sakaeda1 and Katsuhiko Okumura1, 3

1Department of Hospital Pharmacy, School of Medicine, Kobe University, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017 and 2Department of Hospital Pharmacy, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582

The interaction of the novel anticancer drug KRN5500, a spicamycin derivative, with human P-glycoprotein (P-gp) was analyzed from the viewpoint of cellular pharmacokinetics, i.e. by means of [3H]azidopine photoaffinity labeling, cellular accumulation and transcellular transport experiments. In this study, P-gp-overexpressing LLC-GA5-COL150 cells, porcine kidney epithelial LLC-PK1 cells transformed with human MDR1 cDNA, were used, since this cell line constructs monolayers with tight junctions, and would provide sufficient information for analyzing the cellular pharmacokinetics. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that the growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells was comparable to that in LLC-PK1 cells (IC50 = 79.4 and 72.7 nM, respectively), but the inhibition of [3H]azidopine binding by KRN5500 was concentration-dependent in the membrane fraction of LLC-GA5-COL150 cells. The cellular accumulation of [14C]KRN5500 after its basal application in LLC-GA5-COL150 cells was slightly lower than that in LLC-PK1 cells, and was restored by the multidrug resistance (MDR) modulator SDZ PSC 833. The basal-to-apical transport of [14C]KRN5500 in LLC-GA5-COL150 cells was also slightly higher than that in LLC-PK1 cells, and was inhibited by SDZ PSC 833. However, the basal-to-apical transport of [14C]KRN5500 in LLC-GA5-COL150 cells was only a little higher than the apical-to-basal transport. Consequently, these results demonstrated that KRN5500 interacted with, but was hardly transported via, P-gp. These observations suggested that KRN5500 may be useful even for the treatment of tumors exhibiting P-gp-mediated MDR.

Key words: KRN5500 — P-Glycoprotein — Multidrug resistance — Cellular pharmacokinetic analysis

The novel anticancer drug KRN5500 (6-[4-deoxy-4-(2E,4E)-tetradecadienoylglycyl]amino-L-glycero-β-L-manno-heptopyranosyl][amino-9H-purine), developed in 1993, has a unique molecular structure not belonging to any category of anticancer drugs currently in clinical use (Fig. 1).1, 2) KRN5500 was demonstrated to be highly active in a number of experimental solid tumors, including stomach, colon and esophageal cancer.3, 4) KRN5500 exerts its growth-inhibitory effect after conversion to the active metabolite 4′-N-glycylspicamycin aminonucleoside (SAN-Gly) in the intracellular space of tumor cells.3, 5) SAN-Gly inhibits protein synthesis, a mechanism of action different from those of other anticancer drugs.3, 5, 6) Currently, KRN5500 is under clinical investigation in Japan and the USA.7)

Multidrug resistance (MDR) is one of the most serious problems responsible for the failure of chemotherapy. A well-characterized cellular phenotype of MDR is mediated by the multidrug transporter P-glycoprotein (P-gp), and P-gp is known to be frequently acquired in patients given chemotherapy.8) P-gp, which is encoded by the MDR1 gene, expels drugs from cells by utilizing the energy of ATP hydrolysis, and it has been found to be an efflux pump with broad substrate specificity.9, 10) It is important to determine whether anticancer drugs are substrates for P-gp, since substrates can be unexpectedly ineffective in such patients. The substrates for P-gp include vinblastine11, 12) and doxorubicin,12) as well as the cardiac glycoside digoxin13, 14) and the immunosuppressive agent cyclosporin A.15) It has been demonstrated in vitro that the cellular accumulation of vinblastine and doxorubicin is reduced11, 12) and they are not effective in P-gp-expressing MDR cells. Even for drugs that are substrates for P-gp, it should be clarified whether they are indeed transported by P-gp. Anticancer drugs that are not transported by P-gp will show little reduction in cellular accumulation in multidrug-resistant cells, and may therefore be effective in such cells. The MDR modulator PSC833,12, 16) Ca2+ antagonist nitrendipine17) and the steroid hormone proge-
sterone\textsuperscript{18,19} are known not to be transported by P-gp, resulting in comparable levels of cellular accumulation in multidrug-resistant and sensitive cells.

