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Induction of cellular resistance to nucleoside reverse transcriptase inhibitors by the wild-type breast cancer resistance protein

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Abstract

Breast cancer resistance protein (BCRP/ABCG2) is a novel member of ATP-binding cassette transporters, which induce multidrug resistance in cancer cells. We previously reported that a high level of BCRP expression in $CD4^+$ T cells conferred cellular resistance to nucleoside reverse transcriptase inhibitors (NRTIs) of human immunodeficiency virus type 1 (HIV-1). However, this BCRP was found to have a mutation of Arg to Met at position 482 ($BCRP_{R482M}$). The present study demonstrated that the wild-type BCRP ($BCRP_{WT}$) also conferred cellular resistance to NRTIs. MT-4 cells (a $CD4^+$ T-cell line) highly expressing $BCRP_{WT}$ (MT-4/BCRP) were generated and the expression of $BCRP_{WT}$ was confirmed by genotypic and phenotypic analyses. Compared to the parental MT-4 cells, MT-4/BCRP cells displayed resistance to zidovudine (AZT) in terms of antiviral activity as well as drug cytotoxicity. In addition, other NRTIs were also less inhibitory to HIV-1 replication in MT-4/BCRP cells than in MT-4 cells. Significant reduction of intracellular AZT accumulation was observed in MT-4/BCRP cells. An analysis for intracellular metabolism of AZT suggested that the resistance was attributed to the increased efflux of AZT and its metabolites in MT-4/BCRP cells. Furthermore, the BCRP-specific inhibitor fumitremorgin C completely restored the reduction of AZT in MT-4/BCRP cells. These results indicate that, like $BCRP_{R482M}$, $BCRP_{WT}$ also plays an important role in cellular resistance to NRTIs.

Keywords: BCRP/ABCG2; Wild type; T-cell line; HIV-1; NRTI; Resistance

1. Introduction

Significant advances in the treatment of human immunodeficiency virus type 1 (HIV-1) infection have been achieved with highly active antiretroviral therapy (HAART), which is conducted by combination with drugs that block different steps in the viral replication cycle, such as reverse transcription and protein processing. However, the decrease of efficacy with increasing a treatment period becomes a major concern associated with long-term therapy with antiretroviral agents. In fact, HAART often results in treatment failure due to the emergence of drug-resistant HIV-1 mutants [1]. On the other hand, some patients display a sign of drug resistance without emergence of resistant mutants [2], suggesting that certain cellular factors in part account for the failure of antiretroviral therapy.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; ABC, ATP-binding cassette; P-gp, P-glycoprotein; PI, protease inhibitor; CNS, central nervous system; AZT, zidovudine; MRP, multidrug resistance protein; NRTI, nucleoside reverse transcriptase inhibitor; PMEA, phosphornyl-methoxyethyladenine; BCRP, breast cancer resistance protein; DOX, doxorubicin; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; APMSF, *p*-amindinophenylmethanesulfonylfluoride; mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IC_{50}, 50% inhibitory concentration; EC_{50}, 50% effective concentration; AZTMP, AZT 5′-monophosphate; AZTDP, AZT 5′-diphosphate; AZTTP, AZT 5′-triphosphate; NNRTI, non-nucleoside reverse transcriptase inhibitor; BBB, blood–brain barrier

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A common mechanism of resistance to anticancer agents is drug-induced expression of the ATP-binding cassette (ABC) transporters, which act as an ionic pump and prevent intracellular accumulation of anticancer agents in tumor cells. P-glycoprotein (P-gp/ABCB1), one of the ABC transporters, has proved to be an important determinant for the oral bioavailability of HIV-1 protease inhibitors (PIs) and their entrance into central nervous system (CNS). P-gp also appears to affect drug penetration into other tissues serving as sanctuaries for HIV-1 [3]. It was reported that overexpression of P-gp was associated with reduced antiviral activity of zidovudine (AZT) against HIV-1 [4]. In addition, overexpression of multidrug resistance protein (MRP/ABCC) 4 was found to severely impair the anti-HIV-1 activity of AZT and other nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), such as phosphonyl-methoxyethyladenine (PMEA) [5].

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 [6]. BCRP is the second member of the G (white) subfamily of ABC transporters and is also known as the mitoxantrone-resistance protein MXR [7] or the placental ABC transporter ABCP [8]. This glycosylated plasma membrane protein is a half-size transporter evolutionarily distinct from other full-size ABC transporters [9]. Cells highly expressing BCRP showed resistance to mitoxantrone, and to a lesser extent, to DOX, daunorubicin, and topotecan. We have recently established a DOX-resistant $CD4^+$ T-cell line MT-4/DOX_{500} and found that the cells are expressing a high level of BCRP but not other multidrug resistance proteins [10]. This BCRP was found to have a mutation of Arg to Met at position 482 ($BCRP_{R482M}$). Using this cell line, it was demonstrated that high level expression of $BCRP_{R482M}$ in $CD4^+$ T cells brought about reduced anti-HIV-1 activity of AZT and lamivudine (3TC). However, it still remains to be elucidated whether the wild-type BCRP ($BCRP_{WT}$) similarly interacts with NRTIs and affects their antiviral activity and cytotoxicity.

