Impaired 2′,3′-dideoxy-3′-thiacytidine accumulation in T-lymphoblastoid cells as a mechanism of acquired resistance independent of multidrug resistant protein 4 with a possible role for ATP-binding cassette C11

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Cellular factors may contribute to the decreased efficacy of chemotherapy in HIV infection. Indeed, prolonged treatment with nucleoside analogues, such as azidothymidine (AZT), 2′,3′-deoxycytidine or 9-(2-phosphonylmethoxyethyl)adenine, induces cellular resistance. We have developed a human T lymphoblastoid cell line (CEM_{3TC}) that is selectively resistant to the anti-proliferative effect of 2′,3′-dideoxy-3′-thiacytidine (3TC) because the CEM_{3TC} cells were equally sensitive to AZT, as well as the antimitotic agent, vinblastine. The anti-retroviral activity of 3TC against HIV-1 was also severely impaired in the CEM_{3TC} cells. Despite similar deoxycytidine kinase activity and unchanged uptake of nucleosides such as AZT and 2′-deoxycytidine, CEM_{3TC} had profoundly impaired 3TC accumulation. Further studies indicated that CEM_{3TC} retained much less 3TC. However, despite a small overexpression of multidrug resistance protein (MRP) 4, additional studies with cells specifically engineered to overexpress MRP4 demonstrated there was no impact on either 3TC accumulation or efflux. Finally, an increased expression of the MRP5 homologue, ATP-binding cassette C11 (ABCC11) was observed in the CEM_{3TC} cells. We speculate that the decreased 3TC accumulation in the CEM_{3TC} might be due to the upregulation of ABCC11.

Key words: ABC transporter, HIV, retrovirus, nucleoside analogues.

INTRODUCTION

Long-term anti-retroviral therapy is the main strategy in clinical treatment of HIV-1 infected patients. It is known that the best results in the efficacy of HIV therapy are obtained when various combinations of drugs are administered. Generally, combination anti-retroviral therapies consist of one or more nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors, and or non-nucleoside reverse transcriptase inhibitors [1–3]. In fact, the combination of two nucleoside-based reverse transcriptase inhibitors and a protease inhibitor, referred to as 'highly active anti-retroviral therapy' (HAART), dramatically suppresses plasma HIV-RNA levels to < 50 copies/ml [4–6]. Despite the efficacy of such therapeutic regimens, long-term treatment with HAART leads to the emergence of drug-resistant HIV strains, and genetic mutations in the reverse transcriptase gene have been isolated in many patients treated with HAART [7–9].

The presence of HIV mutations has been associated with virological failure; however, individuals also display signs of drug resistance in the absence of drug-resistant virus [10,11]. This observation is consistent with the concept that 'cellular' factors contribute to the failure of anti-retroviral therapy [12–15]. Indeed, most anti-HIV agents, specifically dideoxynucleosides, are phosphorylated by cellular kinases to compounds that inhibit HIV replication. Consequently, decreasing the cellular levels of these compounds could lead to an inability to suppress viral replication and contribute to the failure of anti-retroviral therapy. In this regard, it has been shown that long-term treatment of cell lines with NRTIs [such as 3′-azido-3′-deoxythymidine (AZT) and 2′-3′-dideoxycytidine (ddC)] results in diminished amounts of the phosphorylated forms of NRTIs. In these cases, decreased activity of the cellular kinases leads to antiviral resistance because of an impaired ability to accumulate phosphorylated metabolites [16–18].

Another cellular mechanism has previously been described to explain decreased drug accumulation and resistance to retroviral inhibitors: the increased efflux of phosphorylated drug [19]. Subsequently, we demonstrated that overexpression of a functionally uncharacterized ATP-binding cassette (ABC) drug-transporter [multidrug resistance protein (MRP) 4] was genetically linked to the decreased drug accumulation and resistance to some, but not all NRTIs [20] (for an overview of the ABC-family members and nomenclature see http://nutrigene.4t.com/humanabc.htm). The ABC transporters are mostly plasma membrane localized and show ATP-dependent transport of a broad range of compounds. Most MRP substrates are organic

Abbreviations used: ABC, ATP-binding cassette; AZT, 3′-azido-3′-deoxythymidine; CNT, concentrative nucleoside carrier; dCK, deoxcytidine kinase; dCyd, deoxycytidine; ddC, 2′-3′-dideoxycytidine; ENT, equilibrative nucleoside carrier; GFP, green fluorescence protein; HAART, highly active anti-retroviral therapy; [^{3}H]Cyd, [5-^{3}H]cytidine; ID_{50}, 50% inhibitory dose; MDR, multidrug resistance; [^{3}H]AZT, [*Me*-^{3}H]AZT; MRP, multidrug resistant protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NRTI, nucleoside reverse transcriptase inhibitor; Pgp, P-glycoprotein; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; RT, reverse transcriptiase; 3TC, 2′-3′-dideoxy-3′-thiacytidine (also called lamivudine); [^{3}H]3TC, [*Me*-^{3}H]3TC.

