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ATP-Dependent Efflux of CPT-11 and SN-38 by the Multidrug Resistance Protein (MRP) and Its Inhibition by PAK-104P

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ABSTRACT

Non-P-glycoprotein-mediated multidrug-resistant C-A120 cells that overexpressed multidrug resistance protein (MRP) were 10.8- and 29.6-fold more resistant to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) and SN-38, respectively, than parental KB-3-1 cells. To see whether MRP is involved in CPT-11 and SN-38 resistance, *MRP* cDNA was transfected into KB-3-1 cells. The transfectant, KB/MRP, which overexpressed MRP, was resistant to both CPT-11 and SN-38. 2-[4-Diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide (PAK-104P) and MK571, which reversed drug resistance in MRP overexpressing multidrug-resistant cells, significantly increased the sensitivity of C-A120 and KB/MRP cells, but not of KB-3-1 cells, to CPT-11 and SN-38. The accumulation of both CPT-11 and SN-38 in C-A120 and KB/MRP cells was lower than that in KB-3-1 cells. The treatment with 10 μM PAK-104P increased the accumulation of CPT-11 and SN-38 in C-A120 and KB/MRP cells to a level similar to that found in KB-3-1 cells. The ATP-dependent efflux of CPT-11 and SN-38 from C-A120 and KB/MRP cells was inhibited by PAK-104P. DNA topoisomerase I expression, activity, and sensitivity to SN-38 were similar in the three cell lines. Furthermore, the conversion of CPT-11 to SN-38 in KB-3-1 and C-A120 cell lines was similar. These findings suggest that MRP transports CPT-11 and SN-38 and is involved in resistance to CPT-11 and SN-38 and that PAK-104P reverses the resistance to CPT-11 and SN-38 in tumors that overexpress MRP.

Camptothecin (CPT) is an antitumor agent isolated from extracts of the Chinese tree *Camptotheca acuminata* (Wall et al., 1966). Its target is DNA topoisomerase (topo) I (Hsiang et al., 1985). CPT-11 ([7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin]), one of the analogs of camptothecin, has high antitumor activity against refractory solid tumors, such as carcinomas of the lung, cervix, ovary, colon, rectum, and non-Hodgkin's lymphoma (Slichenmyer et al., 1993). CPT-11 is a prodrug that is converted to an active form, SN-38 (7-ethyl-10-hydroxy-camptothecin), in vivo by enzymes such as carboxylesterase (Senter et al., 1996). SN-38 is 1000-fold more potent than the parent compound in vitro.

Elucidation of the mechanisms for resistance to CPT-11 is important because tumor cell resistance to CPT-11 reduces the success of chemotherapy. Many cells resistant to CPT analogs have been isolated from different cell lines in vitro and some mechanisms of resistance to CPT analogs have been elucidated. These mechanisms include decreased conversion of CPT-11 to SN-38 (Niimi et al., 1992), altered topo I with less sensitivity to CPT-11 (Tanizawa et al., 1993), and decreased expression of topo I and/or topo II (Chang et al., 1992). Cells selected for resistance to adriamycin (ADM; Jansen et al., 1998), cisplatin (Niimi et al., 1992), melphalan (Friedman et al., 1994), 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (Prost and Riou, 1994), or mitoxantrone (Yang et al., 1995) developed cross-resistance to CPT analogs.

We found that multidrug-resistant C-A120 cells and KB/multidrug resistance protein (MRP) cells derived from epi-

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ABBREVIATIONS: CPT, camptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxy camptothecin; SN38-G, SN-38 glucuronide; topo, DNA topoisomerase; ADM, adriamycin; VCR, vincristine; MRP, multidrug resistance protein; MDR, multidrug resistance; cMOAT, canalicular multispecific organic anion transporter; P-gp, P-glycoprotein; LTC$_4$, leukotriene C$_4$; BSO, buthionine sulfoximine; MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GST, glutathione S-transferase.

dermoid carcinoma KB-3-1 cells, which overexpress MRP, are resistant to CPT-11 and SN-38, and that MRP transports CPT-11 and SN-38.

Few agents reverse MRP-mediated multidrug resistance (MDR). Cole (1992) reported that the ADM resistance of H69AR cells could not be reversed by most MDR-reversing agents. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, enhanced the toxicity of anticancer agents in MRP-expressing MDR cells by inhibiting enhanced drug efflux (Versantvoort et al., 1994a). PAK-104P (Sumizawa et al., 1997), MK571 (Versantvoort et al., 1994a), and genistein (Versantvoot et al., 1994b) increased the sensitivity to drugs of the cells that overexpressed MRP. In this study, we examined whether or not the reversing agents 2-[4-Diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide (PAK-104P), MK571, BSO, and piperine reverse the resistance to CPT-11 and SN-38 in MRP-mediated MDR cells.

Materials and Methods

Chemicals. Minimal essential medium (MEM) was purchased from Nissui Seiyaku Co. (Tokyo, Japan) and newborn calf serum was obtained from Cell Culture Laboratories (Cleveland, OH). CPT-11 and SN-38 were produced by Daiichi Seiyaku (Tokyo, Japan). PAK-104P was obtained from Nissan Chemical Industries (Chiba, Japan). The Leukotriene D_4 receptor antagonist MK571 (Jones et al., 1989) was kindly provided by Dr. A. W. Ford-Hutchinson (Merck-Fross Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). M_2III-6, a monoclonal antibody against canalicular multispecific organic anion transporter (cMOAT) was kindly provided by Drs. Marcel Kool and Piet Borst (the Netherlands Cancer Institute, Amsterdam, the Netherlands). CPT, BSO, and other drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Cell Lines. Cultured human KB-3-1 cells (Akiyama et al., 1985) were propagated in MEM containing 10% newborn calf serum, 1 mg/ml bactopeptone, 0.292 mg of glutamine/ml and 100 U penicillin/ml. Non-P-glycoprotein (P-gp)-mediated ADM-resistant C-A120 cells were originally selected from KB-3-1 cells with increasing concentrations of ADM in the presence of 1 µg/ml cepharanthine and 100 nM mezerein and were maintained in a medium containing 120 ng/ml ADM, 1 µg/ml cepharanthine, and 100 nM mezerein (Sumizawa et al., 1994). KB/MRP cells were stably transfected with the *MRP* gene as described previously (Taguchi et al., 1997).