In the present study, we have generated MT-4 cells highly expressing $BCRP_{WT}$ (MT-4/BCRP) and examined NRTIs for their cytotoxicity and anti-HIV-1 activity in MT-4/BCRP cells. The results clearly show that, like $BCRP_{R482M}$, $BCRP_{WT}$ also confers cellular resistance to NRTIs through increasing drug efflux from the cells.

2. Materials and methods

2.1. Compounds

DOX, paclitaxel, mitoxantrone, and rhodamine 123 were purchased from Sigma. 7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38) were obtained from Daiichi Pharmaceuticals. AZT, stavudine (d4T), didanosine (ddI), and zalcitabine (ddC) were also purchased from Sigma. 3TC was kindly provided by Mitsubishi Chemical Corporation. The BCRP-specific inhibitor fumitremorgin C [11,12] was a generous gift from Dr. Rabindran, Wyeth-Ayerst Research.

2.2. Cells

The human $CD4^+$ T-cell line MT-4 [13] was grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin (culture medium). MT-4 cells were transduced with an HaBCRP retrovirus supernatant, and the cells were treated and maintained with 4 ng/ml mitoxantrone [14]. The obtained mitoxantrone-resitant cells, MT-4/BCRP, were used in this study. MT-4/DOX_{500} cells [10] were maintained in the presence of 500 ng/ml DOX. Prior to cytotoxicity and antiviral assays, MT-4/BCRP and MT-4/DOX_{500} cells were cultured in the absence of any compounds at least for 7 days.

2.3. 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 [15].

2.4. Drug accumulation and retention assay

Intracellular drug accumulation and retention in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells were determined by a slight modification of the flow cytometric method described previously [16]. Briefly, the cells were exposed to either 10 μg/ml DOX, 10 μg/ml mitoxantrone, or 100 ng/ml rhodamine 123 for 30 min (accumulation phase) and washed with ice-cold phosphate buffered saline (PBS). The cells were resuspended in culture medium in the absence of compounds, further incubated for 60 min at 37 °C (retention phase), and analyzed for intracellular drug concentrations by flow cytometry (FACScan™, Becton Dickinson). To determine the effect of fumitremorgin C, the cells were exposed to 10 μg/ml mitoxantrone in the absence or presence of 5 μM fumitremorgin C for 20 min at 37 °C, and its intracellular concentration was evaluated by flow cytometry.

2.5. Preparation of crude membrane fractions

Crude membrane fractions were prepared from MT-4 and MT-4/BCRP cells, according to the method described previously [17,18]. 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 $MgCl_2$, 10 mM Tris–HCl (pH 7.4), 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM *p*-amindinophenylmethanesulfonylfluoride (APMSF), and 2 μg/ml aprotinin]. After 10 min on ice, the cells were homogenized with approximately 80 strokes of Dounce homogenizer. The intact cells and nuclei in the homogenate were removed by centrifugation (1500 × *g*) for 10 min. To prepare membrane-enriched fractions, the supernatants were ultracentrifuged (100,000 × *g*) 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 APMSF]. Protein concentrations were determined by Bradford's method [19], and each protein was kept at −80 °C until use.

2.6. Western blot analysis

The crude membranes were subjected to the analysis for BCRP expression. An anti-BCRP antibody was prepared, according to the procedures described previously [14]. The antibody was generated by immunizing rabbits with a peptide that corresponds to amino acids 340–359 of the human BCRP protein. For Western blot analysis, the extracted proteins (100 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride membrane [10]. The transferred proteins were treated with the anti-BCRP antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG monoclonal antibody (Amersham Pharmacia Biotech). Antibody binding was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech).

2.7. Anti-HIV-1 assay

The activity of the compounds against HIV-1 replication was based on the inhibition of virus-induced cytopathicity in MT-4 and MT-4/BCRP cells, as previously described [20]. Briefly, the cells (1×10^5 cells/ml) were infected with HIV-1 (III_B strain) at a multiplicity of infection of 0.3 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.

2.8. Determination of intracellular concentration of AZT and its metabolites

MT-4 and MT-4/BCRP cells were treated with [methyl-^{3}H]AZT (15.4 Ci/mmol) for different periods of time, rapidly washed with ice-cold buffer, and extracted with 60% (v/v) methanol. The methanol extracts were heated at 95 °C for 1.5 min, and their radioactivity was determined. The intracellular concentrations of AZT and its metabolites was also determined in MT-4 and MT-4/BCRP cells exposed to [methyl-^{3}H]AZT at various concentrations ranging from 62.5 to 1000 nM.

