0022-3565/05/3143-1202–1209$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 314:1202–1209, 2005

Vol. 314, No. 3
86272/3045878
Printed in U.S.A.

In Vitro Synergy and Enhanced Murine Brain Penetration of Saquinavir Coadministered with Mefloquine

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Received March 14, 2005; accepted May 26, 2005

ABSTRACT

Highly active antiretroviral therapy has substantially improved prognosis in human immunodeficiency virus (HIV). However, the integration of proviral DNA, development of viral resistance, and lack of permeability of drugs into sanctuary sites (e.g., brain and lymphocyte) are major limitations to current regimens. Previous studies have indicated that the antimalarial drug chloroquine (CQ) has antiviral efficacy and a synergism with HIV protease inhibitors. We have screened a panel of antimalarial compounds for activity against HIV-1 in vitro. A limited efficacy was observed for CQ, mefloquine (MQ), and mepacrine (MC). However, marked synergy was observed between MQ and saquinavir (SQV), but not CQ in U937 cells. Furthermore, enhancement of the antiviral activity of SQV and four other protease inhibitors (PIs) by MQ was observed in MT4 cells, indicating a class specific rather than a drug-specific phenomenon. We demonstrate that these observations are a result of inhibition of multiple drug efflux proteins by MQ and that MQ also displaces SQV from orosomucoid in vitro. Finally, coadministration of MQ and SQV in CD-1 mice dramatically altered the tissue distribution of SQV, resulting in a >3-fold and >2-fold increase in the tissue/blood ratio for brain and testis, respectively. This pharmacological enhancement of in vitro antiviral activity of PIs by MQ now warrants further examination in vivo.

That combination antiretroviral therapy has substantially improved clinical outcome in HIV infection is beyond question. Nevertheless, there are limitations to its efficacy, such as the poor penetration of some components of the regimen into sanctuary sites (e.g., central nervous system and genital tract) as well as the high costs of therapy that preclude widespread implementation in many parts of the developing world.

Recently, there has been much interest in the role of antimalarials such as chloroquine (CQ) in HIV therapy. CQ suppresses HIV-1 and -2 replication in vitro (Tsai et al., 1990; Savarino et al., 2001a) (as does its analog hydroxyCQ; Sperber et al., 1997; Boelaert et al., 2001), possibly by inhibition of HIV gp120 (Tsai et al., 1990). In vitro studies examining CQ in HIV-infected cells has shown some additivity with zidovudine (Boelaert et al., 2001) and synergy with numerous protease inhibitors (PIs) in T cell lines (Savarino et al., 2004). Clinical trials are now underway to assess CQ in HIV infection (www.iatec.com). To date, no studies have systematically assessed other antimalarial compounds for antiretroviral activity. Other quinolines such as amadiaquine (AQ) and mefloquine (MQ), acridine derivatives such as mepacrine (MC, and 9-phenanthrenes such as hyfantrin (HF), cross the blood-brain barrier and as such may be able to achieve adequate drug concentrations in sites that provide sanctuary for virus, thereby preventing or slowing the development of resistance.

The accumulation of HIV protease inhibitors into HIV-infected cells or sanctuary sites is a complex process (Hoggard and Owen, 2003; Owen and Khoo, 2004) governed by physicochemical characteristics of the drug, ion trapping, protein binding, metabolism, and affinity for drug transport proteins (Hoggard and Owen, 2003; Owen and Khoo, 2004). It is clear that for some of these factors (e.g., protein binding, metabolism, and drug transport) variations in host phenotype play a major role in determining interindividual varia-

This work was supported by a grant from the British Society for Antimicrobial Chemotherapy. B.C. and O.J. are funded by British Society for Antimicrobial Chemotherapy.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.086272.

ABBREVIATIONS: HIV, human immunodeficiency virus; CQ, chloroquine; PI, protease inhibitor; AQ, amadiaquine; MQ, mefloquine; MC, mepacrine; HF, hyfantrin; SQV, saquinavir; Pgp, P-glycoprotein; FCS, fetal calf serum; TCID, tissue culture-infective dose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; FIC, fractional inhibitory concentration; RTV, ritonavir; NFV, nelfinavir; IDV, indinavir; APV, amprenavir; MRP, multidrug resistance associated protein; CAR, cellular accumulation ratio; MDR, multidrug resistance.

tion in response to treatment. For PIs such as saquinavir (SQV), the transporters P-glycoprotein (Pgp) and MRP1 have been shown to limit intracellular accumulation (Jones et al., 2001a,b; Meaden et al., 2002; Williams et al., 2002) and brain penetration (Glynn and Yazdanian, 1998; Choo et al., 2000; Washington et al., 2000; Huisman et al., 2001) of drug and the use of a reversal agent has been suggested as a viable cotherapy in HIV (Choo et al., 2000). Indeed, we recently showed a linear correlation between Pgp expression in lymphocytes and the EC_{50} of SQV (Owen et al., 2004). Of interest, the antimalarial compounds CQ and MQ also interact with Pgp and/or MRP1, and both drugs have been shown to enhance intracellular accumulation of substrates in cell lines that overexpress these transporters (Riffkin et al., 1996; Vezmar and Georges, 1998; Fujita et al., 2000; Vezmar and Georges, 2000).