Cell Survival by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay. The MTT colorimetric assay was used to assess the sensitivity of the cells to agents in vitro (Carmichael et al., 1987). To examine the effects of BSO, PAK-104P, MK571, or piperine on drug resistance, cells were preincubated with or without 100 µM BSO for 24 h, 10 µM PAK-104P, 20 µM MK571, or 100 µM piperine for 30 min and then incubated with various concentrations of drugs for 4 days. Surviving cells were determined as described (Sumizawa et al., 1997).

Immunoblotting. To detect MRP, we prepared a polyclonal antibody against a synthetic peptide with the sequence KEDTSEQVVPVLVKN, which was selected from a unique region of MRP (amino acids 246–260; Krishnamachary and Center, 1993). Monoclonal antibody M_2lll-6, generated against amino acids 1340–1541 of the rat cMOAT protein, was used to detect cMOAT. Polyclonal human antibody against human topo I (Topogen, Columbus, OH) was used to detect topo I. Either 10 µg of protein membrane vesicles or 10 µg of nuclear protein was mixed with an equal volume of SDS sample buffer consisting of 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glyc-

erol, and 0.005% bromophenol blue. Electrophoresis on SDS 7.5% (w/v) polyacrylamide minigels was performed according to the method of Laemmli (1970). Transfer to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) was performed electrophoretically for 30 min at 15 V (constant voltage) using a Transblot SD apparatus (Bio-Rad, Richmond, CA) as described by Kyhse-Anderson (1984). The membranes were incubated with antibody for 1 h at room temperature and then with horseradish peroxidase-linked second antibody for 1 h at room temperature. Membranes were developed by chemiluminescence following the enhanced chemiluminescence protocol (Amersham, Buckinghamshire, UK). Glutathione *S*-transferase (GST)-π Western blot analyses were conducted as described above, except that 15% instead of 7.5% (w/v) polycrylamide minigels were used and the filters were incubated with rabbit antibodies to human GST-π.

CPT-11 and SN-38 Accumulation. To measure drug accumulation, confluent KB-3-1, C-A120, and KB/MRP cells in 150-mm plastic dishes were incubated overnight in MEM and then incubated with 5 to 160 µM CPT-11 or 10 to 400 µM SN-38 for 2 h at 37°C. The cells were washed three times with cold PBS and immediately harvested with a rubber scraper. The harvested cells were again washed three times with cold PBS and were counted with a hemocytometer before the last wash. After the addition of methanol (1 ml/10^6 cells), the cells were suspended and centrifuged at 3000 rpm for 10 min, and the supernatants were evaporated with a concentrator. A modified reverse-phase HPLC method reported by Kaneda and Yokokura (1990) was used to analyze the content of CPT-11, SN-38, and SN-38 glucuronide (SN38-G). To examine the effect of PAK-104P on drug accumulation, cells were preincubated with or without 10 µM PAK-104P for 30 min and then incubated with various concentrations of CPT-11.

Efflux of CPT-11 and Its Product, SN-38. Cells were incubated in MEM with 160 µM CPT-11 for 1 h at 37°C. For depletion of ATP, cells were preincubated in ATP-depletion medium as described previously (Chen et al., 1998), CPT-11 was added to the medium, and the cells were incubated for 1 h at 37°C. The cells were washed three times with a total volume of 20 ml of PBS at 37°C. The cells were further incubated in medium without CPT-11 at 37°C for the indicated times. The medium was collected for measuring the effluxed CPT-11 and SN38-G, and the cells were washed three times with cold PBS. Levels of CPT-11, SN-38, and SN38-G in the cells and the medium were determined as described by Kaneda and Yokokura (1990). To examine the effects of PAK-104P on drug efflux, cells were preincubated for 30 min with or without 10 µM PAK-104P, and then 160 µM CPT-11 was added and the cells were incubated for 1 h at 37°C. Next, each dish was washed three times with PBS and then fresh medium with or without 10 µM PAK-104P was added. Cells were then incubated for the indicated times at 37°C and harvested, and the levels of CPT-11, SN-38, and SN-38G were determined.

Preparation of Nuclear Extracts. Crude nuclear extracts were prepared as described previously (Nakagawa et al., 1992). Protein concentration in the extract was determined by the method of Bradford (1976). An equal volume of glycerol was added to the supernatant, which was then kept at −20°C.

topo I Activity Assay. topo I activity was determined by the supercoiled *Escherichia coli* DNA (plasmid pBR322) relaxation assay (Liu and Miller, 1981). To examine the inhibition of topo I activity by CPT-11 or SN-38, different amounts of the agents were added to the protein in the reaction. Relaxed and supercoiled DNA were separated in a 1% agarose gel by electrophoresis and visualized by staining with 2 µM ethidium bromide.

Statistical Analysis. Differences between groups were tested by one-way ANOVA or Student's *t* test. Significance levels given are those for the two-tailed Student's paired *t* test. Data are presented as means ± S.D. Differences were considered significant when $P < .05$.

Results

Immunoblotting of MRP, cMOAT, GST-π, and topo I.
We examined the expression of MRP, cMOAT, GST-π, and
topo I in KB cell lines. As shown in Fig. 1, MRP was overex-
pressed in the membrane vesicles prepared from C-A120 and
KB/MRP cells. cMOAT was detected in membrane vesicles
from KB-3-1, C-A120, and KB/MRP cells, and the expression
level of cMOAT in C-A120 cells was about 2-fold higher than
that found in KB-3-1 and KB/MRP cells. The expression
levels of GST-π and topo I were similar in KB-3-1, C-A120,
and KB/MRP cells.