2.9. Effect of fumitremorgin C on the efflux of AZT and its metabolites

The effect of fumitremorgin C on the efflux of intracellular AZT and its metabolites from MT-4 and MT-4/BCRP were analyzed by a slight modification based on high performance liquid chromatography (HPLC), as previously reported [21]. Five million cells were incubated with 1 μM [methyl-^{3}H]AZT in the absence or presence of fumitremorgin C (5 μM). After a 2-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 heated at 95 °C for 1.5 min. The extracts were clarified by centrifugation (12,000 × *g*) for 6 min. Separation and detection of AZT and its metabolites were performed with a 25-cm Whatman Partisil-10 SAX column (Gilson) by HPLC. After injection of the samples (25 μl), a buffer gradient was applied, starting at zero time with 5 mM potassium phosphate and increasing linearly to 750 mM potassium phosphate over 55 min at 1 ml/min. Then, 750 mM potassium phos-phate was further pumped for 10 min. The elution was fractionated at 1-min intervals (1 ml) and analyzed for radioactivity.

3. Results

3.1. Establishment of MT-4/BCRP cells

To determine whether $BCRP_{WT}$ affects the anti-HIV-1 activity and cytotoxicity of NRTIs, we have established MT-4/BCRP cells expressing a high level of $BCRP_{WT}$. $BCRP_{WT}$ in the crude membrane fractions of the cells was detected with the anti-BCRP polyclonal antibody 3488. As show in Fig. 1, a limited level of BCRP protein was expressed in the parental MT-4 cells, while a high level of BCRP protein expression was observed in MT-4/BCRP cells. Sequence analysis for the full-length BCRP cDNA obtained from MT-4 and MT-4/BCRP cells revealed that only $BCRP_{WT}$ mRNA was expressed in these cell lines (data not shown).

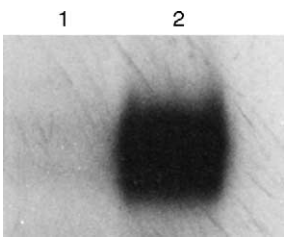

Fig. 1. BCRP expression in MT-4 and MT-4/BCRP cells. Crude membranes (100 μg) from MT-4 and MT-4/BCRP cells were separated by SDS-PAGE and transferred to a polyvinyldenedifluoride membrane. The transferred proteins were immunoblotted with a mAb against human BCRP. Lanes 1 and 2 are the proteins obtained from MT-4 and MT-4/BCRP cells, respectively.

Table 1
Cytotoxicity of anticancer agents and AZT in MT-4, MT-4/BCRP, and MT-4/DOX500 cells[a]

Compound	IC_{50}[b]		
	MT-4	MT-4/BCRP	MT-4/DOX_{500}
Doxorubicin (ng/ml)	4.5 ± 1.0	7.2 ± 2.1	653 ± 25
Mitoxantrone (ng/ml)	0.10 ± 0.07	22 ± 6.2	678 ± 172
SN-38 (μM)	4.1 ± 0.1	44 ± 8.5	133 ± 32
CPT-11 (μM)	2.3 ± 0.2	14 ± 3.7	78 ± 21
Paclitaxel (ng/ml)	0.89 ± 0.16	1.03 ± 0.24	0.77 ± 0.23
AZT (μM)	94 ± 16	154 ± 12	240 ± 12

[a] The cells were cultured for 4 days in the presence of the compounds. The number of viable cells was determined by the MTT method, as described in Section 2. All data represent means ± standard deviations for three separate experiments.

[b] Fifty percent inhibitory concentration, required to reduce cell proliferation and viability by 50%.

3.2. Resistance profile of MT-4/BCRP cells

Several anti-cancer agents were examined for their cytotoxicity in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells. The level of BCRP expression was approximately 10-fold lower in MT-4/BCRP cells than that in MT-4/DOX_{500} cells (data not shown), although the BCRPs expressing in the former and the latter were $BCRP_{WT}$ and $BCRP_{R482M}$, respectively. MT-4/DOX_{500} cells were found to be resistant to DOX, mitoxantrone, CPT-11, and SN-38 but remained sensitive to paclitaxel (Table 1), which was in agreement with our previous results [10]. MT-4/BCRP cells proved to be resistant to mitoxantrone, CPT-11 and SN-38 but not resistant to paclitaxel. Furthermore, MT-4/BCRP cells showed little, if any, resistance to DOX compared with the parental MT-4 cells (Table 1).