In this study, we screened five antimalarial compounds (CQ, MQ, MC, HF, and AQ) for anti-HIV activity and for any interactions with HIV PIs. Inhibition of HIV replication was evaluated in both acutely and persistently (to assess postintegration effects) HIV-infected cells. Interactions between antimalarials and HIV PIs were assessed at HIV protease level (using a cell-free system containing purified recombinant HIV protease) and at cellular level. For the latter, isobolograms were first constructed to assess synergy/antagonism, and various cell lines (including those expressing the transporters Pgp, MRP1, and MRP2) were used to assess the effect of antimalarials on PI transport as well as any effects in modulating the expression of these transporters. Expression of these transporters was assessed using Western blotting and flow cytometry. In addition, the ability of MQ to displace SQV from orosomucoid and albumin and to increase free drug concentration in serum was investigated. Finally, we investigated the tissue distribution of SQV in CD-1 mice when coadministered with MQ.

Materials and Methods

Cells and Virus

U937, CEM (parental), CEM_{VBL} (overexpressing Pgp; Owen et al., 2003b), CEM_{E1000} (overexpressing MRP1; Owen et al., 2003b), and MT4 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM L-glutamine and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

A laboratory-adapted HIVIIIB strain (X4-tropic) was titrated by limiting dilution assay, and the $TCID_{50}$ was calculated. Viral production was measured immunoenzymatically using commercially available p24 antigen enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (Sim et al., 1998).

For all infectivity assays, the cells were resuspended, and a fraction was removed for cytotoxicity assays. Cell viability and cytotoxicity (throughout this article) were assessed using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as validated previously within our laboratory (Sim et al., 1998). For viral quantitation, an aliquot (100 μl) was centrifuged at 13,000*g* for 10 min, and the supernatant taken for p24 antigen assay as described above.

Antiviral Activity of Antimalarial Compounds

The U937 cell line was used to screen for anti-HIV-1 activity of the antimalarials (CQ, MQ, MC, HF, or AQ). For acute infection, U937 cells were inoculated with viral suspensions (1×10^{-2} $TCID_{50}$ per cell) at 37°C for 1 h in the presence of SQV, CQ, MQ, MC, HF, or AQ (0.1–100 μM in third logs). Cells were then washed three times before resuspension at 2.5×10^5 cells in 2 ml of fresh culture medium in the presence and absence of SQV, CQ, MQ, MC, HF, or AQ (0.1–100 μM in third logs). Persistently infected U937 cells (to assess the effects on the postintegration steps in the HIV-1 life cycle) were suspended in culture medium in the presence or absence of SQV, CQ, MQ, MC, HF, or AQ (0.1–100 μM in third logs). Viral replication was then allowed to occur in the presence and absence of test compounds for 7 days.

In both acute and persistent infection models, IC_{50} (toxicity) and EC_{50} (antiviral activity) values were calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA), and the selectivity indices were calculated as the IC_{50}/EC_{50} ratio.

Isobolograms and Modulation of PI Activity by CQ and MQ

To investigate the potential for combination of CQ or MQ with SQV, isobolograms were constructed. The effect of the combination of CQ with SQV and MQ with SQV on viral p24 production (after acute infection) was tested by titration of the two drugs at fixed ratios proportional to their EC_{50} values. After 7-day incubation, viral replication and cellular toxicity were assessed as described above. This allowed calculation of the fractional inhibitory concentrations (FICs) of the resulting EC_{50} values for each drug, which were plotted as isobolograms as described previously (Berenbaum, 1978). These were interpreted according to recently published guidelines (Odds, 2003) i.e., FIC interpretations of "synergy" (FIC $\leq$ 0.5), "antagonism" (FIC $>$ 4.0), or "no interaction" (FIC = 0.5–4.0).

The antiviral efficacy of SQV, ritonavir (RTV), nelfinavir (NFV), indinavir (IDV), and amprenavir (APV) in the presence and absence of MQ or CQ (10 μM) were also assessed against HIVIIIB in MT4 cells. MT4 cells are highly sensitive to virally induced cytopathic effects that allow infection to be quantified directly by cytotoxicity assay, allowing EC_{50} to be easily calculated. MT4 cells were centrifuged (400*g*; 5 min), and the supernatant fraction was discarded. Cells were resuspended in RPMI 1640 medium containing 10% FCS, counted using a hemocytometer, and the concentration was adjusted to 1×10^6/ml. HIVIIIB cell-free supernatant was added to the cell suspension (1×10^{-2} $TCID_{50}$ per cell). Cell suspension was added to all wells (50 ml, final cell concentration 5×10^5/ml) containing PI in doubling dilutions (100–0.015 nM) with or without MQ or CQ (10 μM), except cell-free negative controls, which contained media only. Drug-free positive controls containing cells alone were also included. After incubation (37°C, 5% CO_2, 7 days), plates were assayed for cytotoxicity as described above.

Interactions with HIV Protease

A fluorescent-based, cell-free assay was developed, and the effect of SQV on recombinant HIV-1 protease both alone and in combination with MQ or CQ was assessed. Recombinant HIV-1 protease (0.22 μg/ml; National Institute for Biological Standards and Control) was combined with SQV (0.3 nM to 10 μM) with or without MQ or CQ (10 μM) in protease buffer (0.1 M NaAc, 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% dimethyl sulfoxide, and 1 mg/ml bovine serum albumin, pH 4.7). To start the reaction, a final concentration of 0.1 μM HIV protease substrate 1 (Molecular Probes, Leiden, The Netherlands) was added. The sequence of this substrate includes the HIV protease cleavage site, along with two covalently modified amino acid residues, one that has been linked to a fluorophore, 5-(aminoethyl)aminonaphthalene sulfonate, and the other to an acceptor chromophore,4-dimethylaminoazobenzene-4-carboxylate), resulting in quenching of the nearby fluorophore through resonance energy transfer. Excitation was therefore performed at 340 nm and the emission simultaneously measured at 490 nm for 10 min. The mean velocity for formation of the cleaved substrate was calculated for each drug concentration and normalized to controls. Comparisons were then made between SQV inhibition in the presence and absence of MQ or CQ (10 μM).