**Cross-Resistance to CPT-11 and SN-38 in KB Sub-
lines.** The IC$_{50}$ values for CPT-11 of KB-3-1, C-A120, and
KB/MRP cells were 3.5, 37.8, and 22.8 μM, respectively.
C-A120 and KB/MRP cells were 10.8-fold and 6.5-fold more
resistant to CPT-11, respectively, than the parental KB-3-1
cells (Table 1). The IC$_{50}$ values for SN-38 of KB-3-1, C-A120,
and KB/MRP cells were 8, 222, and 114 nM, respectively.
C-A120 and KB/MRP cells were 29.6-fold and 14.5-fold more
resistant, respectively, to SN-38 than were the parental KB-
3-1 cells (Table 1).

Accumulation of CPT-11 and SN-38. To investigate
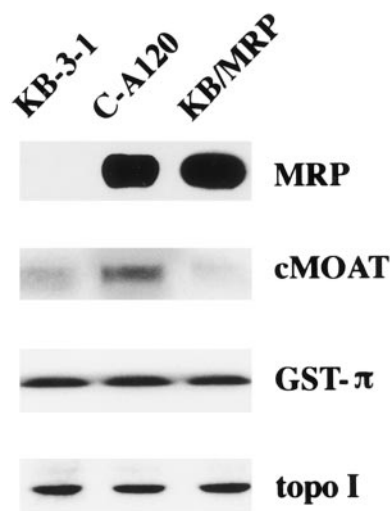whether or not MRP is involved in the resistance to CPT-11

and SN-38, we examined the accumulation of CPT-11 and
SN-38 in KB cell lines and the effect of PAK-104P, which
inhibits the transporting activity of MRP, on the accumula-
tion of CPT-11 and SN-38. The accumulation of CPT-11 in
C-A120 and KB/MRP cells was lower than that in KB-3-1
cells when the cells were incubated in a medium containing 5
or 20 to 160 μM CPT-11. When the cells were incubated with
160 μM CPT-11, the accumulation of CPT-11 in C-A120 and
KB/MRP cells was 66.4 and 68.3%, respectively, of that in
KB-3-1 cells (Fig. 2). The addition of 10 μM PAK-104P en-
hanced the accumulation of CPT-11 in C-A120 and KB/MRP
cells to a level similar to that in KB-3-1 cells without PAK-
104P. The accumulation of SN-38 in KB/MRP cells and C-
A120 cells was lower than that in KB-3-1 cells. When the
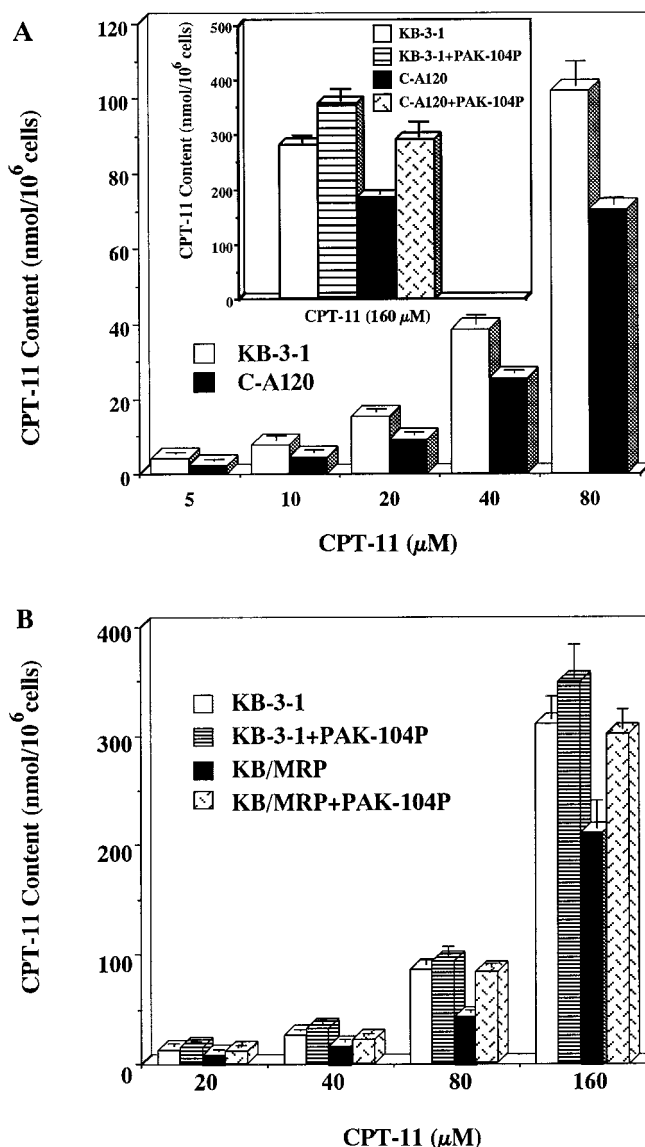cells were incubated in a medium containing 400 nM SN-38,

Fig. 1. Immunoblots for MRP, cMOAT, GST-π, and topo I. Membrane
vesicles (10 μg of protein for MRP and cMOAT), cytosols (50 μg of protein
for GST-π), or nuclear protein (10 μg of protein for topo I) were separated
by SDS-polyacrylamide gel electrophrosis and transferred to a polyvinyli-
dene difluoride membrane. The transferred proteins were immunoblotted
with polyclonal antibodies against MRP or GST-π, polyclonal human
antibody against topo I, and monoclonal antibody against cMOAT (M$_2$III-
6).