To further confirm the difference in substrate specificity between $BCRP_{WT}$ and $BCRP_{R482M}$, the intracellular accumulation and retention of rhodamine 123, DOX, and mitoxantrone were examined in MT-4/BCRP and MT-4/DOX_{500} cells by flow cytometry. The intracellular accumulation and retention of rhodamine 123 and DOX were not apparent in MT-4/BCRP cells (Fig. 2). However, significant reduction of intracellular mitoxantrone was observed in MT-4/BCRP cells after removal of the compound from culture medium. Furthermore, much lower levels of rhodamine 123, DOX, and mitoxantrone were identified in MT-4/DOX_{500} cells compared with MT-4 and MT-4/BCRP cells (Fig. 2). The BCRP-specific inhibitor fumitremorgin C was found to increase the mitoxantrone accumulation up to a similar level in all of the three cell lines (Fig. 3). These results were in agreement with those reported in several previous studies on the substrate specificity of $BCRP_{WT}$ and mutant BCRPs.

3.3. Cytotoxicity and anti-HIV-1 activity of NRTIs in MT-4/BCRP cells

When the cytotoxicity of AZT was evaluated in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells, its 50% inhibitory concentrations (IC_{50}s) were 94, 154, and 240 μM, respectively (Table 1). The IC_{50} of other NRTIs could not be determined because of their low cytotoxicity in these cell lines (data not shown). The anti-HIV-1 assays were also conducted for AZT and other NRTIs to determine whether

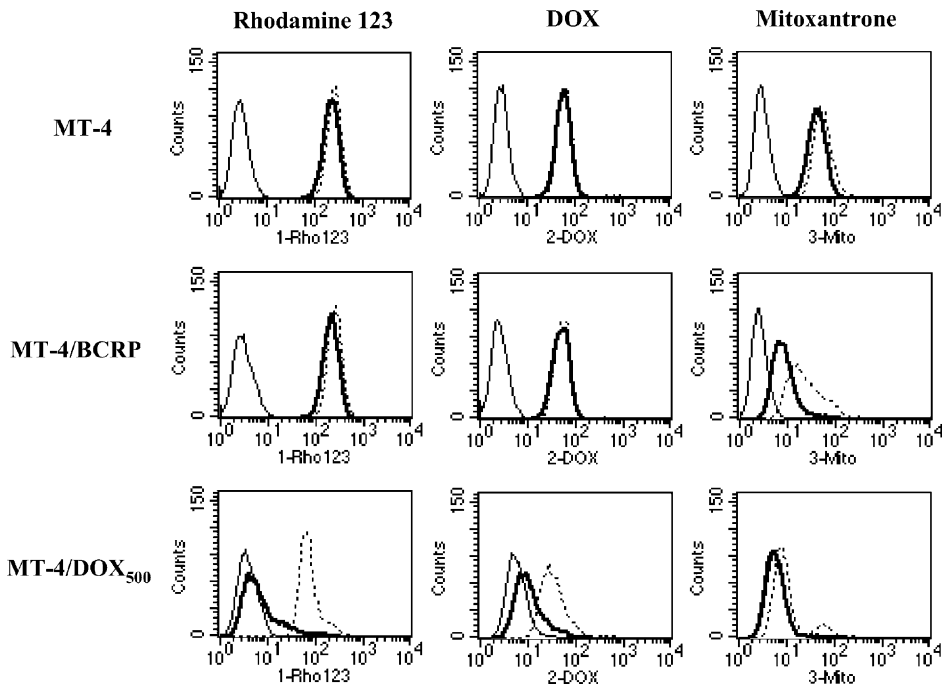

Fig. 2. Accumulation and retention of DOX, mitoxantrone, or rhodamine 123 in MT-4 and MT-4/BCRP cells. The cells were incubated for 30 min in media alone (—), or incubated with 10 μg/ml DOX, 10 μg/ml mitoxantrone, or 100 ng/ml rhodamine 123 (- - -); washed, and further incubated for 60 min in media alone (▬). The intracellular DOX, mitoxantrone, or rhodamine 123 concentration was determined by flow cytometry.

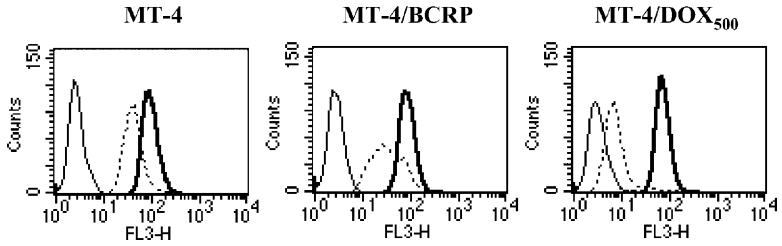

Fig. 3. Accumulation of mitoxantrone in MT-4, MT-4/BCRP, and MT-4/DOX_{500} in the absence or presence of fumitremorgin C. The cells were incubated for 60 min with 10 μg/ml mitoxantrone in the absence (- - -) or presence of 5 μM fumitremorgin C (▬). The intracellular concentrations of mitoxantrone were determined by flow cytometry.

