

Breast Cancer Resistance Protein (BCRP/ABCG2) Induces Cellular Resistance to HIV-1 Nucleoside Reverse Transcriptase Inhibitors

XIN WANG, TATSUHIKO FURUKAWA, TAKAO NITANDA, MIKA OKAMOTO, YOSHIKAZU SUGIMOTO, SHIN-ICHI AKIYAMA, and MASANORI BABA

Division of Human Retroviruses, Center for Chronic Viral Diseases (X.W., T.N., M.O., M.B.), and Department of Cancer Chemotherapy, Institute for Cancer Research (T.F., S.A.), Faculty of Medicine, Kagoshima University, Kagoshima, Japan; and Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan (Y.S.)

Received May 28, 2002; accepted September 23, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a novel member of ATP-binding cassette transporters, which induce multidrug resistance in cancer cells. We found that a high level of BCRP expression in CD4⁺ T cells conferred cellular resistance to human immunodeficiency virus type-1 (HIV-1) nucleoside reverse transcriptase inhibitors. The cell line MT-4/DOX₅₀₀ was established through the long-term culture of MT-4 cells in the presence of doxorubicin (DOX) and had reduced sensitivity to not only DOX but also zidovudine (AZT). MT-4/DOX₅₀₀ cells showed reduced intracellular accumulation and retention of DOX and increased ATP-dependent rhodamine 123 efflux. The cells were also resistant to several anticancer agents such as mitoxantrone, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, and 7-ethyl-10-hydroxycamptothecin. AZT was 7.5-fold less inhibitory

to HIV-1 replication in MT-4/DOX₅₀₀ cells than in MT-4 cells. Furthermore, the anti-HIV-1 activity of lamivudine was severely impaired in MT-4/DOX₅₀₀ cells. In contrast, the antiviral activity of non-nucleoside reverse transcriptase inhibitors and protease inhibitors was not affected in the cells. MT-4/DOX₅₀₀ cells expressed glycosylated BCRP but not P-glycoprotein (ABCB1), multidrug resistance protein 1, 2, or 4 (ABCC1, -2, or -4), or lung resistance-related protein. In addition, the BCRP-specific inhibitor fumitremorgin C completely abolished the resistance of MT-4/DOX₅₀₀ cells to AZT as well as to DOX. An analysis for intracellular metabolism of AZT suggests that the resistance is attributed to the increase of ATP-dependent efflux of its metabolites, presumably AZT 5'-monophosphate, in MT-4/DOX₅₀₀ cells.

At present, seven nucleoside or nucleotide reverse-transcriptase inhibitors (NRTIs), three nonnucleoside reverse-transcriptase inhibitors (NNRTIs), and six protease inhibitors (PIs) are available for the treatment of human immunodeficiency virus type-1 (HIV-1) infection. Highly active antiretroviral therapy (HAART) with these inhibitors has achieved high-level suppression of viral load in HIV-1-infected patients. The emergence of drug-resistant HIV-1 mutants during long-term HAART may result in the failure of therapy (Berger et al., 1998). However, some patients showed a sign of drug-resistance in the absence of drug-resistant viruses (Groschel et al., 1997). Investigations on the host cellular factors responsible for the resistance to antiviral agents revealed that the increased expression of several ATP-binding cassette (ABC) transporters might play a role in drug resistance to anti-HIV-1 agents. It was reported that

overexpression of the ABC transporter P-glycoprotein (P-gp/ABCB1) was associated with the reduced antiviral activity of zidovudine (AZT) against HIV-1 replication (Antonelli et al., 1992). This transporter was also shown to interact with HIV-1 PIs and reduce their therapeutic efficacy (Washington et al., 1998). In addition, it was demonstrated that overexpression of multidrug resistance protein (MRP/ABCC) 4 severely impaired the antiviral activity of AZT and other NRTIs, including phosphonylmethoxyethyladenine (Schuetz et al., 1999).

Recently, breast cancer resistance protein (BCRP/ABCG2), a new member of the ABC transporter superfamily, was identified in the atypical multidrug-resistant human breast cancer cell line MCF-7, which was selected in the presence of doxorubicin (DOX) and verapamil (Doyle et al., 1998). BCRP is the second member of the G (white) subfamily of ABC

ABBREVIATIONS: BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; P-gp, P-glycoprotein; AZT, zidovudine; MRP, multidrug resistance protein; DOX, doxorubicin; 3TC, lamivudine; NFV, nelfinavir; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; LRP, lung resistance-related protein; Topo II, topoisomerase II; mAb, monoclonal antibody; PNGase F, N-glycosidase F; HPLC, high-performance liquid chromatography; AZTMP, AZT 5'-monophosphate.

transporters and is also known as the mitoxantrone resistance protein MXR (Miyake et al., 1999) or the placental ABC transporter ABCP (Allikmets et al., 1998). This glycosylated plasma membrane protein is a half-size transporter, which is evolutionarily distinct from other full-size ABC transporters (Rocchi et al., 2000). Cells overexpressing BCRP show resistance to mitoxantrone and, to a lesser extent, to DOX, daunorubicin, and topotecan. However, it has not been shown whether BCRP interacts with anti-HIV-1 agents and affects their antiviral activity and cytotoxicity.

In this study, we established the DOX-resistant CD4⁺ T-cell line, which expresses BCRP but not other multidrug-resistant proteins. Using this cell line, we demonstrated that a high level of BCRP expression in CD4⁺ T cells brings about reduced anti-HIV-1 activity of NRTIs, such as AZT and lamivudine (3TC).

Materials and Methods

Compounds. DOX, cisplatin, vincristine, etoposide, paclitaxel, mitoxantrone, and actinomycin D were purchased from Sigma (St. Louis, MO). 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin and 7-ethyl-10-hydroxycamptothecin were obtained from Daiichi Pharmaceuticals (Tokyo, Japan). AZT, stavudine, and didanosine were purchased from Sigma. 3TC and the NNRTIs emivirine (Baba et al., 1994) and nevirapine were synthesized by Mitsubishi Chemical Corporation (Yokohama, Japan). The PIs nelfinavir (NFV) and indinavir were provided by Japan Tobacco (Takatsuki, Japan) and Takeda Pharmaceutical Industries (Osaka, Japan), respectively. The BCRP-specific inhibitor fumitremorgin C (Rabindran et al., 1998, 2000) was a generous gift from Dr. Rabindran (Wyeth-Ayerst Research, Pearl River, NY).

Cells and Virus. The human CD4⁺ T-cell MT-4 cells (Miyoshi et al., 1982) were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G, and 100 μ g/ml streptomycin (culture medium). The DOX-resistant cell lines MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ were established by exposing MT-4 cells to increasing concentrations of the compound. MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ cells were maintained in the presence of 100 and 500 ng/ml DOX, respectively. Before cytotoxicity and antiviral assays were performed, MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ cells were cultured in the absence of DOX for at least 7 days. HIV-1_{IIIB} was used for the infection of MT-4 cells. The virus was propagated and titrated in MT-4 cells and stored at -80°C until use.

Cytotoxicity Assay. The cells (1×10^5 cells/ml) were cultured in the presence of various concentrations of test compounds. After a 4-day incubation at 37°C , the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988).