Cellular Accumulation Studies

To determine whether transport of SQV was inhibited by MQ, SQV accumulation was assessed in U937, CEM, CEM_{E1000} (overexpressing MRP1), and CEM_{VBL} (overexpressing Pgp) cells in the presence of MQ. After preincubation for 10 min with 0, 1, 3, 10, 30, or 50 μM MQ, cells were incubated in the presence of 1 μM [^{3}H]SQV (specific activity 26 μCi/mg) for 30 min at 37°C in serum-free media.

At the end of the incubation period, the samples were removed and centrifuged (15,000*g* for 2 min) in a chilled microcentrifuge. An aliquot (50 μl) of the supernatant was taken for scintillation counting, and the cell pellets were washed three times in ice-cold phosphate-buffered saline before the cells were solubilized by adding 100 μl of a cocktail containing 5 parts tissue solubilizer, 2 parts H_2O_2, and 2 parts glacial acetic acid. The samples were then analyzed by liquid scintillation counting. The cellular accumulation ratio (CAR) of SQV is the concentration of SQV in the cell to the concentration of SQV in the extracellular media after incubation.

Transporter Expression

Real-Time Reverse Transcriptase-Polymerase Chain Reaction. Quantification of mRNA transcripts for MDR1, MRP1, and MRP2 was achieved by real-time polymerase chain reaction using an Opticon2 sequence detection system. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene. cDNA (40 ng) was combined with Universal master mix, sense and antisense primers (0.4 μM each), and oligonucleotide probe (0.2 μM) in a final volume of 25 μl. Amplification was carried out for 40 cycles with a combined annealing/extending temperature of 60°C. Quantification of MDR1, MRP1, and MRP2 was then achieved using the comparative C(t) method. Primers and probes were obtained via the Assays-on-Demand Gene Expression products available through the Applied Biosystems (Foster City, CA) Web site.

Western Blot Analysis. Infected and noninfected U937 cells (typically 5×10^6 cells/ml) were incubated in the presence or absence of SQV (10 nM), CQ (10 μM), MQ (10 μM), CQ/SQV, or MQ/SQV for 3 days. The cells were then collected and Western blot analysis was carried out for Pgp, MRP1, and MRP2 as described previously (Owen et al., 2003b). In addition, U937 membrane preparations were used to enrich the transporter-containing fraction of the cells. Briefly, U937 cells were homogenized in ice-cold homogenization buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and the nuclei were pelleted at 500*g* for 10 min. The supernatant fraction was then further centrifuged at 100,000*g* for 30 min to pellet the membrane fraction.

Briefly, 50 μg of total protein from each cell line was electrophoresed on 3 to 12% Tris-acetate gels. After transfer, membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing Tween 20. Monoclonal antibodies C219, MRPm5, and MRP2I-4 were used for specific detection of Pgp, MRP1, and MRP2, respectively (1:2000). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody specific for the primary (1:10,000), and transporters were detected using enhanced chemiluminescence reagent.

Flow Cytometry. Flow cytometric analysis was carried out for Pgp and MRP1 in U937 cells to characterize these cells with respect to efflux transporters known to transport SQV. MRP2 flow cytometry was also attempted, but the antibody was found to be noncompatible with this technique (data not shown). Pgp flow cytometry was carried out using monoclonal antibody UIC2 as validated previously in our laboratory (Chandler et al., 2003). Briefly, cells (1×10^6) were fixed with CellFIX (4°C; 30 min) and incubated with UIC2 (room temperature; 60 min). After three washes with Hanks' balanced salt solution, cells were stained with phycoerythrin-conjugated secondary antibody (room temperature; 60 min). After another three washes, cells were resuspended in CellFIX for analysis by flow cytometry.

The expression of MRP1 was determined by modification of a previously reported method (Chen et al., 2002). Briefly, cells were fixed with CellFIX (30 min; 4°C) and permeablized with saponin (0.1 mg/ml in HBSS). Cells were then labeled with the MRP1-specific mouse anti-human primary antibody, QCRL-1 (200 μl; 2.5 μg/ml), and detection was achieved with fluorescein isothiocyanate-conjugated IgG secondary antibody (200 μl; 5.0 μg/ml). Cells were then fixed with CellFIX for analysis by flow cytometry.

For all analyses, the forward and side scatters of the cells were measured simultaneously on an EPICS-XL flow cytometer (Coulter Electronics, Luton, Beds, UK), and the lymphocyte population was electronically gated to exclude debris. The fluorescence of the cells was plotted against the number of events, and the data were registered on a logarithmic scale. The median fluorescence for an appropriate isotype control antibody was then deducted from the median fluorescence of the test antibody to calculate the antibody-specific fluorescence.

Assessment of SQV Protein Binding in the Presence and Absence of MQ

To determine any effects of MQ on SQV protein binding, [^{3}H]SQV (10 μM) was incubated with recombinant orosomucoid (150 mg/dl; median physiological concentration), recombinant albumin (4.5 g/dl; median physiological concentration), or FCS (10%) in RPMI 1640 medium. In addition, the ability of MQ to increase SQV free drug concentration in human serum was assessed ($n = 6$). A range of MQ concentrations (0–100 μM) were preincubated with protein-containing RPMI 1640 medium or serum for 10 min before the addition of SQV. After 30-min incubation at 37°C, the samples (500 μl) were applied to an Amicon Centrifree filter system (molecular mass cut-off 30,000 kDa; Millipore Corporation, Bedford, MA) and centrifuged (1500*g*, 60 min; constant temperature of 37°C to prevent altered drug protein binding). Each sample provided approximately 200 μl of ultrafiltrate containing the unbound drug. SQV concentrations in the total and unbound fractions were then assessed by liquid scintillation counting.