TABLE 1
Cross-resistance to CPT-11 and SN-38 in KB cell lines

Agent	IC$_{50}$[a] for KB-3-1 Cells	Fold Resistance	
		C-A120	KB/MRP
	μM		
CPT	0.5 ± 0.1[b]	13.8 ± 1.7[c]	5.2 ± 0.8[c]
CPT-11	3.5 ± 0.5	10.8 ± 1.9[c]	6.5 ± 1.1[c]
SN-38	0.0075 ± 0.0009	29.6 ± 4.1[c]	14.5 ± 1.9[c]

[a] Cell survival was determined by MTT assay.
[b] Data are means $\pm$ S.D. of three determinations obtained from triplicate cul-
tures.
[c] Significantly different ($p < 0.05$) compared with KB-3-1 cells, as determined by
paired Student's t test.

Fig. 2. The accumulation of CPT-11 and the effect of PAK-104P on the
accumulation of CPT-11. A, KB-3-1 and C-A120 cells were incubated with
5 to 80 μM CPT-11 or 160 μM CPT-11 (inset) for 2 h in the absence or
presence of 10 μM PAK-104P. B, KB-3-1 and KB/MRP cells were incu-
bated with 20 to 160 μM CPT-11 for 2 h in the absence or presence of 10
μM PAK-104P. The intracellular level of CPT-11 was determined by
HPLC as indicated in *Materials and Methods*. Columns represent means
of triplicate determinations; bars represent S.D.

the accumulation of SN-38 in C-A120 and KB/MRP was 31.3 and 56.8%, respectively, of that in KB-3-1 cells (Fig. 3). The addition of 10 μM PAK-104P enhanced the accumulation of SN-38 in KB/MRP cells to a level similar to that in KB-3-1 cells without PAK-104P. The addition of 10 μM PAK-104P also increased the intracellular SN-38 in C-A120 cells, but the level was lower than that in KB-3-1 cells without PAK-104P. SN38-G was not detected in the cells incubated with CPT-11 or SN-38 (data not shown).

Efflux of CPT-11 and Its Active Metabolite, SN-38, from KB Sublines. The difference between efflux of CPT-11 and SN-38 from KB-3-1 cells in ATP-repleted and ATP-depleted cells was marginal. We consider that CPT-11 and SN-38 are only slightly dependent on ATP for efflux from KB-3-1 cells, if at all. The ATP-dependent efflux of CPT-11 from C-A120 and KB/MRP cells was greater compared with KB-3-1 cells. When the cells were incubated with 160 μM CPT-11 for 1 h at 37°C, then without CPT-11 for an additional hour, 33.3% and 38.5% of CPT-11 was retained in the C-A120 and KB/MRP cells, respectively. However, 67.6% of CPT-11 was retained in KB-3-1 cells (Figs. 4A and 5A). When intracellular ATP was depleted, the CPT-11 retained in C-A120 (Fig. 4A) and KB/MRP cells (data not shown) was similar to that in KB-3-1 cells not treated with ATP-depleting agents. The ATP-dependent efflux of SN-38 from C-A120 and KB/MRP cells was considerably higher compared with KB-3-1 cells. When the cells were incubated with 160 μM CPT-11 for 1 h at 37°C (efflux time 0), then without CPT-11 for an additional hour, the active metabolite of CPT-11, SN-38, in C-A120, KB/MRP, and KB-3-1 cells was decreased by 30.6, 35.7, and 68.4% respectively, compared to efflux time 0 (Figs. 4B and 5B). To examine the effects of PAK-104P on CPT-11 and SN-38 efflux, cells were preincubated with or without PAK-104P and then incubated with CPT-11 in the absence of ATP-depleting agents. We found that PAK-104P inhibited the efflux of CPT-11 from KB-3-1 and KB/MRP cells, but the
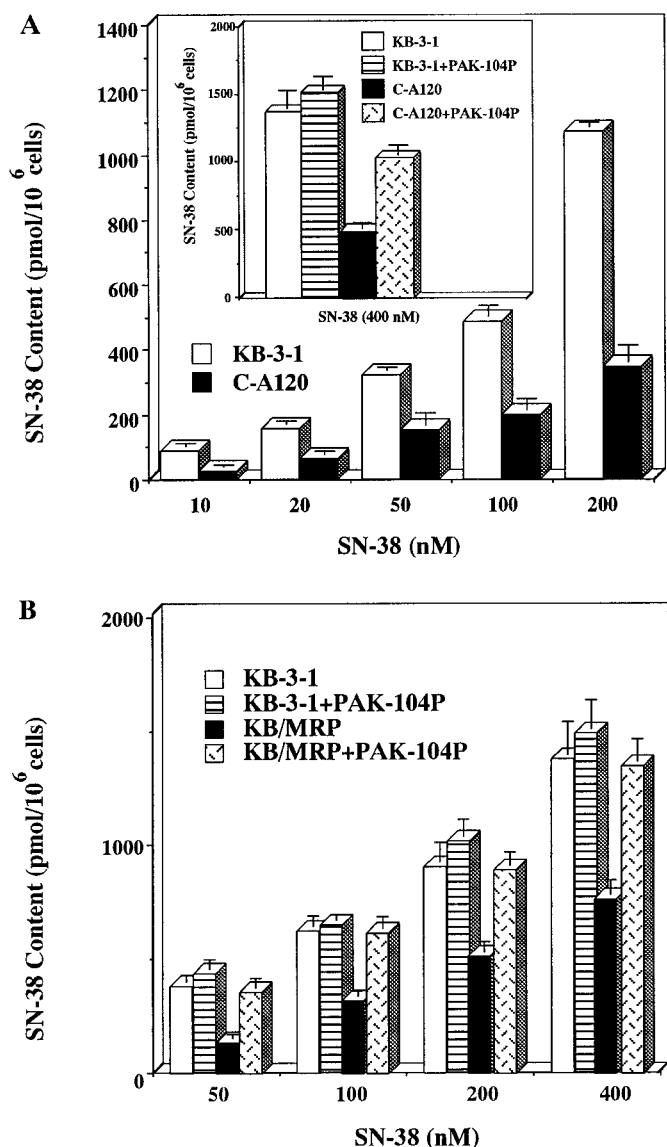