Determination of Intracellular DOX and Effect of ATP-Depletion. Intracellular accumulation and retention of DOX in MT-4, MT-4/DOX₁₀₀, and MT-4/DOX₅₀₀ cells were determined by a slight modification of the flow cytometric method described previously (Krishan et al., 1997). Briefly, the cells were exposed to 10 μ g/ml of DOX for up to 180 min (accumulation phase), washed with ice-cold phosphate-buffered saline (PBS), and resuspended in warm culture medium in the absence of the compound (retention phase). At certain intervals, the cells were examined for their intracellular DOX concentrations by the use of flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). The intracellular drug concentration was expressed as the mean channel fluorescence. To determine the effect of ATP depletion on the intracellular accumulation of compounds, the cells were incubated in glucose-free medium containing 50 mM 2-deoxy-D-glucose and 15 mM sodium azide for 20 min at 37°C (Doyle et al., 1998). Rhodamine 123 (100 ng/ml) (Sigma) was added and

further incubated for 30 min. After washing with glucose-free medium, the cells were incubated under ATP-depleting conditions for an additional 30 min on ice, and rhodamine retention was determined by the use of flow cytometry.

Preparation of Crude Membrane Fractions, Cytosols, and Nuclear Extracts. The preparation of crude membrane fractions, cytosols, and nuclear extracts from MT-4 and the DOX-resistant MT-4 cells was described previously (Nakagawa et al., 1992; Grant et al., 1994). To prepare the crude membrane fractions, the cells were washed with 1% aprotinin-containing PBS and treated with lysis buffer [10 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM *p*-aminidinophenylmethanesulfonylfluoride, and 2 μ g/ml aprotinin]. After 10 min on ice, the cells were homogenized with approximately 80 strokes with a Dounce homogenizer. The intact cells and nuclei in the homogenate were removed by centrifugation at 1,500g for 10 min at 4°C . To prepare membrane-enriched fractions, the supernatants were ultracentrifuged at 100,000g for 30 min at 4°C , and the pellets were resuspended in dilution buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM *p*-aminidinophenylmethanesulfonylfluoride). To prepare the cytosols and nuclear extracts, the cells were washed with ice-cold PBS, resuspended in 2 ml of buffer A [10 mM KCl, 10 mM HEPES-KOH, pH 7.8, 0.1 mM EDTA, pH 8.0, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride, and 2 μ g/ml aprotinin] and kept on ice for 10 min. The cells were resuspended with 1.2 ml of buffer A and homogenized with approximately 80 strokes of Dounce homogenizer on ice. The supernatants were harvested as the cytosol extracts after centrifugation at 500g for 5 min at 4°C . The pellets were resuspended in 500 μ l of buffer B (420 mM KCl, 50 mM HEPES-KOH, pH 7.8, 0.1 mM EDTA, pH 8.0, 5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride, and 2 μ g/ml aprotinin). The nuclear proteins were extracted at 4°C followed by centrifugation at 24,000g for 30 min. Protein concentrations were determined by use of the methods described by Bradford (1976), and each protein was kept at -80°C until use.

Western Blot Analysis and Deglycosylation Assay. The crude membranes, cytosols, and nuclear extracts were subjected to the analyses of P-gp, MRP1, MRP2, MRP4, BCRP, lung resistance-related protein (LRP), and DNA topoisomerase II (Topo II). Membrane vesicles from KB-C2, KB/MRP, LLC-PK1-cMOAT, K6, and MCF-7 AdVp 3000 cells were used as the positive controls for P-gp, MRP1, MRP2, MRP4, and BCRP (Akiyama et al., 1985; Taguchi et al., 1997; Chen et al., 1999; Lee et al., 2000). The cytosolic fraction and nuclear extract obtained from SW620 cells after a 2-week treatment with 2 mM sodium butyrate were used as the positive controls for LRP and Topo II (Kitazono et al., 1999). The anti-P-gp monoclonal antibody (mAb) C-219, the anti-MRP1 mAb MRPm6, the anti-MRP2 mAb M₂I-4, and an anti-human Topo II rabbit antibody were purchased from Zymed Laboratories (South San Francisco, CA), Kamiya Biomedical (Thousand Oaks, CA), Monosan (Uden, Netherlands), and TopoGen Inc. (Columbus, OH), respectively (Kartner et al., 1985; Flens et al., 1994). An anti-MRP4 mAb was a gift from Dr. Kruh (Fox Chase Cancer Center, Philadelphia, PA). Anti-BCRP and anti-LRP antibodies were prepared according to the procedures described previously (Kitazono et al., 1999; Kage et al., 2002). The anti-BCRP antibody was generated by immunizing rabbits with a peptide that corresponds to amino acids 340 to 359 of the human BCRP protein.

For Western blot analysis (Chen et al., 1999), the extracted proteins (100 μ g) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidenedifluoride membrane. The transferred proteins were reacted with each antibody and treated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG mAb (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Antibody binding was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham). For deglycosylation assay, crude membranes (10 μ g of protein) from MT-4/DOX₅₀₀ and MCF-7 AdVp 3000 cells were incubated with or without 500 units of *N*-glycosidase F (PN-

Gase F) (New England BioLabs, Beverly, MA) for 60 min at 37°C, according to the manufacturer's instructions. The samples were analyzed by immunoblotting with the anti-BCRP antibody.

Northern Blot Analysis. Total RNA was extracted from MT-4 and MT-4/DOX₅₀₀ cells with an RNA extraction kit (RNAzol B; Tel-Test, Friendswood, TX). Total RNA was also extracted from MCF-7 AdVp 3000 cells and used as the positive control. A *SacII*/*HincII*-digested fragment of BCRP cDNA (approximately, 1200 base pairs) was used as a hybridization probe. Each fragment was labeled with a random primer labeling kit (Stratagene, La Jolla, CA) and [³²P]dCTP (ICN Biomedicals, Costa Mesa, CA). Free [³²P]dCTP was removed with MicroSpin S-300HR column (Amersham). RNA (1.5 µg) was electrophoresed on a formamide gel and transferred to a membrane (Hybond-N⁺; Amersham) in 20× sodium chloride-sodium citrate buffer overnight. After crosslinking with UV light, the membrane was prehybridized in hybridization buffer (5× sodium chloride-sodium phosphate-EDTA, 5× Denhardt's solution, 0.5% SDS, and 20 µg/ml denatured salmon sperm DNA) for 2 h at 65°C, and hybridized with each probe in hybridization buffer at 65°C overnight. After washing thoroughly, the membrane was exposed to X-ray film for 1 or 3 days.

Anti-HIV-1 Assay. The activity of the compounds against HIV-1 replication was determined from the inhibition of virus-induced cytopathicity in MT-4 and MT-4/DOX₅₀₀ cells, as described previously (Baba et al., 1991). Briefly, the cells (1 × 10⁵ cells/ml) were infected with HIV-1 at a multiplicity of infection of 0.02 and cultured in the presence of various concentrations of the test compounds. After a 4-day incubation at 37°C, the number of viable cells was determined by the MTT method.