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anions and they are often conjugated to glutathione, glucuronide or sulphate. Notably, two members of the MRP family (MRP4 and 5) efflux nucleotide analogues such as the nucleotide analogue, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), azidothymidine-monophosphate, and thioguanine-monophosphate [21,22]. Further, the cells that overexpressed MRP4 had decreased antiviral efficacy for 2′,3′-dideoxy-3′-thiacytidine (3TC), a finding strongly implicating MRP4 as a contributor to 3TC cellular resistance. In order to evaluate whether the prolonged treatment with 3TC was able to induce cellular resistance by this mechanism, we cultured a T-lymphoblastoid cell line in the presence of increasing concentrations of this nucleoside analogue. Our findings indicate that these cells acquire stable resistance to 3TC by a mechanism whereby 3TC accumulation is substantially decreased. Furthermore, the cells harbor no defect in the enzyme activating 3TC to a nucleotide, nor is there a general impairment in nucleoside uptake. However, despite a small overexpression of MRP4 in these cells, it is clear that another mechanism is responsible because MCF-7 cells engineered to overexpress MRP4 do not show impaired 3TC accumulation or increased 3TC efflux.

MATERIALS AND METHODS

Chemicals

The 3TC, kindly provided by Glaxo Wellcome (Stevenage, Herts., U.K.), was dissolved in PBS and kept at −20 °C. AZT, [*Me*-^{3}H]AZT ([^{3}H]AZT, 3 Ci/mmol), ddC and [5-^{3}H]cytidine ([^{3}H]Cyd, 17.4 Ci/mmol) were purchased from Sigma Chemical Co (Milan, Italy). [*Me*-^{3}H]3TC ([^{3}H]3TC, 17.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, U.S.A.). Commercial reagents and solvents were of analytical grade, unless otherwise stated. [^{3}H]2′-deoxycytidine ([^{3}H]dCyd, 18–30 Ci/mmol) was from Amersham (Milan, Italy).

Selection of 3TC-resistant cell lines

3TC-resistant cells were obtained by exposure of CEM cells, the parental cell line, to increasing concentrations of 3TC. CEM cells were initially propagated in the presence of 10 μM 3TC. Doubling concentrations of 3TC were added to the culture medium and the cells were allowed to grow until they reached a cell density of 10^6 cells/ml. After approx. 4 months, a stably resistant 3TC CEM line grew in the presence of 1 mM 3TC, with a doubling time similar to non-drug selected CEM cells. These cells were called CEM_{3TC}.

Assay to determine the anti-growth activity of drugs in CEM and CEM_{3TC}

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to evaluate the anti-growth activity of drugs in CEM and CEM_{3TC} [23]. Briefly, CEM and CEM_{3TC} were seeded in 96-well microtitre plates at a concentration of 50000 cells/well. Different concentrations of drugs were added to triplicate cultures. Four days later, 20 μl of MTT solution was added to each well and the cultures were incubated at 37 °C. The viability of cells was examined spectrophotometrically and the values were used to calculate the 50 % toxic concentration (TC_{50}) of the various test compounds.

Assay of drug sensitivity

CEM and CEM_{3TC} cells (3×10^5) were incubated with the HIV-PNL43 strain at a multiplicity of infection ('MOI') of 1 $TCID_{50}$ (50 % tissue culture infectious dose)/cell. After 1 h at 0 °C, the cultures were washed three times with medium, resuspended in medium containing 3TC (or AZT or ddC) at the appropriate concentrations, and incubated at 37 °C. After 5 days the amount of viral antigens produced by infected cells was determined by ELISA (Abbott Laboratories, Abbott Park, IL, U.S.A.). The values for the 50 % inhibitory dose (ID_{50}) were calculated from plots of the percentage reduction of viral antigens.

Determination of intracellular accumulation of 3TC or other nucleosides

CEM and CEM_{3TC} cells were treated with [^{3}H]3TC (0.1 μM; 2 μCi/ml) at different times (as indicated in the Figures) and then rapidly washed with ice-cold buffer and, after lysis, the amount of radioactivity was determined. The intracellular uptake of 3TC was also determined using a range of drug concentrations (from 0.02 μM to 0.2 μM). To study the intracellular accumulation of AZT and dCyd, CEM and CEM_{3TC} cells were exposed to [^{3}H]AZT (0.6 μM; 2 μCi/ml) and [^{3}H]dCyd (0.1 μM; 2 μCi/ml). At the indicated time, cells were washed, lysed and the radioactivity was determined.

3TC retention

CEM and CEM_{3TC} were preincubated with [^{3}H]3TC (2 μCi/ml) for 2 h, washed with ice-cold PBS by centrifugation, resuspended in drug-free medium, and maintained at 37 °C. After 1 h, the intracellular radioactivity and radiolabelled drug released into the medium was assessed by scintillation counting.

Reverse transcription (RT) and PCR

RNA from 5×10^6 CEM or CEM_{3TC} was isolated using Trizol reagents (Gibco BRL, NY, U.S.A.). The RT-PCR analysis of the RNA sample was performed as follows. RNA (10 μg) was incubated with 2 μl of random primers (150 μg/ml) at 72 °C for 10 min, then combined with a mixture containing 4 μl of 5 % reaction buffer [250 mM Tris (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$; Roche Molecular Biochemicals, Milan, Italy], 25 units of human placental ribonuclease inhibitor, 1 μl of 10 mM dNTP (Roche Molecular Biochemicals), 8 units of Moloney murine leukaemia virus RT (Roche Molecular Biochemicals). After 90 min at 42 °C, 5 μl of cDNA was subjected to the PCR-mediated amplification for P-glycoprotein (Pgp) according to conditions described previously [24].

Cell extracts

CEM and CEM_{3TC} cell pellets, prepared as described above, were resuspended in 5 vols of 20 mM Bis-Tris, pH 6.5, containing 1 mM dithiothreitol (DTT) and 0.5 mM PMSF, and sonicated for 5 s on ice at 50 W. Sonication was repeated five times at intervals of 10 s. Cell extracts were centrifuged at 4 °C at 5000 ***g*** in a benchtop centrifuge for 15 min. Supernatants were collected and assayed for protein concentration using the spectrophotometric-based Bio-Rad Protein Assay.