Murine Tissue Distribution of SQV in the Presence and Absence of MQ

Male CD-1 mice were administered a bolus intravenous dose of MQ (10 mg/kg) 10 min before a bolus intraperitoneal dose of SQV (10 mg/kg). After 1 h, the animals were sacrificed with a rising concentration of CO_2, and a cardiac puncture was performed to obtain blood samples. The brain, testis, liver, and kidneys were then removed and frozen in liquid nitrogen. On the following day, tissues were homogenized, and SQV concentrations were assessed by liquid chromatography/tandem mass spectrometry as reported previously (Khoo et al., 2002).

Data Analysis

Unless otherwise stated, results are presented as the mean of $n = 4$ experiments (conducted in triplicate). Statistical analyses were carried out for normally distributed data (assessed by Shapiro-Wilk test) using an unpaired t test. For non-normally distributed data, a Mann-Whitney statistical test was used.

Results

Antiviral Activity of Antimalarial Drugs. Selective inhibition of HIV-1 was observed for SQV and to a much lesser extent for CQ, MQ, and MC (Table 1). The rank order, as illustrated by the selectivity index (IC_{50}/EC_{50}), was SQV (2716) $\ggg$ MQ (2.5) $>$ CQ (2.2) $>$ MC (1.6). Neither HF nor AQ exhibited any selectivity toward HIV (selectivity index <1). In persistently HIV-infected cells, only SQV was found to have an effect on viral replication with a selectivity index of 488. None of the antimalarials screened exhibited selectiv-

TABLE 1
Effects of SQV, CQ, MQ, MC, HF, and AQ (0.1–100 μM) on viral p24 (EC_{50}) and U937 MTT (toxicity; IC_{50}) in cells acutely and persistently infected with HIV-1 after incubation for 7 days
Data are calculated from the mean of $n = 4$ experiments.

Drug	Acutely Infected U937 Cells			Chronically Infected U937 Cells		
	EC_{50} (Antiviral)	IC_{50} (Toxicity)	Selectivity Index	EC_{50} (Antiviral)	IC_{50} (Toxicity)	Selectivity Index
	μM			μM		
SQV	0.03	73.8	2716	0.13	61.7	488
CQ	11.1	24.6	2.2	22.9	11.3	0.5
MQ	3.3	8.0	2.5	7.5	7.0	0.9
MC	7.4	12.1	1.6	4.8	3.9	0.8
HF	3.7	1.2	0.3	62.7	4.8	0.08
AQ	12.9	9.3	0.7	22.9	19.9	0.9

ity against HIV (selectivity index <1 for CQ, MQ, MC, HF, and AQ).

Isobolograms for MQ/CQ in Combination with SQV. To investigate the potential for combination of CQ or MQ with SQV, isobolograms were constructed. Combination of CQ with SQV had an antagonistic effect on viral p24 production in U937 cells (Fig. 2A), but no effect on cellular toxicity as measured by MTT (data not shown). Conversely, the combination of MQ with SQV resulted in a synergistic effect on viral p24 in U937 cells (Fig. 1B), despite no effect on cellular toxicity (data not shown). In both cases MTT, data followed the line of additivity (data not shown).

Effect of MQ/CQ on Antiviral Activities of PIs in MT4 Cells. To investigate whether the synergy observed between MQ and SQV was a drug-specific or a class-specific occurrence, the effect of MQ and CQ on RTV, SQV, NFV, IDV, and APV was investigated in HIVIIIB-infected MT4 cells (Fig. 2A). A significant decrease in the EC_{50} (relative to control) by MQ was observed for SQV (0.3 ± 0.06 versus 0.1 ± 0.07; $p = 0.01$; 95% CI = 0.06–0.27), RTV (4.2 ± 1.3 versus 0.8 ± 0.7; $p = 0.005$; 95% CI = 1.56–5.19), NFV (0.7 ± 0.3 versus 0.1 ± 0.07; $p = 0.05$; 95% CI = 0.12–0.89), IDV (8.5 ± 2.0 versus 3.7 ± 0.8; $p = 0.005$; 95% CI = 2.10–7.47), and APV (6.6 ± 1.4 versus 2.3 ± 0.8; $p = 0.005$; 95% CI = 2.40–6.35). The fold enhancement of EC_{50} were in the rank order of RTV (5.2) > NFV (3.7) > APV (2.9) > SQV (2.4) = IDV (2.3). CQ did not significantly alter the EC_{50} of any of the PIs tested in these experiments (Fig. 2B). Toxicity was not observed in noninfected cells incubated with these concentrations (data not shown).

Effect of MQ/CQ on Recombinant Protease Assay. A fluorescence-based recombinant HIV protease assay was developed to assess for direct interactions between drugs and effects on HIV protease. Production of fluorescent cleavage product by recombinant HIV protease was inhibited by SQV with an EC_{50} of 97.4 ± 11.7 μM. Addition of MQ (10 μM) had no appreciable effect (EC_{50} = 116.1 ± 23.2 μM). Addition of CQ did not attenuate the inhibitory effect of SQV, suggesting that a direct chemical interaction was unlikely to account for the observed antagonism between the two compounds.