Efflux Analysis of AZT and Its Metabolites. Intracellular AZT and its metabolites in MT-4 and MT-4/DOX₅₀₀ were analyzed by a slight modification of the high-performance liquid chromatography (HPLC), as described previously (Perno et al., 1989). Five million cells were incubated with 1 µM [*methyl*-³H]AZT. After a 3-h incubation, the cells were washed three times with ice-cold medium and immediately frozen in dry ice. The cells were then extracted with 60% (v/v) methanol, and the methanol extracts were further heated at 95°C for 1.5 min. The extracts were clarified by centrifugation at 12,000g for 6 min. Separation and detection of AZT and its metabolites were performed by use of a 25-cm Whatman Partisil-10 SAX column (Gilson Medical Electronics, Middleton, WI) by HPLC. After injection of the samples (25 µl), the buffer gradient was applied, starting at 0 time with 5 mM potassium phosphate and increasing linearly to 750 mM potassium phosphate over 55 min at a rate of 1 ml/min. Then, 750 mM potassium phosphate was further pumped for 10 min. The elution was fractionated at 1-min intervals (1 ml) and analyzed for radioactivity.

TABLE 1

Cytotoxicity of various anticancer and anti-HIV-1 compounds in MT-4 and MT-4/DOX₅₀₀ cells

All data represent means ± S.D. for three separate experiments.

Compound	Units	IC ₅₀		Fold resistance
		MT-4	MT-4/DOX ₅₀₀	
Doxorubicin	ng/ml	5.1 ± 2.0	755 ± 37	148
Mitoxantrone	ng/ml	0.081 ± 0.024	445 ± 26	5494
CPT-11	µM	1.6 ± 0.7	56 ± 13	35
SN-38	µM	4.2 ± 0.7	137 ± 41	33
Etoposide	ng/ml	75 ± 26	1038 ± 171	14
Actinomycin D	ng/ml	0.046 ± 0.022	0.12 ± 0.03	2.6
Paclitaxel	ng/ml	1.2 ± 0.1	0.96 ± 0.23	0.80
Vincristine	ng/ml	0.71 ± 0.42	0.29 ± 0.04	0.41
Cisplatin	mg/ml	43 ± 6	11 ± 3	0.26
AZT	µM	118 ± 10	300 ± 71	2.5
NFV	µM	11 ± 2	12 ± 4	1.1

fold resistance, ratio of IC₅₀ in MT-4 cells to IC₅₀ in MT-4/DOX₅₀₀ cells; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin.

Results

Cytotoxicity of Compounds in DOX-Resistant Cells.

The DOX-resistant T-cell line MT-4/DOX₅₀₀ was established from MT-4 cells by exposure to increasing concentrations of DOX (up to 500 ng/ml) for 1 year. When several anticancer agents were examined for their cytotoxic effects on MT-4/DOX₅₀₀ cells and compared with those on MT-4 cells, MT-4/DOX₅₀₀ cells were found to be highly resistant to DOX and mitoxantrone, moderately resistant to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, 7-ethyl-10-hydroxycamptothecin, and etoposide, and slightly resistant to actinomycin D, whereas the cells remained sensitive to paclitaxel, vincristine and cisplatin (Table 1). When the anti-HIV-1 agents AZT and NFV were evaluated for their IC₅₀ values in MT-4/DOX₅₀₀ cells, AZT was 2.5-fold less cytotoxic to MT-4/DOX₅₀₀ cells than to MT-4 cells (Table 1). However, such reduction in cytotoxicity was not observed with NFV.

Anti-HIV-1 Activity of Compounds in DOX-Resistant Cells. Because MT-4/DOX₅₀₀ cells showed some resistance to the cytotoxicity of AZT, anti-HIV-1 assays were conducted to determine whether the activity of AZT and other anti-HIV-1 agents was also reduced. AZT proved to be 7.5-fold less inhibitory to HIV-1 replication in MT-4/DOX₅₀₀ cells than in MT-4 cells (Table 2). The 50% effective concentrations (EC₅₀) of AZT were 0.013 and 0.094 µM in MT-4 and MT-4/DOX₅₀₀ cells, respectively. Furthermore, the anti-HIV-1 activity of

TABLE 2

Anti-HIV-1 activity of various compounds in MT-4 and MT-4/DOX₅₀₀ cells

All data represent means ± S.D. for three separate experiments.

Compound	EC ₅₀		Fold resistance
	MT-4	MT-4/DOX ₅₀₀	
	µM		
AZT	0.013 ± 0.003	0.094 ± 0.062	7.5
3TC	1.3 ± 0.7	>100	>77
ddI	12.3 ± 2.7	32.5 ± 5.0	2.7
d4T	0.33 ± 0.05	0.53 ± 0.08	1.6
NVP	0.10 ± 0.02	0.11 ± 0.02	1.1
EMV	0.015 ± 0.006	0.018 ± 0.008	1.2
IDV	0.041 ± 0.028	0.018 ± 0.009	0.5
NFV	0.023 ± 0.003	0.018 ± 0.008	0.8

Fold resistance, ratio of EC₅₀ in MT-4 cells to EC₅₀ in MT-4/DOX₅₀₀ cells; ddI, didanosine; d4T, stavudine; NVP, nevirapine; EMV, emivirine; IDV, indinavir.

3TC was severely (more than 77-fold) impaired in MT-4/DOX₅₀₀ cells. In contrast, the activity of neither NNRTIs (nevirapine and emivirine) nor PIs (indinavir and NFV) was affected in the cells (Table 2). The anti-HIV-1 activity of AZT and 3TC was also determined by the inhibition of p24 antigen production in the culture supernatants. These compounds were also less inhibitory to p24 antigen production in MT-4/DOX₅₀₀ cells than in MT-4 cells (data not shown).

Intracellular DOX Concentration in DOX-Resistant Cells. To gain insight into the mechanism of DOX resistance in MT-4/DOX₅₀₀ cells, its intracellular accumulation and retention were examined. Both MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ cells showed reduced accumulation and retention of DOX compared with MT-4 cells (Fig. 1A). The intracellular steady-state concentrations in MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ were approximately 50% and 40% of that in MT-4 cells, respectively, suggesting increased influx or decreased efflux of DOX in the resistant cells. However, when ATP was depleted, the accumulation and retention of DOX in MT-4/DOX₅₀₀ cells was found to be similar to those observed in MT-4 cells (data not shown). These results suggest that the resistance of the cells was caused by increased influx or decreased efflux of DOX and that the transport was ATP-dependent. The intracellular retention of rhodamine 123 in MT-4/DOX₅₀₀ cells was also reduced to less than 10% of that of MT-4 cells. Depletion of ATP significantly increased the retention of rhodamine 123 in MT-4/DOX₅₀₀ cells to a level comparable with that in MT-4 cells, although ATP depletion also slightly increased the retention of rhodamine 123 in MT-4 cells (Fig. 1B). Therefore, it was assumed that the efflux of DOX was markedly enhanced in the DOX-resistant MT-4 cells and that the expression of an ATP-dependent transporter might be involved in the resistance.