Fig. 3. The accumulation of SN-38 and the effect of PAK-104P on the accumulation of SN-38. A, KB-3-1 and C-A120 cells were incubated with 10 to 200 nM SN-38 or 400 nM SN-38 (inset) for 2 h in the absence or presence of 10 μM PAK-104P. B, KB-3-1 and KB/MRP cells were incubated with 50 to 400 μM SN-38 for 2 h in the absence or presence of 10 μM PAK-104P. The intracellular level of SN-38 was determined by HPLC as indicated in *Materials and Methods*. Columns represent means of triplicate determinations; bars represent S.D.
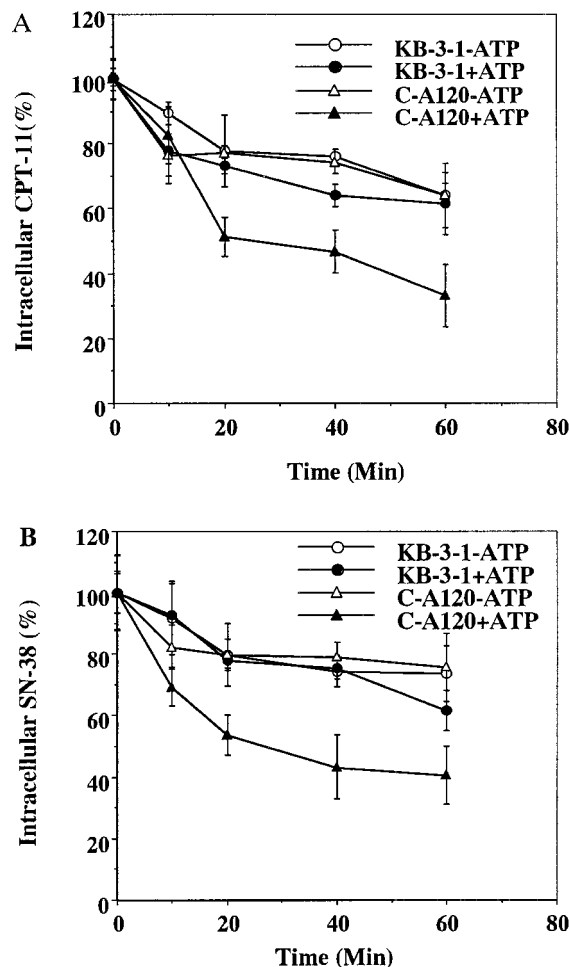

Fig. 4. Efflux of CPT-11 and SN-38. A, CPT-11 retained in KB-3-1 and C-A120 cells when the cells were incubated with 160 μM CPT-11 for 1 h in the absence (solid symbols) or presence (open symbols) of ATP-depleting agents. B, SN-38 converted from CPT-11 and retained in KB-3-1 and C-A120 cells when the cells were incubated with 160 μM CPT-11 in the absence (solid symbols) or presence (open symbols) of ATP-depleting agents. Points represent means of triplicate determinations; bars represent S.D.

extent of inhibition in KB/MRP cells was greater than that in KB-3-1 cells (Fig. 5A). Figure 5B also shows that 10 μM PAK-104P considerably inhibited the efflux of SN-38 from KB/MRP cells, whereas the effect of PAK-104P on KB-3-1 cells was marginal. The efflux of CPT-11 and SN-38 from C-A120 cells was also inhibited by 10 μM PAK-104P (data not shown).

CPT-11 Effluxed in the Medium. CPT-11 is converted into SN-38 by de-esterification, and SN-38 is conjugated to form SN38-G in the liver and is excreted into the bile duct (Kaneda et al., 1990). However, less than 1/1000 of the CPT-11 accumulated in the KB cells was converted to SN-38. Therefore, we examined whether CPT-11 in the KB cell lines was directly excreted into the medium and if SN38-G was effluxed from the cells.

When the cells were incubated with 160 μM CPT-11 for 1 h
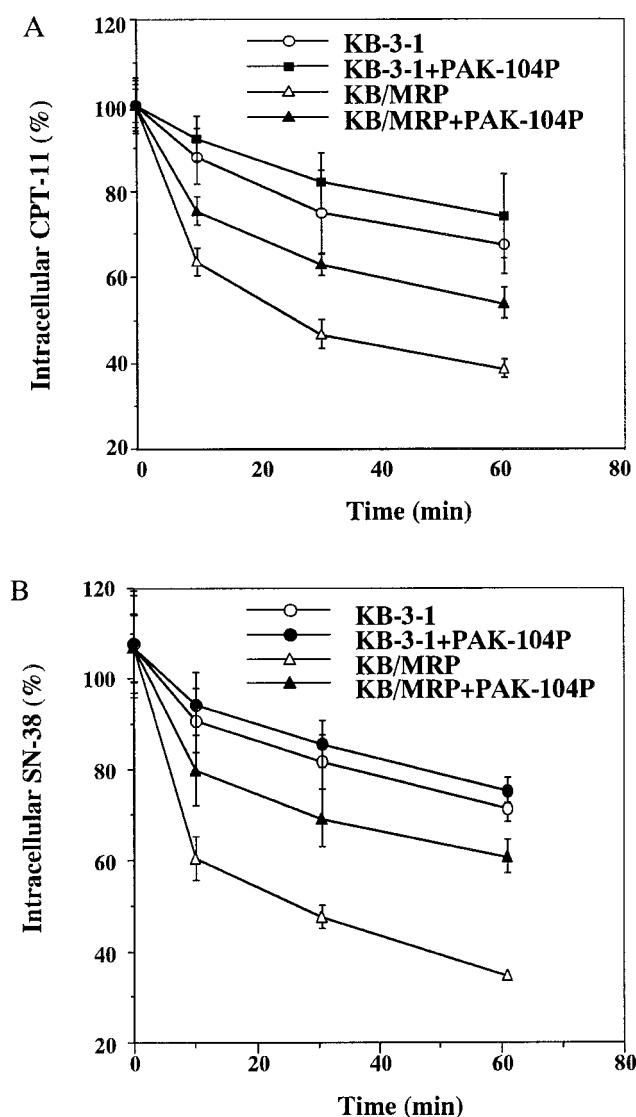