Effect of MQ/CQ on Cellular Accumulation of SQV in U937 Cells. To examine the effects of CQ and MQ on the intracellular accumulation, [^{3}H]SQV (1 μM) was incubated with increasing concentrations of MQ and CQ (0–50 μM) in U937 cells (Fig. 3).

CQ decreased cell-associated SQV in a concentration-dependent manner from a cellular accumulation ratio of 101 ± 13 in controls to 80 ± 21 at 1 μM ($p = 0.08$), 71 ± 13 at 3 μM

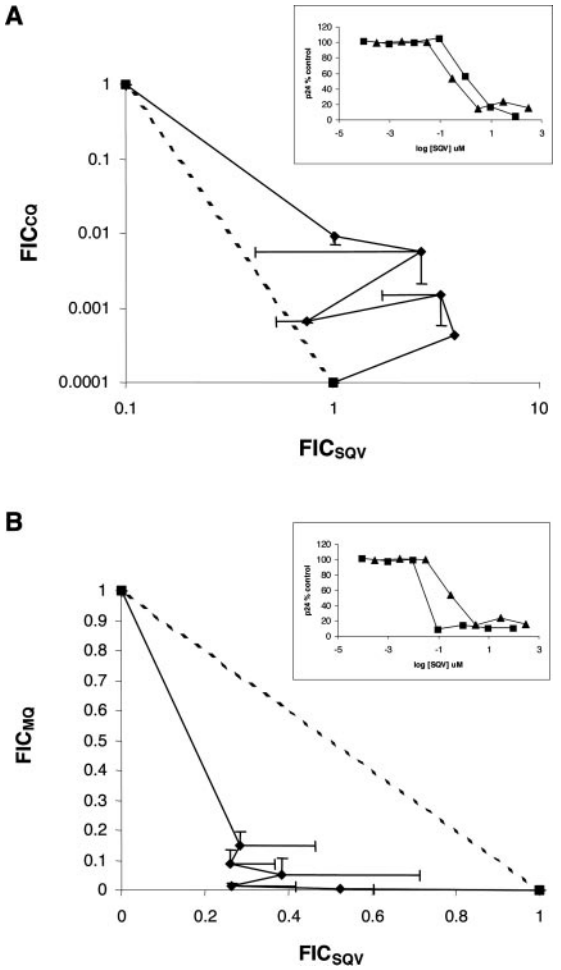

Fig. 1. A, isobologram illustrating the effect of CQ in combination with SQV on viral p24 in U937 cells acutely infected with HIV-1 IIIB. Inset, representative dose-response curves for SQV with CQ (CQ:SQV, 9:1; ■) versus SQV alone (▲). B, isobologram illustrating the effect of MQ in combination with SQV on viral p24 in U937 cells acutely infected with HIV-1 IIIB. Inset, representative dose-response curves for SQV with MQ (MQ:SQV, 7:3; ■) versus SQV alone (▲). Incubations were carried out for 7 days, and data are expressed as the mean and standard deviation of $n = 4$ experiments. The dashed lines illustrate the theoretical line of additivity.

($p = 0.02$; 95% CI = 5.8–50.9), 65 ± 12 at 10 μM ($p = 0.005$; 95% CI = 12.1–57.1), 61 ± 5 at 30 μM ($p = 0.003$; 95% CI = 15.5–60.5), and 62 ± 4 at 50 μM ($p = 0.003$; 95% CI = 14.8–59.8). Conversely, MQ increased cell associated SQV in a concentration-dependent manner from a cellular accumulation ratio of 101 ± 13 in controls to 121 ± 12 at 10 μM (p =

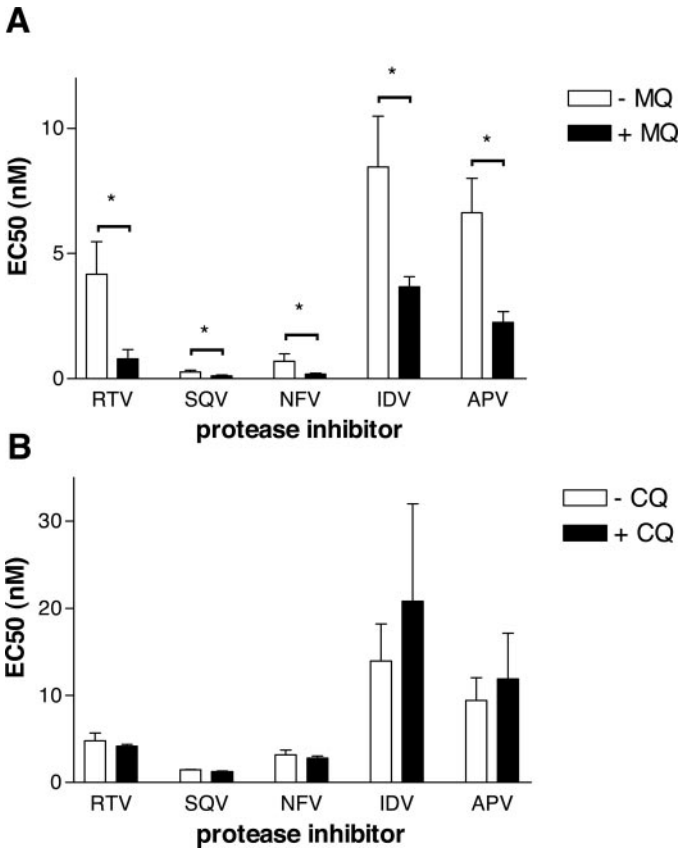

Fig. 2. Effects of MQ (A; 10 μM) and CQ (B; 10 μM) on antiretroviral activity (EC_{50}) of RTV, SQV, NFV, IDV, and APV in MT4 cells infected with HIVIIIB. MT4 cells were infected with HIVIIIB in the presence of MQ and PIs, and syncytia-mediated cell death was assessed by MTT toxicity assay. Data represent the mean and standard deviation of $n = 4$ experiments conducted in triplicate. *, $p < 0.01$. See text for details.