ABC Transporter Expression in DOX-Resistant Cells. To elucidate which ATP-dependent transporter is involved in the resistance, Western blot analyses with the anti-P-gp mAb were conducted for MT-4 and MT-4/DOX₅₀₀ cells. However, no enhancement of P-gp expression was observed in MT-4/DOX₅₀₀ cells (Fig. 2). Therefore, we further examined the expression of MRP1, MRP2, MRP4, BCRP, and LRP. As shown in Fig. 2, BCRP was highly expressed in MT-4/DOX₁₀₀ and MT-4/DOX₅₀₀ cells. Although BCRP was also expressed in MT-4 cells, the expression level was not comparable with those in MT-4/DOX₅₀₀ and MT-4/DOX₁₀₀ cells (data not shown). In contrast, no expression of MRP1, MRP2, MRP4, and LRP was detected in the DOX-resistant or in the DOX-sensitive MT-4 cells (Fig. 2). The expression of BCRP in MT-4/DOX₁₀₀ cells was lower than that in MT-4/DOX₅₀₀ cells. In accordance with the level of BCRP expression, MT-4/DOX₅₀₀ cells were more resistant to DOX than were MT-4/DOX₁₀₀ cells (Fig. 3A). A similar result was also obtained with AZT. Although the magnitude of AZT resistance in the MT-4/DOX₅₀₀ and MT-4/DOX₁₀₀ cells was not comparable with that of their DOX-resistance, MT-4/DOX₅₀₀ cells seemed to be more resistant to AZT than did MT-4/DOX₁₀₀ cells (Fig. 3B).

The 99- and 91-kDa doublet proteins were identified in the DOX-resistant MT-4 cells. These molecular masses differed from that of BCRP (83 kDa) expressed in the positive control MCF-7 AdVp 3000 cells (Fig. 2B). On the other hand, Northern blot analysis revealed that BCRP mRNA was expressed in MT-4/DOX₅₀₀ cells but not in MT-4 cells, and that the sizes

of BCRP mRNA obtained from MT-4/DOX₅₀₀ and from MCF-7 AdVp 3000 cells were identical (Fig. 4A and data not shown). Treatment of the doublet (99- and 91-kDa) and the 83-kDa proteins with PNGase F yielded proteins of the same size (72 kDa), indicating that the difference in their molecular masses was caused by the glycosylation level of BCRP in MT-4/DOX₅₀₀ and MCF-7 AdVp 3000 cells (Fig. 4B).

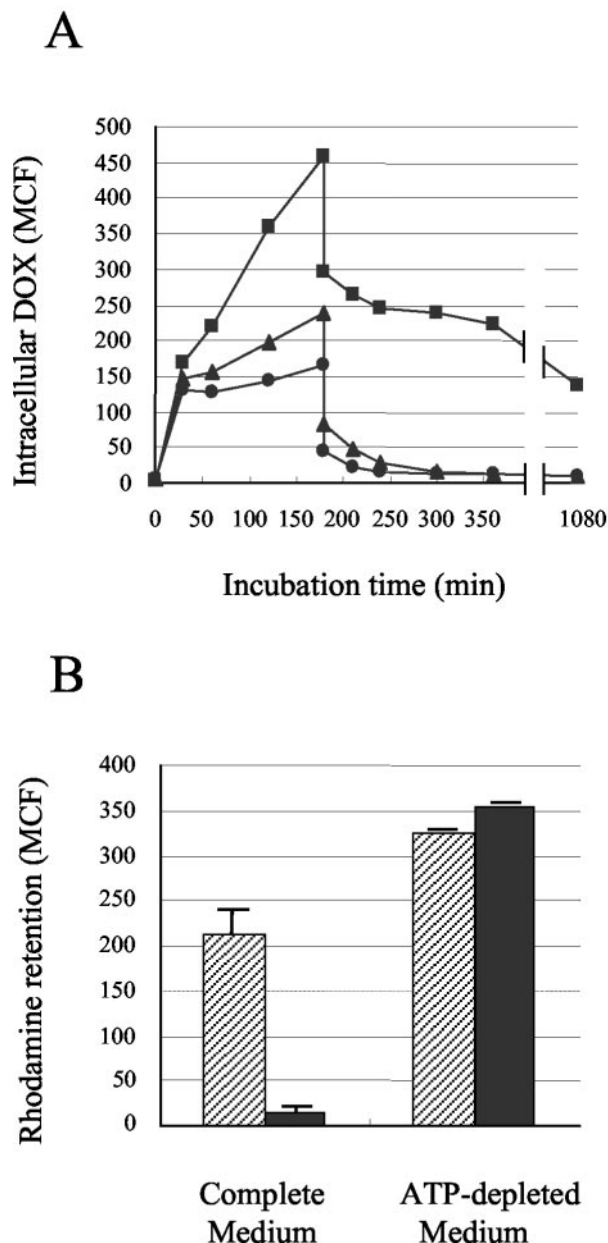


Fig. 1. Kinetics of intracellular DOX and effect of ATP depletion. A, MT-4 (■), MT-4/DOX₁₀₀ (▲), and MT-4/DOX₅₀₀ (●) cells were exposed 10 μ g/ml of DOX for up to 180 min (accumulation phase), washed with ice-cold PBS, and resuspended in warm culture medium in the absence of the compound (retention phase). At certain intervals, the cells were measured for their intracellular DOX concentrations by flow cytometry. The amount of intracellular DOX was expressed as the mean channel fluorescence (MCF). All data points are the mean values of three separate experiments. B, MT-4 (▨) and MT-4/DOX₅₀₀ (■) cells were incubated in glucose-free medium, as described under *Materials and Methods*. Rhodamine 123 (100 ng/ml) was added and further incubated for 30 min. After washing with glucose-free medium, the cells were incubated under ATP-depleting conditions for an additional 30 min on ice, and rhodamine retention was determined by flow cytometry.

Effect of the BCRP-Specific Inhibitor Fumitremorgin C. To confirm the role of BCRP in AZT resistance, the effects of fumitremorgin C on the cytotoxicity of DOX and AZT were examined in MT-4 and MT-4/DOX₅₀₀ cells. As shown in Table 3, fumitremorgin C completely abolished the resistance of MT-4/DOX₅₀₀ cells to DOX and AZT at a concentration of 5 μ M. However, fumitremorgin C alone did not have any inhibitory effect on the viability and proliferation of both MT-4 and MT-4/DOX₅₀₀ cells at this concentration (data not shown). Furthermore, the inhibitor also abolished the resistance of MT-4/DOX₅₀₀ cells to mitoxantrone but not to paclitaxel (data not shown).

AZT and Its Metabolites in DOX-Resistant Cells. To determine whether the reduced anti-HIV-1 activity of AZT in the DOX-resistant cells can be attributed to the increased efflux of the compound or its metabolites from MT-4/DOX₅₀₀ by BCRP, the intracellular metabolism of AZT was investigated in MT-4 and MT-4/DOX₅₀₀ cells. An HPLC analysis showed that the AZT 5'-monophosphate (AZTMP) accumula-

tion was significantly diminished in MT-4/DOX₅₀₀ cells (Fig. 5). Compared with AZTMP level in MT-4 cells, only 7.0% of AZTMP was retained in MT-4/DOX₅₀₀ cells. In addition, the levels of AZT, AZT 5'-diphosphate, and AZT 5'-triphosphate were also reduced to 45.7%, 30.4%, and 50.3% of those in MT-4 cells, respectively (Fig. 5). These results suggest that the impaired anti-HIV-1 activity of AZT in MT-4/DOX₅₀₀ cells was caused by the increased efflux of its metabolites, presumably AZTMP, by BCRP.