2′-Deoxycytidine kinase assay

Deoxcytidine kinase (dCK) activity present in cell extracts was assayed with a radiochemical method which measures the formation of [^{3}H]dCMP from [^{3}H]dCyd. The cell extracts were incubated at 37 °C in 25 μl of a mixture containing 30 mM Hepes-K^+ (pH 7.5), 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM DTT and 2.4 μM [^{3}H]dCyd (2200 c.p.m./pmole) or [^{3}H]3TC

(1500 c.p.m./pmole). The reaction, after 20 min incubation, was terminated by spotting 20 μl of the incubation mixture onto a 25 mm DEAE paper disk (DE-81 paper; Whatman Biosystems Ltd., Maidstone, Kent, U.K.). The disks were washed three times in an excess of 1 mM ammonium formate, pH 3.6, in order to remove unconverted nucleoside, followed by a final wash in ethanol. The filters were dried and radioactive dCMP was estimated by scintillation counting in 1 ml of Betamax scintillating fluid (ICN Pharmaceuticals, Milan, Italy). One unit is defined as the amount of enzyme catalysing the formation of 1 nmol of dCMP in 1 h at 37 °C.

Immunoblot analysis

Crude membranes were prepared from cells as described previously [20]. Proteins were estimated using the Bio-Rad Protein Assay (Bio-Rad, Milan, Italy) and BSA was used as the standard. The crude membrane proteins (200 μg) were resuspended in standard Laemmli sample preparation buffer and loaded onto a 7.5 % denaturing polyacrylamide gel and transferred to nitrocellulose filters. The filters were blocked in 1 × PBS containing 0.1 % Tween 20 and 10 % non-fat dry milk, immunoreacted with polyclonal rabbit anti-(MRP4) IgG followed by peroxidase conjugated anti-(rabbit IgG), and then developed with the Amersham ECL® detection system (Amersham, Airlington Heights, IL, U.S.A.). The immunoblots were stripped with glycine and reprobed with a monoclonal antibody to MRP1 (mPrl; Signet Laboratories, MA, U.S.A.).

Pgp detection

Pgp was also detected by FACS analysis. CEM, CEM_{3TC} and CEMVBL100 (a T-cell line expressing a high level of Pgp) cells were incubated with a Pgp-specific monoclonal antibody that recognizes an external epitope of Pgp (mMRK16, Alexis Italia, Florence, Italy). After incubation (30 min at 18–25 °C) the cells were washed with PBS and incubated with FITC-labelled goat anti-mouse immunoglobulin (Bioline Diagnostics, Turin, Italy) for an additional 30 min. After washing with PBS, the cells were resuspended in PBS and analysed by flow cytometry. This was performed using a FACScan (DAKO-Galaxy, Milan, Italy) flow cytometer. Forward and side light scatter were collected in linear mode and served to exclude unwanted events (i.e. debris, dead cells and aggregates). The fluorescence signal was collected in the log mode.

Generation of MRP4 stable cell lines

The human MRP4 cDNA was cloned into the MSCV-IRES-GFP [25] vector (kindly provided by Dr Robert Hawley, Holland Laboratory, American Red Cross, Rockville, MD, U.S.A.) using the *EcoR*I site. 293T cells were cotransfected with 10 μg each of MSCV-MRP4-IRES-GFP, the helper plasmid pSRa-G, and pEQPAM3-e (kindly supplied by P. Kelly and E. F. Vanin, Department of Hematology/Oncology St Jude Children's Research Hospital, Memphis, TN, U.S.A.) by standard calcium phosphate precipitation [26]. The supernatant was collected 48 h after transfection, filtred, titred and frozen at −80 °C. To confirm transfection, the 293T cells were analysed for green fluorescence protein (GFP) expression. Subsequently, the cells were transduced with MRP4. Briefly, the cells were plated at 5×10^4 cells/60 mm tissue culture dish and then the medium was replaced by the retroviral supernatant supplemented with 6 μg/ml polybrene and placed overnight in an incubator at 37 °C in a 5 % CO_2 humidified atmosphere. The transduction was repeated again twice for a total of three times. The transduced cells were expanded and the GFP-positive cells were selected after FACS [26]. Subsequently, a total lysate was prepared and loaded on a denaturing polyacrylamide gel for MRP4 and MRP1 detection by immunoblot [20].

Semi-quantitative RT-PCR analysis of MRP5 and ABCC11

RNA was isolated from CEM and CEM_{3TC} cells using Trizol. First-strand cDNA was made from 2.5 μg of RNA using the cDNA synthesis kit for PCR (Boehringer Mannheim, Indianapolis, IN, U.S.A.) in a final volume of 20 μl. MRP5 and ABCC11 (also called MRP8) were amplified with 125 ng of cDNA in a final volume of 50 μl containing 200 μM each of dATP, dCTP, dGTP, and dTTP, and 300 nM each of forward and reverse primers using the Expand High Fidelity PCR System (Boehringer Mannheim). Samples were denatured for 5 min at 94 °C, followed by cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min, and a final incubation at 68 °C for 2 min. The number of cycles for MRP5 was 26 and the number of cycles for ABCC11 was 30. Primers were as follows: MRP5, forward primer, 5′-TCCTGCCTTCTGTCCTGGTGT-3′ and reverse primer, 5′-CTGTGCGACGACTGCGGTGAG-3′; ABCC11 forward primer, 5′-AGAATGGCTGTGAAGGCTCAGC- 3′ and reverse primer, 5′ GTTCCTCTCCAGCTCCAGTGC 3′. The predicted sizes for the MRP5 and ABCC11 products were 390 bp and 550 bp respectively. Aliquots (10 μl) of the PCR reactions were loaded onto a 1 % agarose gel containing ethidium bromide.