Fig. 5. The effect of PAK-104P on the efflux of CPT-11 and its metabolite, SN-38. A, CPT-11 retained in KB-3-1 and KB/MRP cells when the cells were incubated with 160 μM CPT-11 for 1 h in the absence (open symbols) or presence (solid symbols) of 10 μM PAK-104P. B, SN-38 retained in KB-3-1 and KB/MRP cells when the cells were incubated with 160 μM CPT-11 for 1 h in the absence (open symbols) or presence (solid symbols) of 10 μM PAK-104P. Points represent means of triplicate determinations; bars represent S.D.

at 37°C and then without CPT-11 for 1 h, 10.1 and 56.5% of the accumulated CPT-11 in KB-3-1 and C-A120 cells, respectively, was effluxed and detected in the medium (Table 2). However, none of the cells or media contained detectable SN38-G (data not shown).

Conversion of CPT-11 to the Active Metabolite, SN-38. To examine the conversion of CPT-11 to SN-38, we incubated KB-3-1 and C-A120 cells in a medium containing 300 μM CPT-11 for 3 h and then measured the intracellular concentration of CPT-11, SN-38, and SN38-G. The level of CPT-11 in C-A120 cells was 29% of that (688.94 nmol/10^6 cells) in KB-3-1 cells, and the level of the active metabolite SN-38 in C-A120 cells was also 29% of that (0.15 nmol/10^6 cells) in KB-3-1 cells (Fig. 6). On a molar basis, the amount of SN-38 in KB-3-1 and C-A120 cells was 0.022 and 0.027%, respectively, compared with CPT-11. These results indicated that the conversion of CPT-11 to the active metabolite SN-38 in C-A120 cells was not decreased in comparison to KB-3-1 cells. Again, SN38-G was not detected in any of the cells (data not shown).

topo I Levels in KB Cell Lines. Decreased expression of topo I or decreased sensitivity of topo I to topo I inhibitors may play an important role in cellular resistance to CPT-11 (Kanzawa et al., 1990). Therefore, we examined topo I levels and the sensitivity of topo I to CPT-11 and SN-38 in the three cell lines. As shown in Fig. 1, there was no significant difference in the expression level of topo I in the three cell lines. Next, we examined the sensitivity of topo I to CPT-11 and SN-38 in nuclear extracts. We first measured the total cellular activity of topo I in KB-3-1, C-A120, and KB/MRP cells by monitoring the relaxation of supercoiled DNA by the catalytic action of topo I by gel electrophoresis. topo I activity in these cell lines was similar (data not shown). The effect of CPT-11 and SN-38 on the catalytic activity of topo I from the three cell lines was then examined. SN-38 inhibited the topo I activity to a similar extent in the three cell lines and completely inhibited it at 5 μM (data not shown). In contrast, CPT-11 had no effect, even at a concentration of 250 μM (data not shown).

Effect of Modulators on the Cytotoxicity of CPT-11 and SN-38. The effects of the MDR-reversing agents PAK-104P and MK571, the γ-glutamylcysteine synthetase inhibitor BSO, and the UDP-glucuronyltransferase inhibitor piperine on the sensitivity of the KB cell lines to CPT-11 and SN-38 were examined. PAK-104P ($\leq$10 μM), MK571 ($\leq$20 μM), BSO ($\leq$100 μM), and piperine ($\leq$100 μM) had no cytotoxic effect on KB-3-1, C-A120, or KB/MRP cells (data not shown). The sensitivity of the cell lines to CPT-11 and SN-38

TABLE 2
The kinetics of CPT-11 in KB cell lines

Cells	Efflux Time	CPT-11 Efflux to Medium	Intracellular CPT-11	Ratio (a/b)
	min	*nmol/10^7 cells[a]*	*nmol/10^7 cells[b]*	*%*
KB-3-1	0		2338.93 ± 182.15	
	20	112.69 ± 10.12		4.82
	60	236.77 ± 9.64		10.12
C-A120	0		1637.63 ± 90.11	
	20	515.29 ± 33.97		31.47
	60	924.50 ± 90.11		56.45

[a] The concentration of CPT-11 effluxed from the cells that were incubated with 160 μM CPT-11 for 1 h and then incubated without CPT-11 for the indicated times. [b] The concentration of CPT-11 retained in the cells that were incubated with 160 μM CPT-11 for 1 h.

with or without modulators was assayed by the MTT method and the data are summarized in Table 3. PAK-104P and MK571 almost completely reversed the resistance to CPT-11 in C-A120 and KB/MRP cells, but BSO did not. PAK-104P and MK571 moderately reversed the resistance to SN-38 in C-A120 and KB/MRP cells and BSO only slightly reversed it. In contrast, piperine did not reverse the resistance to either CPT-11 or SN-38 in C-A120 and KB/MRP cells.