Discussion

Significant advances in the treatment of HIV-1 infection have been achieved with the success of HAART, which is conducted through a combination of drugs that block different steps in the viral replication cycle, such as reverse transcription and protein processing. Unfortunately, the emergence of drug resistance frequently occurs because of rapid

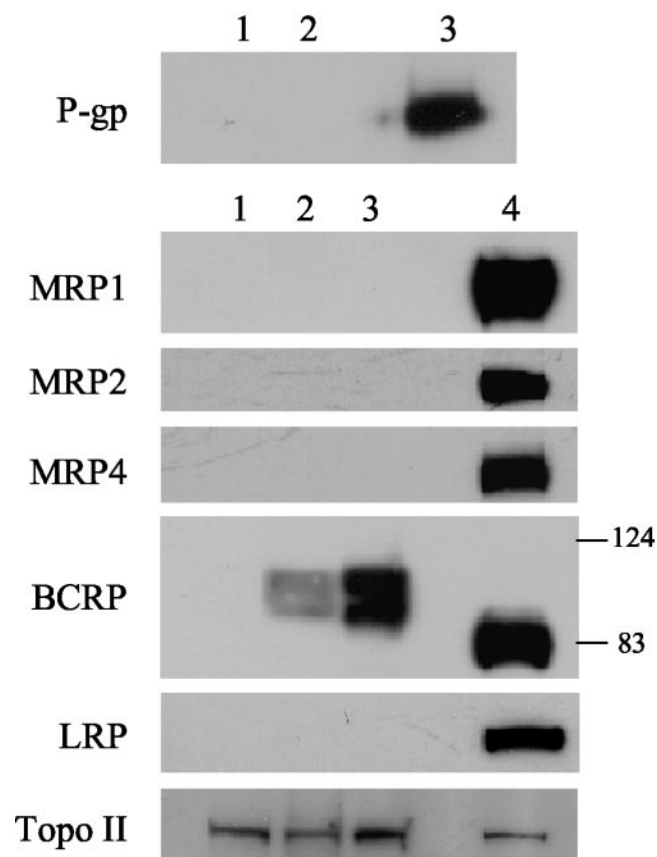


Fig. 2. ABC transporter expression in DOX-resistant cells. Crude membranes (for P-gp, MRP1, MRP2, MRP4, and BCRP), cytosols (for LRP), or nuclear proteins (for Topo II) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidenedifluoride membrane. The transferred proteins were immunoblotted with a mAb against each transporter protein. Membrane vesicles from KB-C2, KB/MRP, LLCPK1-cMOAT, K6, and MCF-7 AdVp 3000 cells were used as the positive controls for P-gp, MRP1, MRP2, MRP4, and BCRP, respectively. Cytosolic fractions and nuclear extracts from sodium butyrate-treated SW620 cells were used as the control protein for LRP and Topo II. In the analysis for P-gp, lanes 1, 2, and 3 are proteins obtained from MT-4, MT-4/DOX₅₀₀, and the control cells, respectively. In the analyses for MRP1, MRP2, MRP4, and BCRP, LRP, and Topo II, lanes 1, 2, 3, and 4 indicate those obtained from MT-4, MT-4/DOX₁₀₀, MT-4/DOX₅₀₀, and the control cells, respectively.

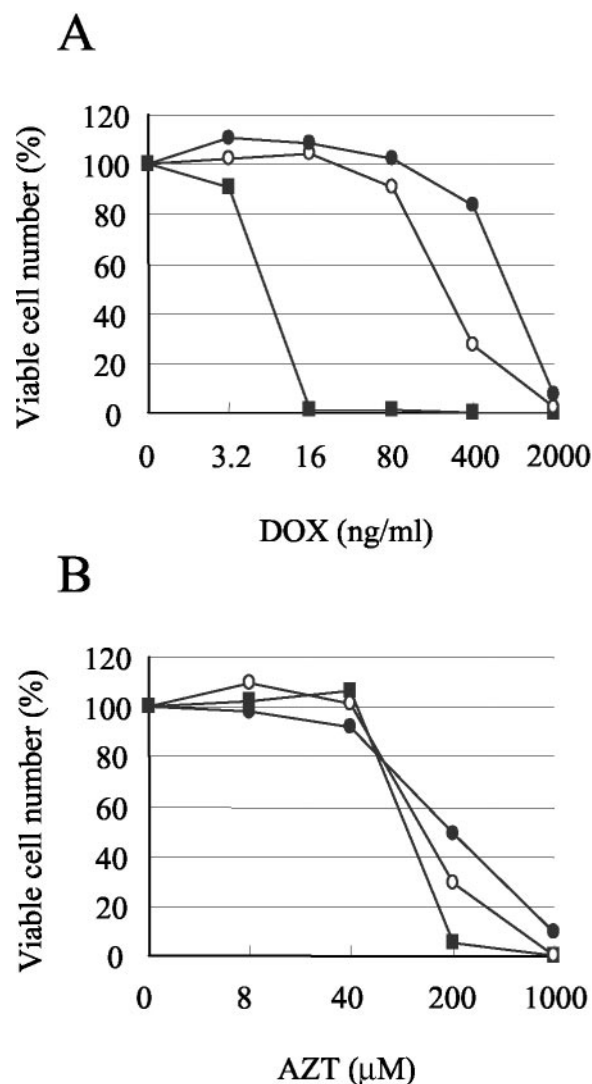


Fig. 3. Cytotoxicity of DOX and AZT in DOX-resistant cells. MT-4 (■), MT-4/DOX₁₀₀ (○) and MT-4/DOX₅₀₀ (●) cells were cultured in the presence of various concentrations of DOX (A) or AZT (B). After a 4-day incubation, the number of viable cells was assessed by the MTT methods. Representative results for three independent experiments are shown. The viable cell numbers of MT-4, MT-4/DOX₁₀₀, and MT-4/DOX₅₀₀ in the presence of 200 μ M AZT were 3.4 ± 1.9 , 35.1 ± 20.3 , and $54.1 \pm 4.5\%$ of those in the absence of the compound, respectively.

mutation of viral genome (Schinazi et al., 2000). In addition, it is assumed that host cellular factors are also involved in the resistance to antiretroviral drugs (Groschel et al., 1997; Swanstrom and Erona, 2000). One factor that may limit the therapeutic efficacy of PIs is the ABC transporters P-gp and MRP1. These transporters are known to interact with several PIs (Washington et al., 1998; Srinivas et al., 1998). Furthermore, if the resistance to NRTIs could be induced by host cellular factors, it would be a more serious impediment to the

TABLE 3

Effects of fumitremorgin C ($5 \mu\text{M}$) on the cytotoxicity of DOX and AZT in MT-4 and MT-4/DOX₅₀₀ cells

All data represent means \pm S.D. for three separate experiments. Parentheses indicate the relative resistance, determined by use of the IC₅₀ value of DOX alone or AZT alone in MT-4 cells.