Table 2
Anti-HIV-1 activity of NRTIs in MT-4 and MT-4/BCRP cells[a]

Compound	EC_{50}[b] (μM)		Fold resistance to MT-4[c]	
	MT-4	MT-4/BCRP	MT-4/BCRP	MT-4/DOX_{500}[d]
AZT	0.013 ± 0.004	0.032 ± 0.020 *	2.5	7.5
3TC	0.88 ± 0.34	1.58 ± 0.41*	1.8	>77
ddI	11.6 ± 1.6	62.7 ± 28.5*	5.4	2.7
ddC	1.3 ± 0.6	2.4 ± 0.4*	1.9	n.d.[e]
D4T	0.29 ± 0.11	0.36 ± 0.16	1.2	1.6

[a] The infected cells were cultured for 4 days in the presence of the compounds. The number of viable cells was determined by the MTT method, as described in Section 2. All data represent means ± standard deviations for five separate experiments. The statistical significance between the EC_{50} of MT-4 cells and EC_{50} of MT-4/BCRP cells was determined by the *t*-test.
[b] Fifty percent effective concentration, required to reduce cell HIV-1-induced cytopathicity by 50%.
[c] Fold resistance, the ratio of EC_{50} in MT-4 cells to EC_{50} in MT-4/BCRP cells or the ratio of EC_{50} in MT-4 cells to EC_{50} in MT-4/DOX_{500} cells.
[d] Data are taken from [10].
[e] Not determined.
* $P < 0.05$.

their activities were also affected by BCRP. AZT proved 2.5-fold less inhibitory to HIV-1 replication in MT-4/BCRP cells than in MT-4 cells (Table 2). The 50% effective concentrations (EC_{50}) of AZT were 0.013 and 0.032 μM in MT-4 and MT-4/BCRP cells, respectively. Furthermore, the activities of 3TC, ddI, and ddC were also impaired to some extent (1.8–5.4-fold) in MT-4/BCRP cells (Table 2). These results indicate that, like $BCRP_{R482M}$, the expression of $BCRP_{WT}$ is associated with cellular resistance to NRTIs.

3.4. AZT accumulation in MT-4/BCRP cells

It was assumed that the resistance of MT-4/BCRP cells to NRTIs could be attributed to the reduction of intracellular drug concentration. A steady-state level of AZT and its metabolites was already achieved after 10 min of drug exposure (Fig. 4). No significant difference was observed between MT-4 and MT-4/BCRP cells at this time point. When the cells were further incubated in the presence of AZT, the intracellular drug concentration retained at the same level in MT-4 cells until 2 h and gradually decreased thereafter. On the other hand, the intracellular drug concentration started to decrease at 30 min in MT-4/BCRP cells and further decreased over an incubation period. The intracellular drug concentrations in MT-4/BCRP cells were always less than half of those in MT-4 cells after 1 h (Fig. 4). Furthermore, similar results were also obtained in MT-4/BCRP cells after a 2 h-exposure to various concentrations of AZT (Fig. 5).

3.5. The effect of the BCRP-specific inhibitor fumitremorgin C

To gain insight into the mechanism of increased efflux of AZT and its metabolites in MT-4/BCRP cells, the intracellular metabolism of AZT was investigated in MT-4 and MT-4/BCRP cells. An HPLC analysis revealed that the intracellular levels of AZT and its metabolites, especially

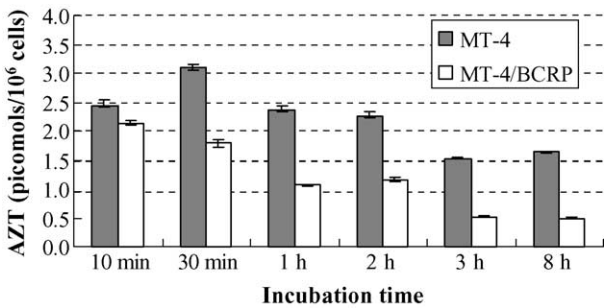

Fig. 4. Intracellular uptake of [methyl-^{3}H]AZT in MT-4 and MT-4/BCRP cells. MT-4 (closed bars) and MT-4/BCRP (open bars) cells were incubated in the presence of 0.5 μM [methyl-^{3}H]AZT. At the indicated times, the cells were extensively washed with ice-cold PBS. Fractions extracted with methanol were determined for their radioactivity. The results represent the means ± standard derivations in three independent samples.