Discussion

Cancer cells treated with certain anticancer agents can acquire cross-resistance to other structurally unrelated anticancer agents. We have established and characterized a MRP-dependent MDR C-A120 cell line. C-A120 cells were resistant to CPT-11 and SN-38, as well as to ADM, vincristine (VCR), and VP-16 (Sumizawa et al., 1994). Because it is possible that drug-selected resistance phenotypes are generally multifactorial, we used KB/MRP, KB-3-1 cells transfected with *MRP* cDNA, as well as C-A120 cells, to elucidate the mechanism of resistance to CPT-11. KB/MRP cells over-

Fig. 6. Conversion of CPT-11 to SN-38. The KB-3-1 and C-A120 cell lines were incubated with 300 μM CPT-11 for 3 h and the concentrations of CPT-11 and SN-38 (inset) were measured by HPLC. Columns represent means of triplicate determinations; bars represent S.D.

expressed MRP, but the expression levels of cMOAT and topo I were similar to that in KB-3-1 cells. KB/MRP cells were resistant to ADM, VP-16, VCR (Taguchi et al., 1997), CPT-11, and SN-38. Previous studies showed that MDR cell lines that overexpressed MRP were cross-resistant to CPT or CPT-11 (Jansen et al., 1998), but MRP seemed not to be involved in the resistance to these agents.

The accumulation of ADM, VCR, and antimony potassium tartrate in C-A120 cells (Sumizawa et al., 1994, 1997; Chen et al., 1997) was significantly less than in the parental cells, and the decreased accumulation played an important role in the acquisition of resistance. In this study, we found that the accumulation of CPT-11 and SN-38 in C-A120 and KB/MRP cells was lower than in KB-3-1 cells. When the doses of CPT-11 and SN-38 that are near to their IC$_{50}$ values were used, the accumulation levels of CPT-11 and SN-38 in C-A120 were 3- to 5-fold higher than those in KB-3-1 cells. The accumulation data were not completely correlated with the cytotoxicity data. These discrepancies may be attributed to the difference in the incubation periods. The cytocidal effect of the agents was determined after a continuous exposure for 4 days, whereas the accumulation data were determined after an incubation for 2 h. Alternatively, there may be other unknown mechanisms in addition to the decreased accumulation for the resistance to CPT-11 and SN-38 in C-A120 cells. CPT-11 and SN-38 were actively effluxed from C-A120 and KB/MRP cells, but only slightly, if at all, from KB-3-1 cells. The efflux of CPT-11 and SN-38 was decreased by PAK-104P that inhibited transporting activity of MRP and reversed drug resistance in C-A120 cells (Sumizawa et al., 1997). These findings strongly suggested that MRP effluxes CPT-11 and SN-38 and is involved in the resistance to CPT-11 and SN-38. Other previous publications also showed that reduced accumulation and/or increased drug efflux is a component of resistance to camptothecins. Reid et al. (1997) found that CPT-resistant yeast overexpressed pleiotropic drug resistance 5, and reduced accumulation of CPT and SN-38 was reported in a topotecan-resistant ovarian cell line (Ma et al., 1998).

The transport of the endogenous GSH conjugate leukotriene C$_4$, S-(2, 4-dinitrophenyl)-glutathione, and glutathione disulfide was ATP dependent in membrane vesicles prepared
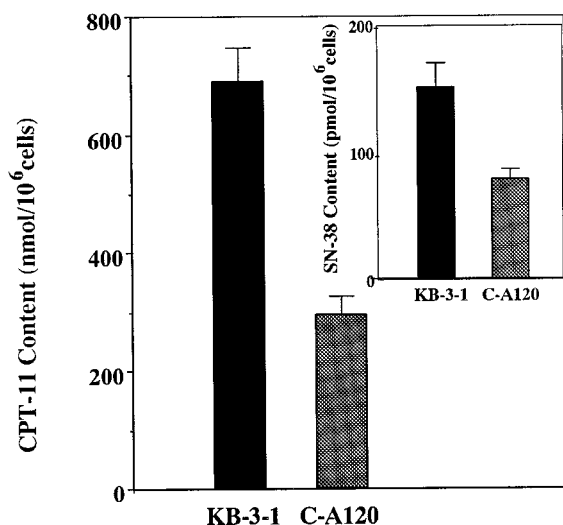
TABLE 3
Effect of reversing agents on CPT-11 and SN-38 cytotoxicity in KB cell lines

Treatment	IC$_{50}$[a]		
	KB-3-1	C-A120	KB/MRP
	μM		
CPT-11	3.5 ± 0.5[b] (1)[c]	37.8 ± 4.7 (10.8)	22.8 ± 2.1 (6.5)
+piperine (100 μM)	3.4 ± 0.4 (1.0)	36.8 ± 4.1 (10.5)	21.4 ± 2.4 (6.1)
+BSO (100 μM)	2.9 ± 0.3 (0.8)	35.7 ± 3.8 (10.2)	20.5 ± 1.6 (5.9)
+MK571 (20 μM)	2.5 ± 0.4 (0.7)	8.4 ± 0.9 (2.4)[d]	7.8 ± 0.6 (2.2)[d]
+PAK-104P (10 μM)	2.4 ± 0.4 (0.7)	7.1 ± 0.9 (2.0)[d]	4.7 ± 0.7 (1.3)[d]
SN-38	0.008 ± 0.001 (1.0)	0.222 ± 0.031 (29.6)	0.114 ± 0.015 (14.5)
+piperine (100 μM)	0.008 ± 0.001 (1.0)	0.232 ± 0.030 (31.0)	0.103 ± 0.092 (12.9)
+BSO (100 μM)	0.007 ± 0.001 (0.9)	0.147 ± 0.011 (18.4)[d]	0.071 ± 0.010 (8.9)[d]
+MK571 (20 μM)	0.007 ± 0.001 (0.9)	0.061 ± 0.010 (7.6)[d]	0.023 ± 0.008 (2.9)[d]
+PAK-104P (10 μM)	0.006 ± 0.001 (0.8)	0.045 ± 0.005 (6.3)[d]	0.022 ± 0.006 (2.8)[d]