Compound	Units	IC ₅₀	
		MT-4	MT-4/DOX ₅₀₀
DOX alone	ng/ml	5.1 ± 2.0 (1.0)	755 ± 37 (148)
DOX + fumitremorgin C	ng/ml	4.0 ± 1.7 (0.8)	5.5 ± 1.5 (1.1)
AZT alone	μM	118 ± 10 (1.0)	300 ± 71 (2.5)
AZT + fumitremorgin C	μM	107 ± 7 (0.9)	35 ± 5 (0.3)

progress of HAART (Lavie et al., 1997). Overexpression of MRP4 has been reported to efflux some NRTIs from CD4⁺ T cells and result in their decreased anti-HIV-1 activity in vitro (Schuetz et al., 1999). The present study clearly demonstrates that the novel ABC transporter BCRP also affects the anti-HIV-1 activity of NRTIs, particularly AZT and 3TC, in cell cultures.

After prolonged treatment of MT-4 cells with DOX, the established cell line MT-4/DOX₅₀₀ displayed multidrug resistance to different classes of anticancer agents (Table 1). More interestingly, MT-4/DOX₅₀₀ cells showed some resistance to NRTIs but not to NNRTIs and PIs in terms of anti-HIV-1 activity and cytotoxicity (Tables 1 and 2). Although the anti-HIV-1 activity of 3TC was severely impaired in MT-4/DOX₅₀₀ cells, it was not possible to determine the IC₅₀ of 3TC in MT-4/DOX₅₀₀ as well as in MT-4 cells because of its low cytotoxicity (data not shown). ATP-dependent retention of rhodamine 123 in MT-4/DOX₅₀₀ cells indicated that an ABC transporter might play an important role in the resistance (Fig. 1B). It has been documented that the ABC transporter P-gp recognizes AZT as a substrate and reduces its antiviral activity against HIV-1 replication (Yusa et al., 1990; Antonelli et al., 1992). However, the P-gp-specific inhibitor verapamil could not block the efflux of DOX or rhodamine 123 in MT-4/DOX₅₀₀ cells (data not shown). Furthermore, the expression of P-gp proved to be lower than a detectable level in MT-4/DOX₅₀₀ and MT-4 cells (Fig. 2), and MT-4/DOX₅₀₀ cells did not acquire resistance to the P-gp substrates vincristine and paclitaxel (Table 1), suggesting that an ABC transporter other than P-gp is involved in the resistance to NRTIs in MT-4/DOX₅₀₀ cells. Extensive analyses revealed that, among the ABC transporters examined, BCRP was the only ABC protein that was significantly expressed in the DOX-resistant MT-4 cells (Fig. 2B). In addition, the BCRP-specific inhibitor fumitremorgin C completely abolished the resistance of MT-4/DOX₅₀₀ cells to AZT as well as to DOX (Table 3), suggesting that BCRP is the molecule responsible for the AZT resistance.

It was reported that the arginine at position 482 (R482) of BCRP was an important determinant for substrate specificity. Increased efflux of doxorubicin and rhodamine 123 was observed with the R482T or R482G mutant but not with the wild type of BCRP (Honjo et al., 2001). Our preliminary analysis for the full-length BCRP cDNA identified the R482M mutation in MT-4/DOX₅₀₀ cells (data not shown). The doxorubicin selection in the presence of the P-gp inhibitor verapamil did up-regulate the BCRP expression in human breast carcinoma cells (Doyle et al., 1998). However, we showed here that the doxorubicin selection in the absence of verapamil could also up-regulate the expression of BCRP,

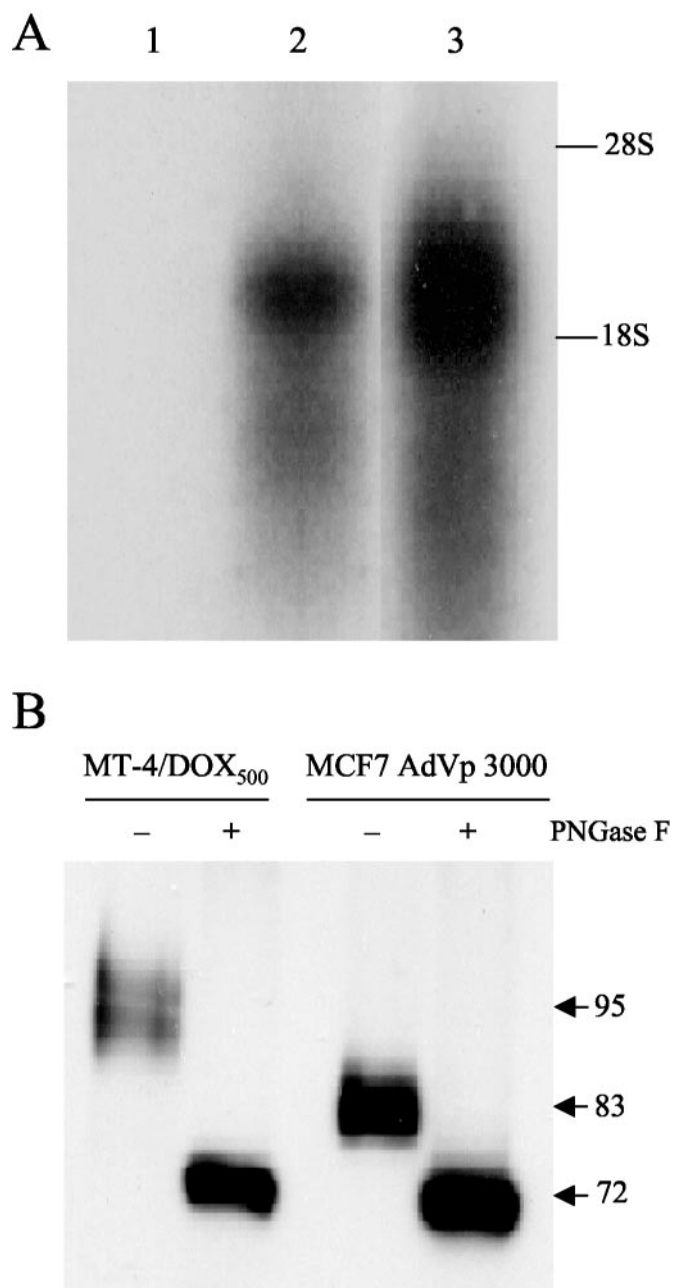


Fig. 4. Expression of BCRP mRNA and glycosylated BCRP in DOX-resistant cells. A, total RNA was extracted from MT-4 (lane 1), MT-4/DOX₅₀₀ (lane 2), and MCF-7 AdVp 3000 (lane 3) cells, electrophoresed, and transferred to a membrane. The membrane was hybridized with a [³²P]dCTP-labeled *SacII-HincII*-digested fragment of BCRP cDNA. B, crude membranes of MT-4/DOX₅₀₀ and MCF-7 AdVp 3000 cells were incubated in the absence or presence of PNGase F (500 units) for 60 min at 37°C. The samples were analyzed by immunoblotting with an anti-BCRP antibody.

albeit a mutant form, in a human CD4⁺ T-cell line. Although this mutation differed from those observed previously in doxorubicin-resistant human cell lines (R482T or R482G), it could not be excluded that the NRTI resistance of MT-4/DOX₅₀₀ cells was caused by the mutation but not by the increased expression of BCRP. Further studies are in progress to determine whether the wild-type BCRP affects the anti-HIV-1 and cytotoxicity to NRTIs.