RESULTS

Selection of 3TC-resistant CEM cells

Cellular factors, such as altered drug activation and/or decreased accumulation may cause failure of anti-retroviral drugs [13–15]. To determine if these cellular factors account for the variable response to the anti-retroviral drug 3TC, we cultured the CEM T-cell line in increasing concentrations of 3TC. After approx. 4 months of culture in the presence of 1 mM 3TC, CEM cells were obtained that were refractory to the growth inhibitory properties of 3TC. These cells are referred to as CEM_{3TC} and their resistance to 3TC was stable for 4 months in the absence of 3TC. Furthermore, the resistance to the cytotoxic effects of 3TC was selective because CEM_{3TC} were equally sensitive to AZT and vinblastine (Table 1).

CEM_{3TC} are impaired for antiviral efficacy

To evaluate whether the CEM_{3TC} cells had an impaired ability to inhibit HIV replication, CEM and CEM_{3TC} cells were infected with HIV (see Materials and methods section) and then treated

Table 1 Sensitivity of CEM and CEM_{3TC} to antiviral and antigrowth activities of 3TC, AZT, ddC and vinblastine

TC_{50} is the concentration producing 50% cytotoxicity and ID_{50} is the dose producing 50% inhibition of HIV replication.

	TC_{50}			ID_{50}		
Cells	3TC (mM)	AZT (mM)	VBL (ng/ml)	3TC (nM)	AZT (nM)	ddC (nM)
CEM	4.00 ± 2	0.30 ± 0.10	0.08 ± 0.06	6 ± 3	7 ± 4	8 ± 3
CEM_{3TC}	> 10†	0.40 ± 0.15	0.10 ± 0.09	60 ± 10*	9 ± 5	10 ± 4

† Owing to 3TC insolubility, concentrations greater than 10 mM were not evaluated.
* CEM versus CEM_{3TC}, $P < 0.05$.

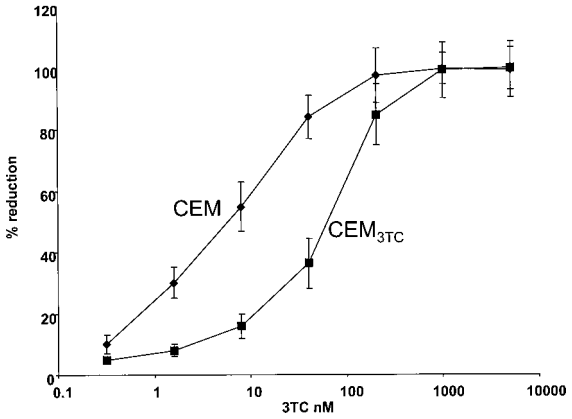

Figure 1 Antiviral activity is reduced in 3TC resistant cells

Antiviral activity of 3TC in CEM (diamonds) and CEM_{3TC} (squares). Cells were infected with HIV-PNL43 and cultured in the presence of different concentrations of 3TC. After 5 days the amount of viral antigen produced by infected cells was measured as described in the Material and methods section. Each point represents the mean and the bars $\pm$one standard deviation from the mean.

with various concentrations of 3TC (Figure 1). The viral yield was determined 5 days later by measuring the viral antigen released into the culture supernatant. We found that CEM_{3TC} were markedly resistant to the antiviral activity of 3TC, with the 3TC ID_{50} value for HIV-1 being approx. 10-fold higher in CEM_{3TC} (ID_{50} = 60 nM) compared with the CEM cell line (ID_{50} = 6.0 nM) (Figure 1). Again, the resistance to the antiviral activity of 3TC was selective because CEM_{3TC} were equally sensitive to the antiviral activity of ddC and AZT (Table 1).

Deoxycytidine kinase activity is not decreased in CEM_{3TC}

After cellular uptake by nucleoside uptake carriers, 3TC is phosphorylated by deoxycytidine kinase [27]. Deoxycytidine kinase effectively phosphorylates both enantiomers of dCyd [28] and dCyd analogues, such as 3TC [29]. Therefore, we evaluated the enzymic activity of dCK in CEM and CEM_{3TC} cells using both 3TC and dCyd as the substrate. The results indicated that the enzymic activity of dCK from CEM_{3TC} was not decreased compared with the parental CEM cells. In fact, using 3TC as substrate, the dCK activity was 0.12 ± 0.01 units/mg of protein in CEM and 0.16 ± 0.02 units/mg in CEM_{3TC}. Similarly, when dCyd was used as substrate the dCK activity was 0.8 ± 0.08 units/mg in CEM and 1.24 ± 0.12 units/mg in CEM_{3TC}. These results indicate, using either dCyd or 3TC, that the 3TC-resistance of CEM_{3TC} cannot simply be ascribed to a reduction in dCK activity.