It has been demonstrated that the growth-inhibitory effect of KRN5500 in mouse leukemia P388 cells is comparable to that in doxorubicin-, vincristine- and mitomycin C-resistant P388 cells.\textsuperscript{53} In contrast, Lee et al. reported that cisplatin-resistant human lung cancer cells showed higher sensitivity to KRN5500 as compared with sensitive cells.\textsuperscript{20} Although these reports suggested that KRN5500 is not a substrate for P-gp, there is no direct evidence, and there is no information on the cellular kinetics of KRN5500, i.e., whether or not it is transported. Such information is necessary to determine appropriate dosage regimens of KRN5500.

Previously, we established the LLC-GA5-COL150 cell line, the cells of which express human P-gp on the apical membranes, by transfection of human \textit{MDR1} cDNA into LLC-PK\textsubscript{1} cells.\textsuperscript{11–15, 17, 18, 21, 22} This cell line is a useful tool for identification of the substrates for P-gp and moreover for quantitative characterization of transport via P-gp.\textsuperscript{11, 15–17, 18, 21, 22} Here, the interaction of KRN5500 with human P-gp was investigated using LLC-GA5-COL150 cells in addition to the parental cell line LLC-PK\textsubscript{1}. \textsuperscript{[3H]}Azidopine photoaffinity labeling, cellular accumulation and transcellular transport were examined.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chemical_structure}\caption{Chemical structure of KRN5500.}
\end{figure}

**Materials and Methods**

Chemicals \textsuperscript{[14C]}KRN5500 (1.28 GBq/mmol) and non-labeled KRN5500 were kind gifts from Kirin Brewery Co., Ltd. (Tokyo). \textsuperscript{[3H]}Azidopine (1.81 TBq/mmol), \textsuperscript{[3H]}vinblastine (422 GBq/mmol), \textsuperscript{[3H]}inulin (25.2 GBq/mmol) and \textsuperscript{[methoxy-14C]}inulin (308 MBq/mmol) were obtained from Amersham International, plc (Buckinghamshire, UK). PSC833 was kindly supplied by Novartis Pharma (Basel, Switzerland). Colchicine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and vinblastine sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were of the highest purity available.

**Cells and cell culture** LLC-GA5-COL150 cells were obtained by transfection of human \textit{MDR1} cDNA into porcine kidney epithelial LLC-PK\textsubscript{1} cells, followed by culturing in the presence of 150 ng/ml colchicine.\textsuperscript{13,14} Cells were maintained in Medium 199 (Dainippon Pharmaceutical Co., Ltd., Osaka) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and 150 ng/ml of colchicine for LLC-GA5-COL150 cells without antibiotics.

**Growth inhibition assay** The growth-inhibitory effects of KRN5500 in LLC-GA5-COL150 and LLC-PK\textsubscript{1} cells were determined by colorimetric assay using MTT reagent.\textsuperscript{18, 23, 24} Cells (3–5×10\textsuperscript{5} cells/well) were seeded onto microporous polycarbonate membrane filters (Transwell\textsuperscript{3414}, Costar, Cambridge, MA) at confluent density, by transfection of human \textit{MDR1} cDNA into porcine kidney epithelial LLC-PK\textsubscript{1} cells.\textsuperscript{11–15, 17, 18, 21, 22} The membrane fractions of the cells were obtained by sucrose gradient centrifugation after breaking up the cells by nitrogen cavitation (Mini-Bomb cell disruption chamber, Kontes Glass Co., Vineland, NJ). Membrane fractions (20 µg of protein) were reacted with \textsuperscript{[3H]}azidopine (0.4 µM, 37 kBq) in the presence or absence of the indicated concentration of KRN5500 or vinblastine at room temperature for 20 min. The reaction mixtures were then irradiated with a UV lamp (Black Ray Type XX-15L, Ultra-Violet Products, Upland, CA) for 30 min on ice. Photolabeled protein (20 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels (\textit{PAGE}, Amersham International). The gels were fixed, treated with fluorographic reagent “Amplify” (Amersham International), dried, and then exposed for 10 days at −80°C using “Hyperfilm” MP (Amersham International).