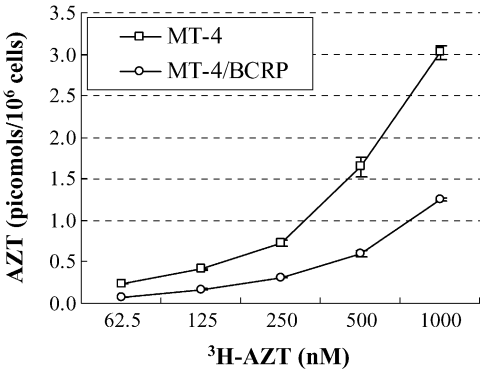

Fig. 5. Concentration-dependent intracelluar uptake of [methyl-^{3}H]AZT in MT-4 and MT-4/BCRP cells. MT-4 (open square) and MT-4/BCRP (open circle) cells were incubated in the presence of various concentrations of [methyl-^{3}H]AZT. After 2 h, the cells were extensively washed with ice-cold PBS. Fractions extracted with methanol were determined for their radioactivity. The points and error bars represent the means ± standard derivations in three independent samples.

Table 3
Effects of fumitremorgin C on intracellular concentrations of AZT and its metabolites in MT-4 and MT-4/BCRP cells[a]

Cells	Fumitremorgin C	Concentration (pmol/10^6 cells)			
		AZT	AZTMP	AZTDP	AZTTP
MT-4	(−)	0.135	1.461	0.101	0.076
MT-4	(+)	0.160	1.596	0.112	0.080
MT-4/BCRP	(−)	0.071	0.410	0.046	0.066
MT-4/BCRP	(+)	0.180	1.599	0.098	0.084

[a] The cells were incubated with [methyl-^{3}H]AZT in the absence or presence of fumitremorgin C (5 μM). After a 2-h incubation, the cells were subjected to HPLC analysis, as described in Section 2. Intracellular concentrations of AZT and its metabolites are expressed as pmol/10^6 cells.

AZT 5′-monophosphate (AZTMP), were highly diminished in MT-4/BCRP cells (Table 3). Only 28% of the AZTMP level in MT-4 cells was identified in MT-4/BCRP cells. In addition, the levels of AZT, AZT 5′-diphosphate (AZTDP), and AZT 5′-triphosphate (AZTTP) were also 52.8, 45, and 86.6% of those in MT-4 cells, respectively (Table 3). The BCRP-specific inhibitor fumitremorgin C could completely restore the decreased levels of AZT and its metabolites in MT-4/BCRP cells at a concentration of 5 μM (Table 3). Fumitremorgin C did not affect the viability and proliferation of MT-4 and MT-4/BCRP cells at this concentration (data not shown). These results suggest that the reduced anti-HIV-1 activity of AZT in MT-4/BCRP cells is due to the increased efflux of AZT and its metabolites by $BCRP_{WT}$ and subsequent decrease of their intracellular concentrations.

4. Discussion

Host cellular factors are assumed to be in part involved in the resistance to anti-retroviral agents [2,22]. One of such factors is the ABC transporter family, which acts as an ionic pump and prevents intracellular accumulation of various drugs. Among the ABC transporters, P-gp was found to be an important determinant for oral bioavailability and CNS penetration of PIs [3]. In addition, it was reported that the overexpression of MRP4 in $CD4^+$ T cells increased the efflux of some NRTIs from the cells and reduced their anti-HIV-1 activity [5]. However, the role of other ABC transporters in NRTI-resistance is still unknown and remains to be elucidated.

We have recently demonstrated that high level of $BCRP_{R482M}$ expression in $CD4^+$ T cells brings about reduced anti-HIV-1 activity of NRTIs but not non-nucleoside reverse transcriptase inhibitors (NNRTIs) or PIs [10]. The arginine at position 482 (R482) is considered to play a crucial role in BCRP function; point mutation of this amino acid residue may significantly change the substrate specificity of BCRP and the phenotype of drug resistance [23]. In fact, this study has shown clear difference in cytotoxicity and efflux of drugs between $BCRP_{WT}$ and $BCRP_{R482M}$ (Table 1 and Figs. 2 and 3). Therefore, it would be possible that the NRTI-resistance could be conferred only by $BCRP_{R482M}$ but not by $BCRP_{WT}$. To exclude this possibility, we have generated the $CD4^+$ T-cell line (MT-4/BCRP) expressing a high level of $BCRP_{WT}$ and examined whether it also affects the anti-HIV-1 activity and cytotoxicity to NRTIs. The present study has clearly demonstrated that, like $BCRP_{R482M}$, $BCRP_{WT}$ also diminishes the anti-HIV-1 activity of NRTIs. Although the reduction rate (fold resistance) seems to be considerably smaller in MT-4/BCRP cells than in MT-4/DOX_{500} cells [10], this may be in part attributed to the 10-fold higher expression of BCRP in MT-4/DOX_{500} cells than in MT-4/BCRP cells. In fact, apart from DOX (a substrate of $BCRP_{R482M}$ but not $BCRP_{WT}$), MT-4/DOX_{500} was found to be much more resistant to mitoxantrone, SN-38, and CPT-11 (Table 1). Furthermore, experiments using MT-4/DOX_{100} cells [10], which express an almost identical level of $BCRP_{R482M}$ compared to the $BCRP_{WT}$ level in MT-4/BCRP cells, revealed that AZT was 3.3-fold less inhibitory to HIV-1 replication in MT-4/DOX_{100} cells than in MT-4 cells (data not shown). Since the anti-HIV-1 activity of AZT was 2.5-fold lower in MT-4/BCRP cells than in MT-4 cells (Table 2), these results suggest that $BCRP_{WT}$ interacts similarly with nucleoside analogs.