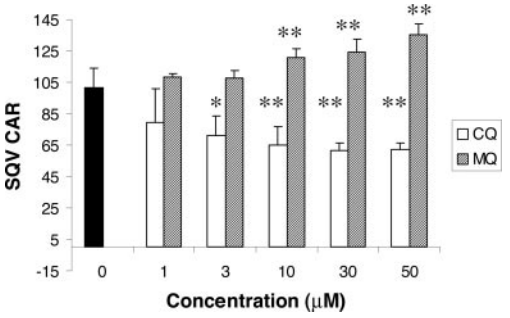

Fig. 3. Effect of MQ on the intracellular accumulation of SQV in U937 cells. SQV (1 μM) was incubated with U937 for 30 min in the presence and absence of MQ (0–50 μM). Data are presented as mean and standard deviation of $n = 4$ experiments conducted in quadruplicate. *, $p < 0.05$; **, $p < 0.01$. See text for details.

0.01; 95% CI = −37 to −5), 124 ± 5 at 30 μM MQ ($p = 0.004$; 95% CI = −41 to −9), and 135 ± 4 at 50 μM MQ ($p = 0.0002$; 95% CI = −52 to −20).

Drug Transporter Expression in U937 Cells. To determine whether transporters known to transport SQV were expressed in U937 cells, real-time reverse transcriptase-polymerase chain reaction was carried out for MDR1, MRP1, and MRP2 (Fig. 4A). Comparisons were made to pooled human liver and pooled human peripheral blood mononuclear cell (PBMC) cDNAs. MDR1 mRNA was ~230- and ~460-fold lower in U937 cells than PBMC and liver, respectively. MRP1

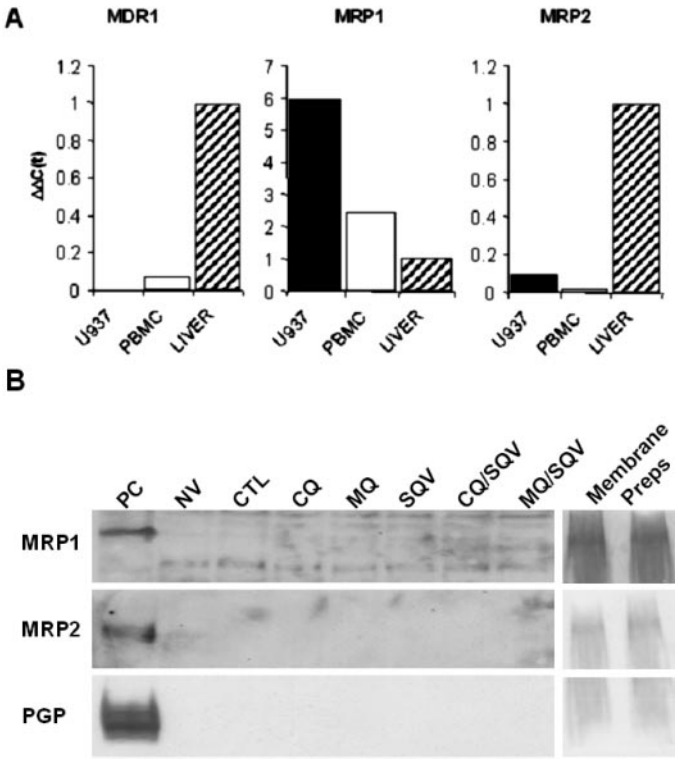

Fig. 4. A, MDR1, MRP1, and MRP2 transcripts in U937 cells relative to pooled cDNA from PBMCs and liver. B, Western blot analysis of Pgp, MRP1, and MRP2 in U937 cells. Separate lanes include noninfected cells (NV) as well as HIVIIIB-infected controls (CTL) and infected cells incubated with MQ (10 μM), CQ (10 μM), SQV (10 μM), or combinations of these for 3 days. Positive controls (PC) for Pgp, MRP1, and MRP2 were also included as indicated. The right panels correspond to Western blots conducted on membrane preparations from noninfected cells.

mRNA was found to be ~3.5- and 7.5-fold higher in U937 cells than PBMC or liver, respectively. For MRP2, the transcript was found to be ~3-fold higher in U937 cells than PBMC but ~7.5-fold lower than liver.

Western blot analysis was carried out for Pgp, MRP1, and MRP2 (Fig. 4B). Although a band of approximately 170 kDa for Pgp (using C219 monoclonal antibody), 190 kDa for MRP1 (using MRPm5 monoclonal antibody), and MRP2 (using M2I-4 monoclonal antibody) was observed in appropriate controls, no Pgp, MRP1, or MRP2 were stained in either noninfected U937 cells or U937 cells treated with CQ (10 μM), MQ (10 μM), SQV (10 nM), CQ/SQV, or MQ/SQV (protein analyses were carried out on infected cells treated with drugs and combinations of drugs to ensure that no increase in transporter expression occurred during the EC_{50} experiments). Furthermore, Pgp and MRP1 transporter proteins were undetectable using flow cytometry with UIC2 and QCRL-1 for Pgp and MRP1, respectively. However, when membrane preparations were used for Western blotting (to enrich the transporter-containing fraction of the cells), MRP1 and MRP2 but not Pgp were detectable in the U937 cells.