Another point that should be considered is the activity of nucleotide kinases in the DOX-resistant cells. If AZT and

3TC would be less phosphorylated in the cells because of a reduced kinase activity, the intracellular concentration of the active form (5'-triphosphate) of AZT and 3TC should be decreased. In this case, the cells might seem to be resistant to these NRTIs. However, this is unlikely, because the resistance of the cells was not induced by prolonged exposure to any nucleoside derivatives but was cultured in the presence of DOX, which is not a substrate of nucleotide kinases. Furthermore, the MT-4/DOX₅₀₀ cells showed little, if any, resistance to another pyrimidine analog stavudine (Table 2 and data not shown).

BCRP has been detected in breast, colon, and gastric cancers and in acute myeloid and lymphoblastic leukemias (Sauerbrey et al., 2002). Furthermore, BCRP is also expressed in some healthy tissues, including placenta, liver, breast, and venous and capillary endothelia (Maliepaard et al., 2001). More importantly, BCRP mRNA was detected in bone marrow and peripheral blood mononuclear cells (Sauerbrey et al., 2002). HIV-1 has been found in several tissues in vivo and can infect many different types of human cells in vitro. It is possible that the interaction of NRTIs with BCRP could reduce the intracellular drug concentrations in these tissues, resulting in insufficient suppression of HIV-1 replication.

The central nervous system disorders associated with HIV-1 infection, such as encephalopathy and dementia, occur at the late stage of the disease (Gendelman et al., 1994). Although the development of HAART has decreased the incidence rates for HIV-1-associated encephalopathy and dementia, its impact on the future incidence and course of dementia remains debatable (Geraci and Simpson, 2001; Sacktor et al., 2001). Most of the licensed anti-HIV-1 drugs have a limited capacity to enter the brain (Brouwers et al., 1997). The expression of BCRP in the capillary endothelium of the blood-brain barrier may contribute to the limited capacity of NRTIs to enter into the brain (Maliepaard et al., 2001). BCRP modulators such as fumitremorgin C may be useful to improve the entry of NRTIs into the brain and increase their concentrations in the cerebrospinal fluid (Rabindran et al., 1998, 2000).

In conclusion, we found that the novel ABC transporter BCRP is a cellular factor involved in the resistance to anti-HIV-1 NRTIs, such as AZT and 3TC. Further studies are needed to determine whether BCRP expression in the target cells is indeed related to the treatment failure and emergence of drug resistance in HIV-1-infected patients.

Acknowledgments

We thank Dr. Susan Bates (National Cancer Institute, Bethesda, MD) for providing MCF-7 AdVp 3000 cells, Dr. Kazumitsu Ueda (Kyoto University, Kyoto Japan) for KB/MRP cells, Dr. Michihiko Kuwano (Kyushu University, Fukuoka-shi, Japan) for LLC PK1-cMOAT cells, and Dr. Gary Kruh (Fox Chase Cancer Center, Philadelphia, PA) for K6, MRP4-transfected NIH3T3 cells, and MRP4-specific antibody. We also thank Dr. Masaki Kawamura, Hiroshi Miyake, Ryuji Ikeda, Ren Xiao-Qin, and Motoi Mukai for their excellent technical assistance and appropriate advice for the experiments.

References

- Akiyama S, Fojo JA, Hanover JA, Pastan IH, and Gottesman MM (1985) Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat Cell Mol Genet* 11:117-126.
- Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, and Dean M (1998) A

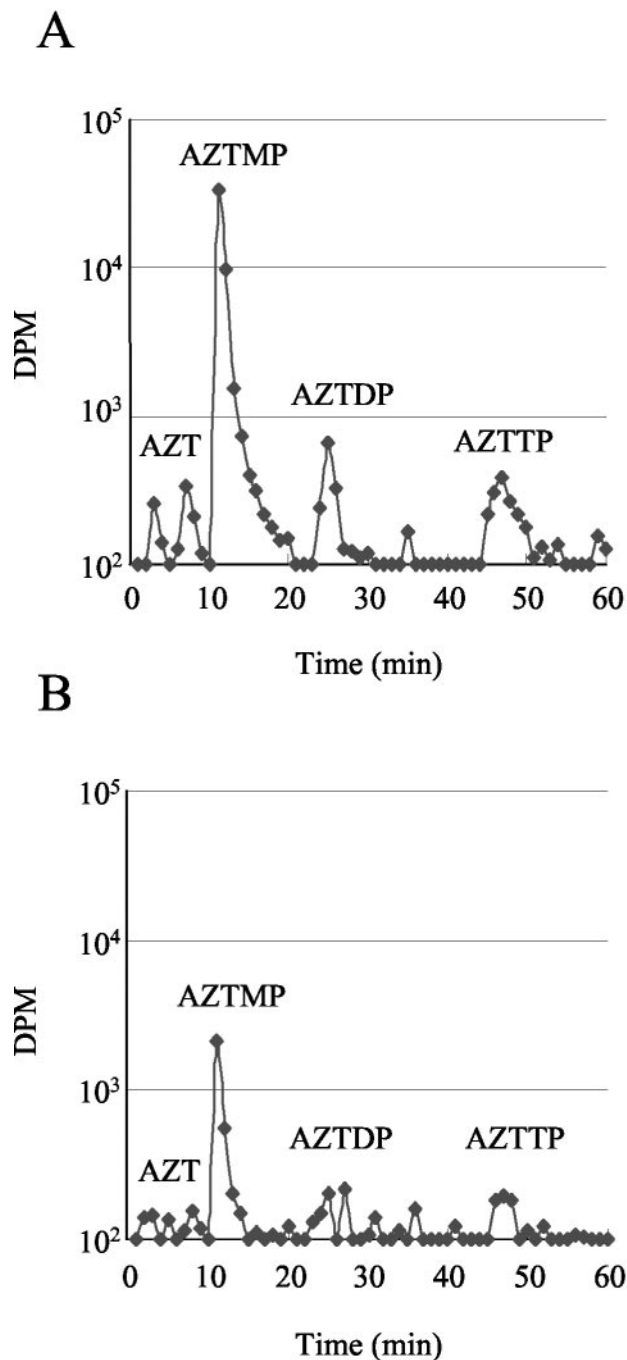


Fig. 5. AZT and its metabolites in DOX-resistant cells. MT-4 (A) and MT-4/DOX₅₀₀ (B) cells were incubated with [*methyl*-³H]AZT. The methanol extracts were separated by HPLC, and each fraction was analyzed for radioactivity. The intracellular concentrations of AZT and its metabolites were expressed as disintegrations per minute (DPM).