HIV-1 has been found in several tissues in vivo and can infect many different types of human cells in vitro. BCRP has also been detected in some normal tissues, including placenta, liver, breast, and venous and capillary endothelium [24]. More importantly, BCRP mRNA could be detected in bone marrow and peripheral blood mononuclear cells [25]. If HIV-1 could upregulate BCRP expression in the infected cells, the intracellular NRTI concentrations would decrease in these tissues, resulting in insufficient suppression of HIV-1 replication and increasing opportunity for the emergence of drug-resistant mutants.

In the brain, HIV-1 infection is restricted, in most cases, to microglia and brain macrophages. The CNS disorders associated with HIV-1 infection, such as encephalopathy and dementia, occur at the late stage of the disease [26]. The development of HAART has decreased the incidence rates for HIV-1-associated encephalopathy and dementia, nevertheless its impact on the future incidence and course of dementia remains debatable [27,28]. Effective treatment of HIV-1-associated dementia or encephalopathy needs efficient distribution of anti-HIV-1 agents into the CNS. The blood–brain barrier (BBB) is well recognized to play a crucial role in restricting the penetration of many drugs and toxins into the brain from the systemic circulation. In the case of PIs, P-gp expression in the BBB enhances the elimination of PIs from CNS, and specific inhibitors of P-gp are able to dramatically increase their penetration into the CNS [29].

Limited distribution of AZT, ddI, and related nucleoside derivatives in the CNS has been demonstrated after their systemic administration [30–32]. Although several forms of organic anion transporters have substantial activities for NRTI transport into the brain, the role of the ABC transporters in penetration of NRTIs through the BBB has not fully been understood yet [32–35]. It was reported that MRP4 acted as a transporter of some NRTIs, such as AZTMP and PMEA [5]. However, even now, there are no solid evidences of MRP4 expression in the BBB. Recently, the brain multidrug resistance protein was discovered in the porcine BBB, and the protein was shown to be highly homologous to the human and mouse BCRP [36,37]. BCRP was also identified in the BBB of both normal and tumor human brain tissues and mainly located at the luminal surface of microvessel endothelium [38]. Although the extracellular concentrations of AZT and its metabolites were not measured in this study, it is assumed that not only $BCRP_{R482M}$ but also $BCRP_{WT}$ are involved in the efflux of AZT and its metabolites, presumably AZTMP [5]. The effect of $BCRP_{WT}$ on the intracellular concentrations of AZT and AZTMP were completely restored by the presence of the BCRP-specific inhibitor fumitremorgin C, also suggesting this possibility (Table 3). Additional experiments are still required to determine whether the same result can be obtained for other NRTIs in the presence of fumitremorgin C. However, if the expression of $BCRP_{WT}$ in the BBB may commonly restrict the entry of NRTIs into the brain, it seems important to reduce the affinity of anti-HIV-1 agents for the ABC transporters to achieve their sufficient concentrations in the brain. A strategy involving either chemical modification of existing anti-HIV-1 agents or discovery of a selective inhibitor of the ABC transporters is needed for effective treatment of the CNS disorders associated with HIV-1 infection. In this point of view, BCRP inhibitors, such as fumitremorgin C, may be useful to improve the entry of NRTIs into the brain and increase their concentrations in the cerebrospinal fluid [11,12].