For MT4 cells, detection of Pgp and MRP1 was achieved using flow cytometry. For Pgp, median FL-2 fluorescence was significantly higher for UIC2 (1.01 ± 0.07) than isotype control antibody (0.73 ± 0.02; $p = 0.005$; 95% CI = −0.36 to −0.19), confirming expression of this transporter. Similarly, for MRP1, median FL-1 fluorescence was significantly higher for QCRL-1 (1.44 ± 0.07) than isotype control antibody

(1.31 ± 0.03; $p = 0.005$; 95% CI = −0.19 to −0.04), confirming expression of this transporter. The expression indexes for these transporters in MT4 cells were 0.28 ± 0.07 and 0.13 ± 0.07 for Pgp and MRP1, respectively. Expression of MDR1, MRP1, and MRP2 mRNA were also confirmed in these cells (data not shown).

Accumulation of SQV in CEM, CEM_{E1000}, and CEM_{VBL} Cells. To assess selective reversal of Pgp- and MRP1-mediated transport of SQV, accumulation experiments were conducted in CEM_{VBL} and CEM_{E1000} cells. In CEM_{E1000} cells, MQ enhanced accumulation of SQV at both 10 μM (53.6 ± 8.0; $p = 0.17$) and 100 μM (64.5 ± 10.5; $p < 0.03$; 95% CI for the difference = −31.3 to −1.3) relative to no MQ controls (48.2 ± 6.6; Fig. 5). Similarly, in CEM_{VBL} cells MQ enhanced accumulation of SQV at both 10 μM (21.0 ± 2.3; $p < 0.0001$; 95% CI = −17.9, −11.6) and 100 μM (54.9 ± 7.9; $p < 0.0001$; 95% CI = −58.5 to −38.8) relative to controls (6.2 ± 1.2; Fig. 5). Collectively, these data indicate that transport of SQV by MRP1 and Pgp is inhibited in a dose-dependent manner by MQ. Counter intuitively, MQ decreased the accumulation of SQV in the CEM parental cell line from 98.2 ± 7.2 to 88.0 ± 0.2 at 10 μM ($p = 0.04$; 95% CI = 0.55–17.76) and 68.5 ± 7.8 at 100 μM ($p < 0.0001$; 95% CI = 20.1–39.3).

Displacement of SQV Protein Binding by MQ. To assess the impact of MQ on SQV free drug concentrations, protein-binding experiments were performed. A significantly higher SQV free drug concentration was observed when orosomucoid and SQV were preincubated with 1 μM (10.1 ± 0.2% unbound; $p = 0.004$; 95% CI = −5.22 to −1.21), 10 μM (13.3 ± 0.3% unbound; $p < 0.0001$; 95% CI = −8.38 to −4.38), or 100 μM (26.4 ± 2.6% unbound; $p < 0.0001$; 95% CI = −21.5 to −17.5) MQ compared with the control (6.9 ± 0.12% unbound). MQ did not displace SQV when incubated in recombinant albumin, FCS, or human serum (data not shown).

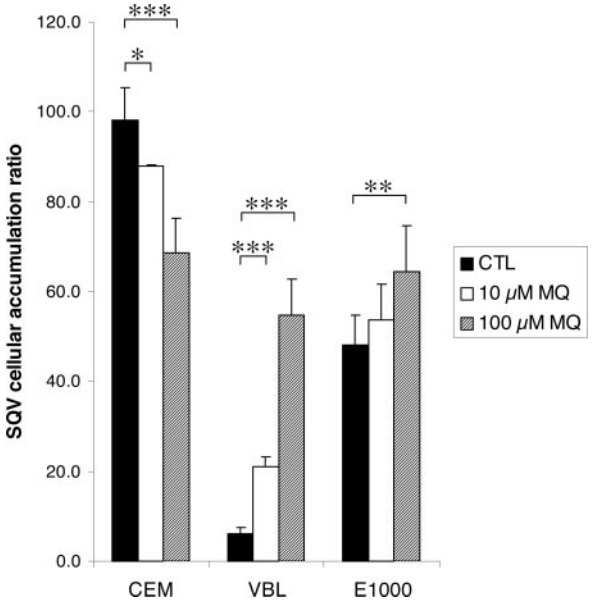

Fig. 5. Cellular accumulation of SQV (1 μM) in CEM_{VBL} (Pgp-overexpressing) and CEM_{E1000} (MRP1-overexpressing) cells and the effect of MQ (10 and 100 μM) on the observed accumulation. Data are presented as mean and standard deviation of $n = 4$ experiments conducted in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. See text for details.

Murine Tissue Distribution of SQV in the Presence and Absence of MQ. To assess the impact of MQ on SQV disposition in vivo, a tissue distribution was conducted for SQV in the presence and absence of MQ. Tissue/blood ratios were found to be consistently higher in the MQ-coadministered animals compared with the SQV alone animals (Fig. 6). A significantly higher tissue/blood ratio was observed for brain (0.07 ± 0.04 versus 0.02 ± 0.01; $p = 0.0003$; 95% CI = −0.09 to −0.02) and testis (0.19 ± 0.15 versus 0.07 ± 0.04; $p = 0.02$; 95% CI = −0.19 to −0.01) but not liver (1.8 ± 1.5 versus 0.8 ± 0.4; $p = 0.16$) or kidney (2.2 ± 1.7 versus 1.0 ± 0.4; $p = 0.19$). Importantly, although differences were observed in blood concentrations between MQ-coadministered animals (117.7 ± 51.3 μg/ml) and controls (240.0 ± 49.9 μg/μl), the brain concentrations of SQV were significantly higher in the former (4.2 ± 1.9 versus 7.1 ± 2.4 pg/g; $p = 0.01$; 95% CI = −5.2 to −0.5).