**Cellular accumulation and transcellular transport of \textsuperscript{14C}KRN5500** Cellular accumulation and transcellular transport of \textsuperscript{[14C]}KRN5500 (1.4 µM, 3.7 kBq) and \textsuperscript{[3H]}vinblastine (100 nM, 37 kBq) were determined as described previously.\textsuperscript{11–15, 17, 18, 21, 22} The transcellular transport assay system using LLC-GA5-COL150 and LLC-PK\textsubscript{1} cells is shown schematically in Fig. 2. The cells were seeded onto microporous polycarbonate membrane filters (“Transwell” 3414, Costar, Cambridge, MA) at confluent cell density and incubated for 3 days. The culture medium was replaced by fresh medium 3 h before the experiments. The medium on the basal or apical side of the monolayer was replaced with medium containing \textsuperscript{[14C]}KRN5500 and \textsuperscript{[3H]}vinblastine together with \textsuperscript{[3H]}inulin (0.73 µM, 37 kBq) and [methoxy-\textsuperscript{14C}]inulin (6 µM, 3.7 kBq), respectively.
and aliquots of the medium on the opposite side were taken at the indicated times. To confirm the formation of tight junctions in the monolayers, paracellular leakage was monitored using radiolabeled inulin, which is a marker of paracellular transport. To examine cellular accumulation, immediately after the last sampling the cells were rapidly washed twice with ice-cold phosphate-buffered saline and lysed in 1 ml of 0.3 N NaOH. PSC833 (2 µM) was added to the medium on both sides of the cell monolayer 1 h before adding [14C]KRN5500 or [3H]vinblastine, and was also contained in the incubation medium throughout the experiments to examine its effects. The levels of radioactivity of the collected media and the lysed cells were counted in 3 ml of ACS II (Amersham International) by liquid scintillation counting (LSC-5100, Aloka Co., Ltd., Tokyo).

**Protein assay** Protein content was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA), with bovine γ-globulin as the standard.

**Statistical analysis** Statistical analysis of the data was performed by one-way analysis of variance followed by Scheffe’s test. Statistical significance was defined as \( P < 0.05 \).

### RESULTS

**Growth-inhibitory effect of KRN5500** The growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells...
was comparable to that in LLC-PK₁ cells (Fig. 3), with IC₅₀ values of 79.4 and 72.7 nM, respectively, suggesting that KRN5500 was not transported by P-gp.

Inhibitory effect of KRN5500 on [³H]azidopine photoaffinity labeling of P-gp

To examine the interaction of KRN5500 with P-gp, the binding ability of KRN5500 for P-gp was examined in terms of the inhibition of [³H]azidopine photoaffinity labeling of P-gp (Fig. 4). [³H]Azidopine binding was observed only in the membrane fractions of LLC-GA5-COL150 cells, but not in LLC-PK₁ cells. Inhibition by KRN5500 was concentration-dependent; i.e., 40 μM KRN5500 showed partial, while 400 μM showed almost complete inhibition of [³H]azidopine binding.

Cellular accumulation of [¹⁴C]KRN5500

The cellular accumulation of [¹⁴C]KRN5500 in LLC-GA5-COL150 and LLC-PK₁ cells was examined following its application on the basal side as well as the apical side (Table I). The cellular accumulation of [¹⁴C]KRN5500 in LLC-GA5-COL150 cells was slightly lower than that in LLC-PK₁ cells after basal application, and co-administration of PSC833 restored it. For apical application, the cellular accumulation of [¹⁴C]KRN5500 in LLC-GA5-COL150 cells was ca. 3-fold lower than that in LLC-PK₁ cells, and PSC833 had no effect on it.