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References

[1] Berger EA, Moss B, Pastan I. Reconsidering targeted toxins to eliminate HIV infection: you gotta have HAART. Proc Natl Acad Sci USA 1998;95:11511–3.
[2] Groschel B, Cinatl J, Cinatl Jr J. Viral and cellular factors for resistance against antiretroviral agents. Intervirology 1997;40:400–7.
[3] Kim RB. Drug transporters in HIV therapy. Top HIV Med 2003;11:136–9.
[4] Antonelli G, Turriziani O, Cianfriglia M, Riva E, Dong G, Fattorossi A, et al. Resistance of HIV-1 to AZT might also involve the cellular expression of multidrug resistance P-glycoprotein. AIDS Res Hum Retroviruses 1992;8:1839–44.
[5] Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, et al. A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999;5:1048–51.
[6] Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998;95:15665–70.
[7] Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res 1999;59:8–13.
[8] Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 1998;58:5337–9.
[9] Rocchi E, Khodjakov A, Volk EL, Yang CH, Litman T, Bates SE, et al. The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. Biochem Biophys Res Commun 2000;271:42–6.
[10] Wang X, Furukawa T, Nitanda T, Okamoto M, Sugimoto Y, Akiyama S, et al. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. Mol Pharmacol 2003;63:65–72.
[11] Rabindran SK, He H, Singh M, Brown E, Collins KI, Annable T, et al. Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. Cancer Res 1998;58:5850–8.
[12] Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 2000;60:47–50.
[13] Miyoshi I, Taguchi H, Kubonishi I, Yoshimoto S, Ohtsuki Y, Shiraishi Y, et al. Type C virus-producing cell lines derived from adult T cell leukemia. Gann Monogr 1982;28:219–28.
[14] Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, et al. Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S–S dependent homodimerization. Int J Cancer 2002;97:626–30.
[15] Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, et al. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods 1988;20:309–21.
[16] Krishan A, Sauerteig A, Andritsch I, Wellham L. Flow cytometric analysis of the multiple drug resistance phenotype. Leukemia 1997;11:1138–46.
[17] Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SP, Deeley RG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res 1994;54:357–61.

[18] Nakagawa R, Hara Y, Arakawa H, Nishimura S, Komatani H. ABCG2 confers resistance to indolocarbazole compounds by ATP-dependent transport. Biochem Biophys Res Commun 2002;299:669–75.
[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[20] Baba M, De Clercq E, Tanaka H, Ubasawa M, Takashima H, Sekiya K, et al. 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 1991;88:2356–60.
[21] Perno CF, Yarchoan R, Cooney DA, Hartman NR, Webb DS, Hao Z, et al. 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′,3′-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. J Exp Med 1989;169:933–51.
[22] Swanstrom R, Erona J. Human immunodeficiency virus type-1 protease inhibitors: therapeutic successes and failures, suppression and resistance. Pharmacol Ther 2000;86:145–70.
[23] Honjo Y, Hrycyna CA, Yan QW, Medina-Perez WY, Robey RW, van de Laar A, et al. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. Cancer Res 2001;61:6635–9.
[24] Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 2001;61:3458–64.
[25] Sauerbrey A, Sell W, Steinbach D, Voigt A, Zintl F. Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia. Br J Haematol 2002;118:147–50.
[26] Gendelman HE, Lipton SA, Tardieu M, Bukrinsky MI, Nottet HS. The neuropathogenesis of HIV-1 infection. J Leukoc Biol 1994;56:389–98.
[27] Geraci AP, Simpson DM. Neurological manifestations of HIV-1 infection in the HAART era. Compr Ther 2001;27:232–41.
[28] Sacktor N, Lyles RH, Skolasky R, Kleeberger C, Selnes OA, Miller EN, et al. HIV-associated neurologic disease incidence changes: Multicenter AIDS Cohort Study, 1990–1998. Neurology 2001;56:257–60.
[29] Choo EF, Leake B, Wandel C, Imamura H, Wood AJ, Wilkinson GR, et al. Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. Drug Metab Dispos 2000;28:655–60.
[30] Anderson BD, Hoesterey BL, Baker DC, Galinsky RE. Uptake kinetics of 2′,3′-dideoxyinosine into brain and cerebrospinal fluid of rats: intravenous infusion studies. J Pharmacol Exp Ther 1990;253:113–8.
[31] Brouwers P, Hendricks M, Lietzau JA, Pluda JM, Mitsuya H, Broder S, et al. 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 1997;11:59–66.
[32] Masereeuw R, Jaehde U, Langemeijer MW, de Boer AG, Breimer DD. In vitro and in vivo transport of zidovudine (AZT) across the blood–brain barrier and the effect of transport inhibitors. Pharm Res 1994;11:324–30.
[33] Dykstra KH, Arya A, Arriola DM, Bungay PM, Morrison PF, Dedrick RL. Microdialysis study of zidovudine (AZT) transport in rat brain. J Pharmacol Exp Ther 1993;267:1227–36.
[34] Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, et al. Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. J Biol Chem 1999;274:13675–80.
[35] Wong SL, Van Belle K, Sawchuk RJ. Distributional transport kinetics of zidovudine between plasma and brain extracellular fluid/cerebrospinal fluid in the rabbit: investigation of the inhibitory effect of probenecid utilizing microdialysis. J Pharmacol Exp Ther 1993; 264:899–909.
[36] Eisenblatter T, Galla HJ. A new multidrug resistance protein at the blood–brain barrier. Biochem Biophys Res Commun 2002;293: 1273–8.
[37] Eisenblatter T, Huwel S, Galla HJ. Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the blood–brain barrier. Brain Res 2003;971:221–31.
[38] Cooray HC, Blackmore CG, Maskell L, Barrand MA. Localisation of breast cancer resistance protein in microvessel endothelium of human brain. NeuroReport 2002;13:2059–63.