[a] Cell survival was determined by MTT assay.
[b] Data are means ± S.D. of three determinations obtained from triplicate cultures.
[c] Relative resistance. IC$_{50}$ for CPT-11 or SN-38 of KB-3-1 with the reversing agents or C-A120 and KB/MRP, with and without the reversing agents, was divided by the IC$_{50}$ of KB-3-1 cells for CPT-11 or SN-38 without the reversing agents.
[d] Significantly different ($p < .05$) compared with the cells without reversing agent, as determined by paired Student's t test.

from human leukemia cells, HL60/ADR, that overexpressed MRP (Leier et al., 1994, 1996). Loe et al. (1996) demonstrated the transport of leukotriene C_4 in membrane vesicles from HeLa cells transfected with *MRP* cDNA. These findings suggest that MRP is an organic anion transporter. The α-hydroxy-δ-lactone ring in CPT-11 and SN-38 is in equilibrium with its carboxylate form, and the equilibrium reaction favors the production of the carboxylate form at physiological pH (Fassberg and Stella, 1992). The carboxylate forms of CPT-11 and SN-38 are negatively charged (Chu et al., 1997), and they may have been transported by MRP overexpressed in C-A120 and KB/MRP cells.

GSH was reported to be necessary for MRP to transport positively charged and neutral drugs such as ADM (Leier et al., 1994, 1996). However, Feller et al. (1995) found that the efflux of calcein was not sensitive to a large decrease in intracellular GSH concentration and suggested that GSH might not be needed or is needed in a very low concentration for the transport of negatively charged molecules by MRP. In the present study, we found that GSH depletion did not enhance the cytotoxicity of CPT-11 and only slightly enhanced the cytotoxic effect of SN-38. These results suggest that GSH may not be needed or a low GSH level may be sufficient for the detoxification of CPT-11 and SN-38 in C-A120 and KB/MRP cells. We also found that the UDP-glucuronyltransferase inhibitor piperine did not reverse the resistance to CPT-11 and SN-38 in C-A120 and KB/MRP cells. In addition, SN38-G was not detected in the cells incubated with CPT-11 or SN-38 (data not shown). These results indicate that SN-38 glucuronide is not a major metabolite of CPT-11 and SN-38 in KB cell lines.

A decreased conversion of CPT-11 to SN-38 was considered to be the cause of resistance to CPT-11 in the human ovarian cancer cell line HAC2/0.1 (Niimi et al., 1992). In our study, the conversion efficiency of CPT-11 to SN-38 in C-A120 cells did not seem to be lower than that in KB-3-1 cells (Fig. 6).

A decreased topo I level and/or activity in resistant cells (Chang et al., 1992) and a reduced sensitivity of topo I to an inhibitor (Tanizawa et al., 1993) were reported to be the most common mechanisms for resistance to CPT and its analogs. Our results show that there was no significant difference in the level and activity of topo I or in the sensitivity of topo I to SN-38 between the parental and the resistant cell lines (data not shown), suggesting that quantitative or qualitative changes of topo I are not involved in the resistance of C-A120 and KB/MRP cells to CPT-11 and SN-38.

Neither P-gp (Chuman et al., 1996; Taguchi et al., 1997) nor GST-π was overexpressed in C-A120 and KB/MRP cell lines, and MRP was overexpressed in these lines (Fig. 1). Thus, the multidrug-resistant phenotype of these two MDR cell lines does not seem to be related to P-gp and GST-π expression. CPT-11 and its metabolites were reported to be substrates for cMOAT (Chu et al., 1997), so cMOAT and/or related transporters might be involved in the active efflux of these drugs from cancer cells. We found that the expression of cMOAT in C-A120 cells was about 2-fold higher than in KB-3-1 cells, but the expression of cMOAT in KB/MRP cells was similar to that in KB-3-1 cells. However, KB/MRP cells were 6.5 and 14.5 times more resistant to CPT-11 and SN-38, respectively, than KB-3-1 cells, suggesting that MRP, but not cMOAT, is involved in the resistance of KB/MRP cells to CPT-11 and SN-38. Because the C-A120 cells are more resis-

tant to CPT-11 and SN-38 than the KB/MRP cells, the 2-fold higher expression of cMOAT in C-A120 than in KB/MRP cells may contribute to the resistance to CPT-11 and SN-38 in C-A120 cells, at least partly. Indeed, Koike et al. (1997) have demonstrated that transfection of the antisense *cMOAT* cDNA to human hepatic HepG2 cells, which stably express the *cMOAT*, increased the sensitivity of the cells to CPT-11 and SN-38.

Many agents that reverse P-gp-mediated drug resistance have been reported, and some, such as MK571, a leukotriene D_4 receptor antagonist (Versantvoort et al., 1994a), and genistein, a protein kinase inhibitor (Versantvoort et al., 1994b), were found to modulate MRP-associated MDR. Our previous study showed that PAK-104P reversed both P-gp- and MRP-associated MDR in KB cell lines. PAK-104P directly interacted with MRP to inhibit its transporting activity (Sumizawa et al., 1997). Therefore, we examined the effects of PAK-104P and MK571 on the sensitivity to CPT-11 and SN-38 and found that they almost completely or partially reversed the resistance of C-A120 and KB/MRP cells to CPT-11 and SN-38. PAK-104P increased the accumulation of CPT-11 and SN-38 and inhibited their ATP-dependent efflux in the resistant cells.

CPT-11, as well as CPT, is used clinically, and it is important to elucidate the mechanism of resistance to CPT-11 and its active metabolite, SN-38. This study demonstrated that CPT-11 and its metabolite, SN-38, are actively effluxed from cells that overexpressed MRP, showing that MRP transports CPT-11 and SN-38 and is involved in the resistance to these agents. PAK-104P reverses the resistance to CPT-11 and SN-38 by inhibiting the transporting activity of MRP. Therefore, PAK-104P may be useful for the reversal of CPT-11 resistance in tumors that overexpress MRP.

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