- human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* **58**:5337–5339.
- Antonelli G, Turriziani O, Cianfriglia M, Riva E, Dong G, Fattorossi A, and Dianzani F (1992) Resistance of HIV-1 to AZT might also involve the cellular expression of multidrug resistance P-glycoprotein. *AIDS Res Hum Retroviruses* **8**:1839–1844.
- Baba M, De Clercq E, Tanaka H, Ubasaba M, Takashima H, Sekiya K, Nitta I, Umezaki K, Nakashima H, Shigeta S, et al. (1991) Potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) by 5-ethyl-6-phenylthiouracil derivatives through their interaction with the HIV-1 reverse transcriptase. *Proc Natl Acad Sci USA* **88**:2356–2360.
- Baba M, Shigeta S, Yuasa S, Takashima H, Sekiya K, Ubasawa M, Tanaka H, Miyasaka T, Walker RT, and De Clercq E (1994) Preclinical evaluation of MKC-442, a highly potent and specific inhibitor of human immunodeficiency virus type 1 in vitro. *Antimicrob Agents Chemother* **38**:688–692.
- Berger EA, Moss B, and Pastan I (1998) Reconsidering targeted toxins to eliminate HIV infection: you gotta have HARRT. *Proc Natl Acad Sci USA* **95**:11511–11513.
- Bradford MA (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**:248–256.
- Brouwers P, Hendricks M, Lietzau JA, Pluda JM, Mitsuya H, Broder S, and Yarchoan R (1997) Effect of combination therapy with zidovudine and didanosine on neuropsychological functioning in patients with symptomatic HIV disease: a comparison of simultaneous and alternating regimens. *AIDS* **11**:59–66.
- Chen ZS, Kawabe T, Ono M, Aoki S, Sumizawa T, Furukawa T, Uchiumi T, Wada M, Kuwano M, and Akiyama S (1999) Effect of multidrug resistance-reversing agents on transporting activity of human canalicular multispecific organic anion transporter. *Mol Pharmacol* **56**:1219–1228.
- Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, and Ross DD (1998) A novel multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* **95**:15665–15670.
- Flens M, Izquierdo MA, Scheffer GL, Fritz JM, Meijer CJ, Scheper RJ, and Zaman GJ (1994) Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* **54**:4557–4563.
- Gendelman HE, Lipton SA, Tardieu M, Bukrinsky MI, and Nottet HS (1994) The neuropathogenesis of HIV-1 infection. *J Leukoc Biol* **56**:389–398.
- Geraci AP and Simpson DM (2001) Neurological manifestations of HIV-1 infection in the HAART era. *Compr Ther* **27**:232–241.
- Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, and Deeley RG (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* **54**:357–361.
- Groschel B, Cinatl J, and Cinatl J Jr (1997) Viral and cellular factors for resistance against antiviral agents. *Intervirology* **40**:400–407.
- Honjo Y, Hrycyna CA, Yan QW, Medina-Perez WY, Robey RW, van de Laar A, Litman T, Dean M, and Bates SE (2001) Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res* **61**:6635–6639.
- Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, and Sugimoto Y (2002) Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int J Cancer* **97**:626–630.
- Kartner N, Evernden-Porelle D, Bradley G, and Ling V (1985) Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature (Lond)* **316**:820–823.
- Kitazono M, Tomoyuki S, Takebayashi Y, Chen ZS, Furukawa T, Nagayama S, Tani A, Takao S, Aikou T, and Akiyama S (1999) Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. *J Natl Cancer Inst* **91**:1647–1653.
- Krishan A, Sauerteig A, Andritsch I, and Wellham L (1997) Flow cytometric analysis of the multiple drug resistance phenotype. *Leukemia* **11**:1138–1146.
- Lavie A, Schlichting I, Vetter IR, Konrad M, Reinstein J, and Goody RS (1997) The bottleneck in AZT activation. *Nat Med* **3**:922–924.
- Lee K, Klein-Szanto AJ, and Kruh GD (2000) Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. *J Natl Cancer Inst* **92**:1934–1940.
- Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, and Schellens JH (2001) Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res* **61**:3458–3464.
- Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, et al. (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* **59**:8–13.
- Miyoshi I, Taguchi H, Kubonishi I, Yoshimoto S, Ohtsuki Y, Shiraishi Y, and Akagi T (1982) Type C virus-producing cell lines derived from adult T cell leukemia. *Gann Monogr* **28**:219–228.
- Nakagawa M, Schneider E, Dixon KH, Horton J, Kelley K, Morrow C, and Cowan KH (1992) Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells. *Cancer Res* **52**:6175–6181.
- Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J, and De Clercq E (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* **20**:309–321.
- Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb DS, Hao Z, Mitsuya H, Johns DG, and Broder S (1989) Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. *J Exp Med* **169**:933–951.
- Rabindran SK, He H, Singh M, Brown E, Collins KI, Annable T, and Greenberger LM (1998) Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res* **58**:5850–5858.
- Rabindran SK, Ross DD, Dolye LA, Yang WD, and Greenberger LM (2000) Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* **60**:47–50.
- Rocchi E, Khodjakov A, Volk EL, Yang CH, Litman T, Bates SE, and Schneider E (2000) The product of the ABC half-transporter gene ABCG2 (BCRP/MXP/ABCP) is expressed in the plasma membrane. *Biochem Biophys Res Commun* **271**:42–46.
- Sacktor N, Lyles RH, Skolasky R, Kleiberger C, Selnes OA, Miller EN, Becker JT, Cohen B, and McArthur JC (2001) HIV-associated neurologic disease incidence changes: Multicenter AIDS Cohort Study, 1990–1998. *Neurology* **56**:257–260.
- Sauerbrey A, Sell W, Steinbach D, Voigt A, and Zintl F (2002) Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia. *Br J Haematol* **118**:147–150.
- Schinazi RF, Larder BA, and Mellors JW (2000) Mutations in retroviral genes associated with drug resistance: 2000–2001 update. *Int Antivir News* **8**:65–91.
- Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kumar A, and Fridland A (1999) MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* **5**:1048–1051.
- Srinivas RV, Middlemas D, Flynn P, and Fridland A (1998) Human immunodeficiency virus protease inhibitors serve as substrates for multidrug transporter proteins MDR1 and MRP1 but retain antiviral efficacy in cell lines expressing these transporters. *Antimicrob Agents Chemother* **42**:3157–3162.
- Swanstrom R and Erona J (2000) Human immunodeficiency virus type-1 protease inhibitors: therapeutic successes and failures, suppression and resistance. *Pharmacol Ther* **86**:145–170.
- Taguchi Y, Yoshida A, Takada Y, Komano T, and Ueda K (1997) Anti-cancer drugs and glutathione stimulate vanadate-induced trapping of nucleotide in multidrug resistance-associated protein (MRP). *FEBS Lett* **401**:11–14.
- Washington CB, Duran GE, Man MC, Sikic BI, and Blaschke TF (1998) Interaction of anti-HIV protease inhibitors with the multidrug transporter P-glycoprotein (P-gp) in human cultured cells. *J Acquir Immune Defic Syndr Hum Retrovir* **19**:203–209.
- Yusa K, Oh-hara T, Yamazaki A, Tsukahara S, Satoh W, and Tsuruo T (1990) Cross-resistance to anti-HIV nucleoside analogs in multidrug-resistant human cells. *Biochem Biophys Res Commun* **169**:986–990.

Address correspondence to: Dr. Masanori Baba, Division of Human Retroviruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan. E-mail: baba@m.kufm.kagoshima-u.ac.jp