Decreased 3TC accumulation in CEM_{3TC} without a general decrease in nucleoside uptake

Resistance to 3TC could be due to the reduced intracellular accumulation of drug, secondary to either transport changes or alterations in enzymic activation. Uptake of radiolabelled 3TC was used to assess variations in 3TC transport. Figure 2(A) shows there was no significant difference between the two cell lines in their initial uptake of 3TC (< 50 min). However, when the cells were incubated for longer intervals (> 1 h) dramatic differences in accumulation emerged. The CEM cells continued to accumulate radiolabelled 3TC, whereas at 8 h, the CEM_{3TC} achieved a steady-state level of drug that was as much as 3-fold

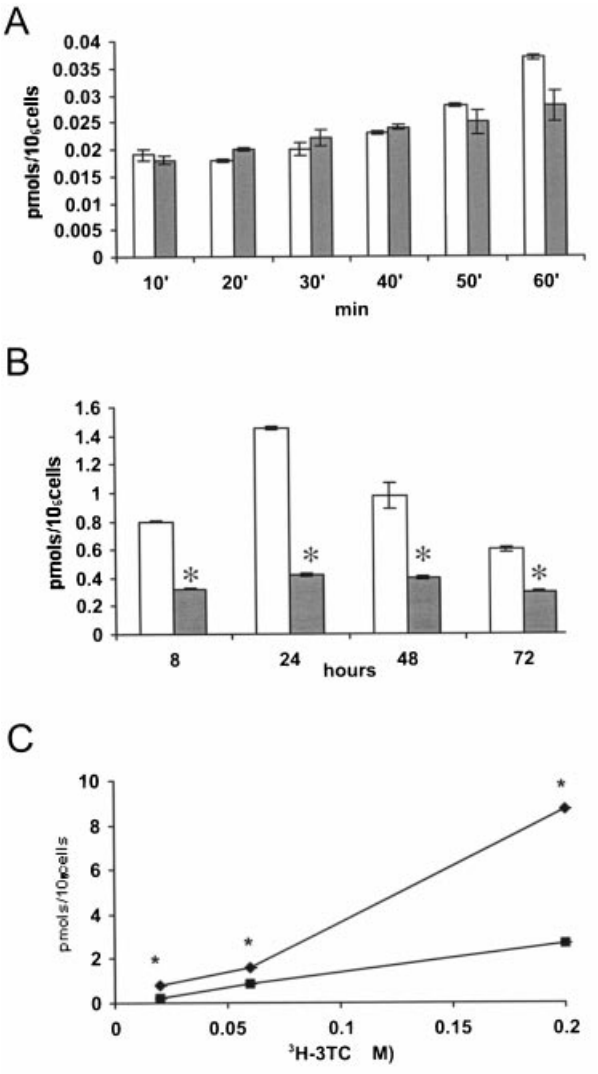

Figure 2 3TC Accumulation is impaired in 3TC resistant cells

(**A**) Intracellular uptake of [^{3}H]3TC in CEM (open bars) and CEM_{3TC} (closed bars). Cell cultures were incubated with 0.1 μM [^{3}H]3TC, and at the indicated times cells were extensively washed with ice-cold PBS, lysed and the radioactivity determined by scintillation counting. The results are the mean ($\pm$SD) from three independent experiments (*$P < 0.05$). (**B**) Long-term 3TC accumulation. Either CEM (open bars) or CEM_{3TC} (closed bars) were incubated with 0.1 μM [^{3}H]3TC for the indicated intervals (*$P < 0.05$). (**C**) Uptake of different concentrations of [^{3}H]3TC. CEM (diamonds) and CEM_{3TC} (squares) were incubated with different concentrations of [^{3}H]3TC. After 8 h, intracellular radioactivity was evaluated by scintillation counting. The points represent the mean of two independent experiments performed in triplicate, with the error bars indicating $\pm$one standard deviation (*$P < 0.05$).

lower than the maximum attained in the CEM cells. It is interesting to note that despite the continued presence of extracellular drug, the 3TC accumulation decreased in the CEM cells after 72 h of 3TC incubation. This suggests that the transporter effluxing 3TC is induced, a phenomenon previously reported for AZT [30] (Figure 2B). Finally, the CEM_{3TC} cells acccumulated much less drug than the CEM cells at multiple concentrations of 3TC (Figure 2C). It is notable that the 5-fold lower 3TC accumulation roughly corresponds with the greater 3TC concentration required to inhibit HIV replication (Figure 1 and Table 1) and supports the idea that impaired 3TC accumulation is responsible for the enhanced survival of these cells in 3TC, as well as the requirement for more 3TC to inhibit HIV replication.

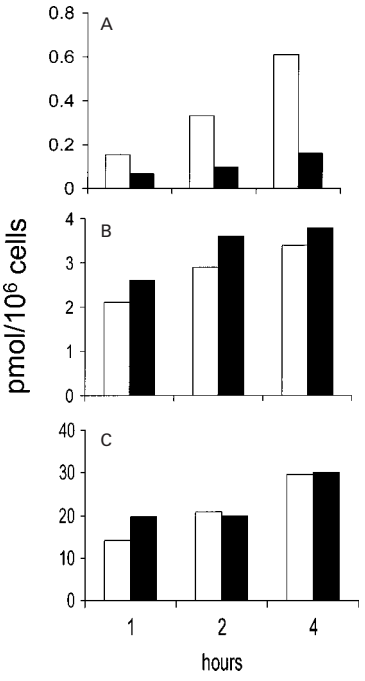

Figure 3 3TC resistant cells have a selective defect in 3TC accumulation

Intracellular accumulation of 3TC, AZT and dCyd in CEM (open bars) and CEM_{3TC} (solid bars). Cells were incubated with (**A**) 0.1 μM [^{3}H]3TC, (**B**) 0.6 μM [^{3}H]AZT or (**C**) 0.1 μM [^{3}H]dCyd. At the indicated times, cells were washed with ice-cold PBS, lysed and the radioactivity determined by scintillation counting. The results are the average of two independent experiments done in triplicate.