Table I. Cellular Accumulation of [¹⁴C]KRN5500 and [³H]Vinblastine in LLC-GA5-COL150 and LLC-PK₁ Cells

| Treatment            | KRN5500 (pmol/mg protein/24 h) | Vinblastine (pmol/mg protein/3 h) |
|----------------------|--------------------------------|----------------------------------|
|                      | Basal apply | Apical apply | Basal apply | Apical apply |
| LLC-PK₁ cells        | 41.0±0.7    | 61.7±9.2     | 48.8±1.2    | 37.2±1.8     |
| LLC-GA5-COL150 cells | 27.8±1.0 b   | 19.8±1.4 b   | 2.8±0.7 b   | 0.5±0.1 b   |
| + 2 μM PSC833        | 41.0±2.1 e   | 13.9±0.8 b   | 28.8±5.4 e   | 25.0±6.2 e   |

a) and b) P<0.05 and P<0.001 significantly different from values of LLC-PK₁ cells with the same treatment, respectively.

Cellular accumulation of [¹⁴C]KRN5500 in LLC-GA5-COL150 cells was ca. 3-fold lower than that in LLC-PK₁ cells, and PSC833 had no effect on it.

Table II. Transcellular Transport of [¹⁴C]KRN5500 and [³H]Vinblastine in LLC-GA5-COL150 and LLC-PK₁ Cells

| Treatment            | KRN5500 (% of dose at 24 h) | Vinblastine (% of dose at 3 h) |
|----------------------|-----------------------------|--------------------------------|
|                      | B-to-A b                     | A-to-B                         | B-to-A                     | A-to-B                         |
| LLC-PK₁ cells        | 4.8±0.1                     | 13.3±2.6                      | 14.4±0.1                   | 9.3±0.2                       |
| LLC-GA5-COL150 cells | 6.8±0.2 c                   | 5.4±0.1 c                     | 21.6±1.3 c                 | 1.9±0.3 c                    |
| + 2 μM PSC833        | 5.3±0.1 c                   | 5.0±0.2 b                     | 11.6±1.6 c                 | 7.0±0.9 b, c                 |

a) B-to-A and A-to-B represent the basal-to-apical transport and the apical-to-basal transport, respectively.

Values represent the mean±SE of four to eight independent experiments.
The transcellular transport of \([^{14}\text{C}]\text{KRN5500}\) after application on the basal side as well as the apical side of LLC-GA5-COL150 and LLC-PK1 cell monolayers was also examined (Table II). The transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells as well as LLC-PK1 cells was time-dependent with almost no alteration of the transport rate up to 24 h (Fig. 5). The basal-to-apical transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells was slightly higher than that in LLC-PK1 cells. PSC833 had a little effect on the basal-to-apical transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells. However, the basal-to-apical transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells was only a little higher than the apical-to-basal transport, resulting in almost no directional transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells. The apical-to-basal transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-PK1 cells was markedly higher than the basal-to-apical transport, resulting in directional transport from the apical to the basal side.

**DISCUSSION**

To determine the optimal dosage regimen of anticancer drugs, it is helpful to clarify whether the anticancer drug is a substrate for P-gp, and moreover whether it is transported by P-gp. Here, the interaction of KRN5500 with P-gp was investigated using LLC-GA5-COL150 cells. The results for KRN5500 were compared with those for vinblastine, which is a substrate for and is transported by P-gp.12)

The growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells was comparable to that in LLC-PK1 cells, with IC50 values of 79.4 and 72.7 nM, respectively, suggesting that KRN5500 was not transported by P-gp. This was different from the case of vinblastine, the IC50 value of which in LLC-GA5-COL150 cells was about 90-fold higher than that in LLC-PK1 cells (697 and 8 nM, respectively). However, KRN5500 inhibited the \([^{3}\text{H}]\text{azidopine}\) binding in a concentration-dependent manner in the plasma membrane fraction derived from LLC-GA5-COL150 cells, although its binding affinity for P-gp was weaker than that of vinblastine, suggesting that KRN5500 was a substrate for P-gp. These results suggested that KRN5500 was a substrate but not transported by P-gp.12)