Table 2 3TC drug accumulation and retention in 3TC resistant cells

The values are the means of two independent experiments, each done in duplicate. For both intracellular radioactivity and radioactivity released into the supernatant, values were significantly different, $P < 0.05$ in each case.

	Percentage of total [^{3}H]3TC	
Cells	Intracellular	Supernatant
CEM	82.4 ± 1.8	17.6 ± 1.8
CEM_{3TC}	65.7 ± 0.8	34.3 ± 0.8

To determine if the impaired accumulation of 3TC was specific for 3TC, and to rule out a general defect in nucleoside uptake carriers, accumulation of [^{3}H]3TC, [^{3}H]AZT and [^{3}H]dCyd was determined (Figure 3). It is known that AZT is a substrate for both the concentrative nucleoside carrier (CNT) and equilibrative nucleoside carrier (ENT2) [31], while deoxycytidine is a known substrate for CNT [32]. The intracellular radioactivity was then measured as described in the Material and methods section. The studies reveal that CEM_{3TC} cells are not impaired for the accumulation [^{3}H]AZT and [^{3}H]dCyd, which indicates that the nucleoside-uptake carriers transporting these nucleosides are not impaired in the CEM_{3TC} cells.

CEM_{3TC} have decreased retention of 3TC, with no change in Pgp and a small increase in MRP4 expression

To explore whether the defect in cellular 3TC accumulation could be associated with a decreased capability of the resistant cell line to retain 3TC, we evaluated 3TC retention. The cells were pre-loaded with [^{3}H]3TC followed by resuspension in drug-free media. Subsequently, the amount of radioactivity in the cells and media was determined. The results, shown in Table 2, indicate that the CEM_{3TC} cells retained much less intracellular radioactivity than the CEM cells. Furthermore, a correspondingly higher percentage of radioactivity was released into the medium from CEM_{3TC} compared with CEM cells. This indicates that CEM_{3TC} have a decreased ability to retain 3TC, and this correlates with the selective impaired accumulation of 3TC (Figure 2).

Next, we evaluated whether the decreased 3TC accumulation could be due to an increased expression of Pgp using FACS analysis with an antibody that detects a surface Pgp epitope (see Materials and methods section). We found that both CEM and CEM_{3TC} have undetectable Pgp, unlike the positive control, $CEMVBL_{100}$, that expresses high amounts of Pgp (results not shown). Furthermore, we demonstrated that neither the CEM nor CEM_{3TC} cells had detectable levels of MDR1 transcript when used amplified to the same extent as the MDR1-positive cell, $CEMVBL_{100}$.

Expression of MRP4 in CEM_{3TC} and transport of 3TC in cells ectopically expressing MRP4

Recent studies indicated that the ABC transporter, MRP4, plays a role in the cellular resistance to anti-retroviral nucleoside drugs, including 3TC [20]. To evaluate MRP4 expression, we performed immunoblot analysis on crude membranes from the CEM_{3TC} cells (Figure 4). We found that the level of immunoreactive MRP4 increased approximately 2-fold in the CEM_{3TC} cells. In contrast, MRP1 was not different in the two cell lines. It is interesting to note that MRP4, which is only a 1325-amino-acid-residue protein, runs at an estimated size of 220 kDa, whereas MRP1, a 1531-amino-acid-residue protein, runs at an estimated size of 190 kDa. This is probably due to the fact that MRP4 is extensively glycosylated with at least seven predicted N-linked asparagine glycosylation sites [25].

To determine whether MRP4 played a role in transport of 3TC, we developed cell lines that ectopically overexpressed MRP4 (Figure 4B). We confirmed the phenotype of these cells by evaluating the uptake of PMEA, a known MRP4 substrate [20] (Figure 4C). These cells were then assessed for the uptake of 3TC (Figure 4D). We evaluated 3TC uptake after a 24 h incubation in concentrations of 3TC from 0.5 to 10 μM. The total accumulation of 3TC radioactivity was the same in the MRP4 cells as in the vector-only transfected cells (Figure 4D). Since longer incubations (48 h) produced slightly lower 3TC accumulation, we assessed whether efflux was faster in the MRP4 cells. The cells were loaded with 3TC, resuspened in drug-free media, and then assessed for both 3TC intracellular-associated radioactivity and the radioactivity released into the media (Figure 4E). For both cells lines, the time to decrease the intracellular radioactivity to one-half the initial level was approx. 20 min and, notably, a corresponding efflux of radioactivity into the media occurred. These studies directly demonstrate in MCF-7 cells overexpressing MRP4 that 3TC efflux is not enhanced by MRP4 overexpression.