Fig. 5. Time course of transcellular transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 and LLC-PK1 cells (A) and effect of PSC833 on transcellular transport of \([^{14}\text{C}]\text{KRN5500}\) in LLC-GA5-COL150 cells (B). A: The open symbols (○, △) indicate LLC-PK1 cells, and closed symbols (□, ▲) show LLC-GA5-COL150 cells. B: The open symbols (○, △) indicate LLC-GA5-COL150 cells, and closed symbols (□, ▲) show LLC-GA5-COL150 cells in the presence of PSC833 (2 µM). The circles indicate the basal-to-apical transport and the triangles show the apical-to-basal transport. Each point represents the mean±SE of four to eight independent experiments.
nitrendipine\(^\text{17}\) and progesterone,\(^\text{18, 19}\) all of which are substrates for, but are not transported by P-gp.

It was unexpected that the cellular accumulation of \(^{14}\text{C}\)KRN5500 after its apical application in LLC-GA5-COL150 cells was markedly lower (ca. 3-fold) than that in LLC-PK\(_1\) cells. The apical-to-basal transport of \(^{14}\text{C}\)KRN5500 in LLC-PK\(_1\) cells was about 2.5-fold higher than that in LLC-GA5-COL150 cells, although the precise mechanism of this directional transport remains unclear. This could be explained by the increase in the incorporation of \(^{14}\text{C}\)KRN5500 into LLC-PK\(_1\) cells due to its higher transport from the apical side.

Our results demonstrated that the novel anticancer drug KRN5500 interacted with, but was hardly transported by human P-gp. These results suggested that KRN5500 may be useful even for the treatment of tumor cells exhibiting P-gp-associated MDR.

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REFERENCES

1) Kamishohara, M., Kawai, H., Odagawa, A., Isoe, T., Mochizuki, J., Uchida, T., Hayakawa, Y., Seto, H., Tsuruo, T. and Otake, N. Structure–antitumor activity relationship of semi-synthetic spicamycin analogues. *J. Antibiot. (Tokyo)*, 46, 1439–1446 (1993).

2) Sakai, T., Kawai, H., Kamishohara, M., Odagawa, A., Suzuki, A., Uchida, T., Kawasaki, T., Tsuruo, T. and Otake, N. Structure–antitumor activity relationship of semisynthetic spicamycin derivatives. *J. Antibiot. (Tokyo)*, 48, 1467–1480 (1995).

3) Kamishohara, M., Kawai, H., Sakai, T., Isoe, T., Hasegawa, K., Mochizuki, J., Uchida, T., Kataoka, S., Yamaki, H., Tsuruo, T. and Otake, N. Antitumor activity of a spicamycin derivative, KRN5500, and its active metabolite in tumor cells. *Oncol. Res.*, 6, 383–390 (1994).

4) Kamishohara, M., Kawai, H., Sakai, T., Uchida, T., Tsuruo, T. and Otake, N. Inhibitory effect of a spicamycin derivative, KRN5500, on the growth of hepatic metastasis of human colon cancer-producing tissue polypeptide antigen. *Cancer Chemother. Pharmacol.*, 38, 495–498 (1996).

5) Kawai, H. Protein synthesis inhibitor—antitumor activity and mode of action of KRN5500. *Jpn. J. Cancer Chemother.*, 24, 1571–1577 (1997) (in Japanese).

6) Burger, A. M., Kaur, G., Hollingshead, M., Fischer, R. T., Nagashima, K., Malspeis, L., Duncan, K. L. K. and Sausville, E. A. Antiproliferative activity in vitro and in vivo of the spicamycin analogue KRN5500 with altered glycoprotein expression in vitro. *Clin. Cancer Res.*, 3, 455–463 (1997).