Expression of MRP4, MRP5 and ABCC11 in CEM_{3TC}

The efflux of nucleotide analogues in mammalian cells has been confirmed for MRP4 and MRP5 [21]. Although we have demonstrated that cells specifically overexpressing MRP4 do not have

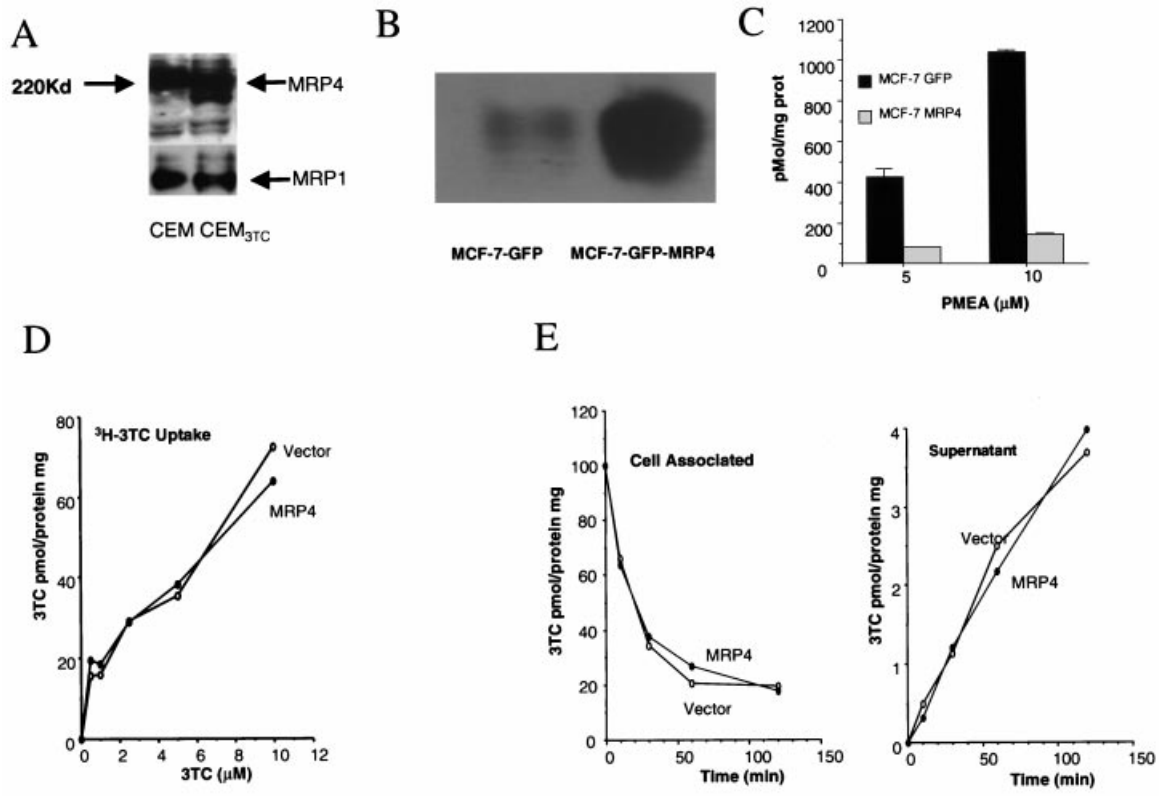

Figure 4 Analysis of MRP1 and MRP4 in CEM_{3TC} and the impact of MRP4 upon 3TC transport

(**A**) Lysates of CEM and CEM_{3TC} cells were analysed on an immunoblot with antiserum against MRP4, and then with antiserum against MRP1. (**B**) Immunoblot analysis of MRP4 expression in cells engineered to overexpress MRP4. (**C**) Functional analysis of MRP4 using PMEA accumulation, as described in the Materials and methods section. (**D**) MCF-7 cells ectopically expressing MRP4 (●) or the control vector (○) were incubated with [^{3}H]3TC (0.5–10 μM). After 24 h, the accumulation of 3TC radioactivity was determined. (**E**) 3TC efflux in cells ectopically expressing MRP4. Control vector (○) and MRP4 expressing cells (●) were loaded with [^{3}H]3TC followed by resuspension in drug-free media. Both intracellular-associated 3TC radioactivity (Cell Associated) and 3TC radioactivity released into the media (Supernatant) were assessed.

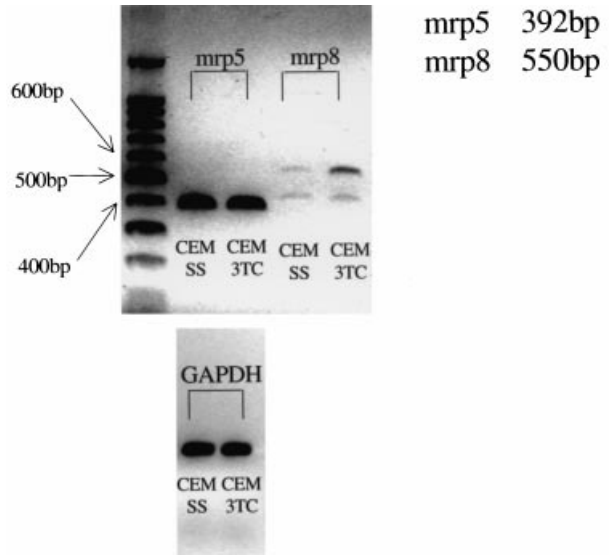

Figure 5 Analysis of MRP5 and ABCC11 (MRP8) expression in CEM_{3TC} cells

Total RNA was isolated from both CEM and CEM_{3TC} cells, followed by RT-PCR. The lower band in MRP8 (ABCC11) was sequenced and found to be a non-specific band. The primers and conditions are described in the Materials and methods section.

decreased accumulation or increased efflux of 3TC, it remains possible that another ABC transporter effluxes 3TC metabolites. Our recent investigations and others studies [33,34] indicate that MRP5 has two closely related homologues on chromosome 16. We evaluated the expression of ABCC11 mRNA levels in CEM and CEM_{3TC} cells by RT-PCR (ABCC12 was not detected). In addition, we assessed the level of MRP5 mRNA (Figure 5). We found that the level of MRP5 was unchanged in the CEM_{3TC} cells. In contrast, semi-quantitative RT-PCR revealed that ABCC11 was increased 6-fold. The magnitude of this increase in ABCC11 mRNA is comparable with the impairment in 3TC antiviral efficacy in these cells. The lack of a direct correspondence may be due to the possibility that the protein is expressed at a much higher level than the mRNA; however, at this time, it is impossible to determine whether ABCC11 protein levels are increased due to the unavailability of a specific antibody.

DISCUSSION

Recent findings have recognized that anti-retroviral drug treatment causes a phenotype described as cellular resistance [12–15]. This phenomenon is consistent with the knowledge that different cell lines require a broad range in the concentration of anti-retroviral drug to inhibit HIV replication [35]. Two main mechanisms contribute to cellular resistance: altered metabolism of nucleoside analogues due to impaired nucleoside phosphoryl-

ation and increased efflux of the compounds by membrane transport mechanisms [20,21].

Our results demonstrate that prolonged treatment with 3TC selects for cells with an acquired, stable resistance to 3TC. Compared with the CEM cells, CEM_{3TC} required about 10-fold more 3TC to inhibit HIV. Moreover, these cells showed increased resistance to the cytotoxic effects of 3TC. However, the CEM_{3TC} were as sensitive to AZT, ddC and vinblastine as the CEM cells, demonstrating that this resistance is specific for 3TC. Notably, 3TC resistance was not due to decreased dCK activity, the principal enzyme required for activation of 3TC [27]. Furthermore, uptake of the natural nucleosides dCyd and azidothymidine was unaltered in CEM_{3TC}. Thus, these findings rule out the possibility of a general defect in nucleoside uptake because such alterations would have undoubtedly have impacted upon AZT and dCyd accumulation, considering that AZT is transported by both ENT2 and CNT, and that dCyd is transported by CNT [32,36]. In contrast, 3TC accumulation was substantially reduced in CEM_{3TC} cells and was associated with decreased intracellular retention. Consequently, we postulated that an efflux transporter was responsible for preventing 3TC accumulation in the resistant cells. In fact, drug efflux pumps are an important part of the cellular defence against cytotoxic compounds. Specifically, cells overexpressing drug-transporting proteins become resistant to a wide range of drugs with different structures and/or cellular targets. This phenomenon is known as multidrug resistance (MDR). The most well characterized of these drug transporters is Pgp [37]. The overexpression of Pgp has been described for many cancer cells with acquired resistance to chemotherapuetics [38]. Several studies have reported that Pgp-expressing cells are also resistant to the anti-growth and antiviral activity of some NRTIs [39–41]. On the basis of these findings, we evaluated Pgp expression in CEM_{3TC}. However, as anticipated, based upon 3TC structure, the CEM_{3TC} cells had no detectable Pgp overexpression.

Recently, it has been reported that one member of the MRP family, MRP4, is overexpressed in cells that acquire resistance to the cytotoxic effects of the modified nucleotide analogue, PMEA [20]. Notably, overexpression of MRP4 impairs the antiviral efficacy of PMEA and other nucleoside analogues, such as 3TC and AZT. In our 3TC resistant cells, we found a small increase in MRP4 (< 2-fold), suggesting that 3TC metabolites could be MRP4 substrates. However, an analysis of MCF-7 cells ectopically expressing MRP4 showed that MRP4 does not affect either the accumulation or the efflux of 3TC. This result contrasts with the previously reported findings; however, it should be noted that only PMEA and AZT-monophosphate were effluxed to a greater extent in the MRP4 overexpressing cells, and it was not directly demonstrated that 3TC metabolites were more readily effluxed in those cells [20]. Thus, based on the current studies, it seems unlikely that MRP4-mediated efflux is directly involved in cellular 3TC resistance and that the impaired 3TC accumulation and decreased retention is due to an additional 3TC transporter in the CEM_{3TC} cells. Since MRP5 has been demonstrated to transport similar substrates as MRP4 [21], we evaluated its mRNA expression, but found no difference in MRP5 expression in the CEM_{3TC} cells. However, recent studies [33] have determined that MRP4 and MRP5 homologues are found on chromosome 16q12. These homologous genes also lack an N-terminal domain that is found in the prototypical ABCC1 (i.e. MRP1). In the CEM_{3TC} cells, we found increases in ABCC11 mRNA expression (6-fold). However, in the absence of an antibody we are unable, at this time, to confirm if ABC11 protein is overexpressed. Nevertheless, it is possible that this transporter contributes to the efflux-mediated resistance to 3TC.

In conclusion, our reuslts are most consistent with the concept that 3TC resistance is mediated by an inability to adequately accumulate 3TC. This is not due to impaired 3TC phosphorylation or initial uptake. It is possible that increased ABCC11 expression decreases 3TC accumulation and increases cellular 3TC resistance. However, at the present time, we cannot directly confirm this possibility. The current studies support the idea that 3TC resistance may be due to ABC11 overexpression. However, we can not exclude the likelihood that a combination of increased MRP4 and ABCC11 underlie the 3TC resistance and impaired accumulation in these cells. This might be analogous to the overexpression and potential role of MRP1, MRP2 and ABCG2 in cells in resistance to the camptothecin class of cancer chemotherapeutic drugs [42]. Future studies will address the possibility of such interactions among ABCC11 and MRP4.

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