7) Saigo, N. New chemotherapeutic agents for the treatment of non-small cell lung cancer: the Japanese experience. *Chest*, 113, 175–23S (1998).

8) Lehnherr, M. Clinical multidrug resistance in cancer: a multifactorial problem. *Eur. J. Cancer*, 32A, 912–920 (1996).

9) Endicott, J. A. and Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.*, 58, 137–171 (1989).

10) Gottesman, M. M. and Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, 62, 385–427 (1993).

11) Tanaka, K., Hirai, M., Tanigawara, Y., Yasuhara, M., Hori, R., Ueda, K. and Inui, K. Effect of cyclosporin analogues and FK506 on transcellular transport of daunorubicin and vinblastine via P-glycoprotein. *Pharm. Res.*, 13, 1073–1077 (1996).

12) Kusunoki, N., Takara, K., Tanigawara, Y., Yamauchi, A., Ueda, K., Komada, F., Yu, K., Kuroda, Y., Saitoh, Y. and Okumura, K. Inhibitory effects of a cyclosporin derivative, SDZ PSC 833, on transport of doxorubicin and vinblastine via human P-glycoprotein. *Jpn. J. Cancer Res.*, 89, 1220–1228 (1998).

13) Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK\(_1\)). *J. Pharmacol. Exp. Ther.*, 263, 840–845 (1992).

14) Okamura, N., Hirai, M., Tanigawara, Y., Tanaka, K., Yasuhara, M., Ueda, K., Komano, T. and Hori, R. Digoxin-cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney. *J. Pharmacol. Exp. Ther.*, 266, 1614–1619 (1993).

15) Saeki, T., Ueda, K., Tanigawara, Y., Hori, R. and Komano, T. Human P-glycoprotein transports cyclosporin A and FK506. *J. Biol. Chem.*, 268, 6077–6080 (1993).

16) Naito, M., Watanabe, T., Tsuge, H., Koyama, T., Oh-hara, T. and Tsuruo, T. Potentiation of the reversal activity of SDZ PSC833 on multi-drug resistance by an anti-P-glycoprotein monoclonal antibody MRK-16. *Int. J. Cancer*, 67, 435–440 (1996).

17) Saeki, T., Ueda, K., Tanigawara, Y., Hori, R. and Komano, T. P-glycoprotein-mediated transcellular transport of MDR-reversing agents. *FEBS Lett.*, 324, 99–102 (1993).

18) Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T. and Hori, R. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J. Biol. Chem.*, 267, 24248–
19) Barnes, K. M., Dickstein, B., Cutler, G. B., Jr., Fojo, T. and Bates, S. E. Steroid transport, accumulation, and antagonism of P-glycoprotein in multidrug-resistant cells. *Biochemistry*, **35**, 4820–4827 (1996).

20) Lee, Y. S., Nishio, K., Ogasawara, H., Funayama, Y., Ohira, T. and Saijo, N. *In vitro* cytotoxicity of a novel antitumor antibiotic, spicamycin derivative, in human lung cancer cell lines. *Cancer Res.*, **55**, 1075–1079 (1995).

21) Hirai, M., Tanaka, K., Shimizu, T., Tanigawara, Y., Yasuhara, M., Hori, R., Kakehi, Y., Yoshida, O., Ueda, K., Komano, T. and Inui, K. Cepharanthin, a multidrug resistant modifier, is a substrate for P-glycoprotein. *J. Pharmacol. Exp. Ther.*, **275**, 73–78 (1995).

22) Tanaka, K., Hirai, M., Tanigawara, Y., Ueda, K., Takano, M., Hori, R. and Inui, K. Relationship between expression level of P-glycoprotein and daunorubicin transport in LLC-PK, cells transfected with human MDR1 gene. *Biochem. Pharmacol.*, **53**, 741–746 (1997).

23) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).

24) Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, **47**, 936–942 (1987).