Discussion

CQ is relatively inexpensive, is widely available, and has been shown to inhibit HIV-1 replication by disrupting the formation of glycoproteins in the viral envelope resulting in a broad spectrum of antiviral activity (Tsai et al., 1990; Savarino et al., 2001a). In keeping with previous reports (Savarino et al., 2001a), we found that CQ exhibited anti-HIV activity. We also present data suggesting similar activity (based on selectivity index) for MQ and MC. However, these effects were extremely modest in comparison with SQV and only observed at high concentrations and in acutely infected cells, suggesting a preintegration site of action. No antiviral effects were observed with HF and AQ. The narrow therapeutic index of these drugs combined with the high concen-

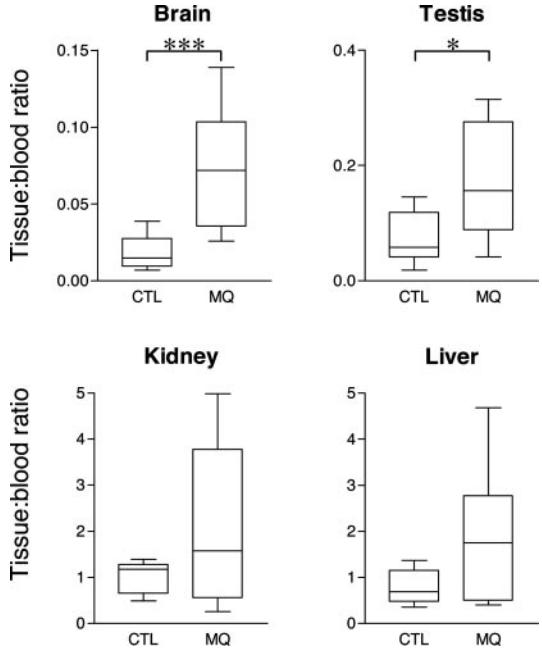

Fig. 6. Tissue distribution of SQV in the presence and absence of MQ. CD-1 mice received an intravenous (tail-vein) dose of MQ (10 mg/kg) or vehicle alone 10 min before an intraperitoneal dose of SQV (1 mg/kg). Tissues were isolated 1 h later and SQV concentrations assessed. Data are presented as the mean and standard deviation of values obtained from 10 mice in each group. *, $p < 0.05$; ***, $p < 0.001$. See text for details.

trations required to inhibit HIV suggest that chronic dosing with these agents is unlikely to prove effective or successful against HIV-1 (Martin et al., 1987). This seems to be confirmed by a study reporting a 1.3 log drop in viral load in treatment naive patients receiving didanosine and hydroxyurea plus hydroxyCQ for 12 weeks, less than the median 1.7 log drop seen with didanosine and hydroxyurea alone (Biron et al., 1996).

We demonstrate MQ-SQV and MQ-PI synergy in U937 and MT4 cells, respectively. In contrast, CQ-SQV antagonism was observed in these cells. We provide evidence that the synergy (MQ) and antagonism (CQ) with SQV in U937 cells was at the level of transport. Indeed, our cell-free recombinant protease assay demonstrated no modulation of SQV activity (as judged by velocity of formation of cleaved fluorescent substrate) with MQ or CQ. This suggests in particular that a direct chemical interaction (e.g., inactivation by complexing) was highly unlikely to account for the observed antagonism between CQ and SQV. This enhancement in the CAR of SQV by MQ is in keeping with previously reported effects of MQ in other cell lines (Fujita et al., 2000). Thus, the increased intracellular accumulation of SQV by MQ in the U937 cells, coupled possibly with similar modulatory effects of MQ on the intracellular accumulation of SQV and other PIs in the MT4 cells may explain the enhanced in vitro antiviral activities of the drugs in these cells.

However, our observations with CQ, when coincubated with SQV, are contrary to recently published findings (Savarino et al., 2004). Interestingly, CQ elicited a decrease in the accumulation of SQV in U937 cells and one can only speculate that this observation may be due to inhibition of as yet uncharacterized influx systems. It has recently been reported that CQ had a synergistic effect with IDV, RTV, and SQV in CD4+ T-cell lines (Savarino et al., 2004). It is important to note that these cells are known to express Pgp and MRP1, indicating that the reported synergy between CQ and SQV may not only be transporter-mediated but also due to cell-specific effects of CQ. Furthermore, it is important to recognize that there are a number of differences between the studies by Savarino et al. (2001a,b, 2004) and our present study. First, we have assessed the ability of CQ (and other antimalarials) to directly inhibit acute and chronic HIV replication, whereas in the previous studies the supernatants from formerly infected and treated cells were used to infect fresh cells to assess the effects of CQ on viral budding and infectivity (Savarino et al., 2001a). Second, the synergy described between CQ and PIs in the previous study were conducted in cells expressing Pgp (Savarino et al., 2004). In our study, we have shown that Pgp is absent from the U937 cell model both at the mRNA and protein level. However, the discord between our and previous studies cannot be explained purely on the basis of Pgp expression since MT4 cells cultured in our laboratory do express this protein. Therefore, one can only speculate that this phenomenon is due to global differences in transporter expression between U937 cells and the cells used in previous studies, coupled with inherent differences in transporter inhibition between MQ and CQ.

If the enhanced in vitro antiviral activities of MQ-PI combinations are at the level of transport, what drug efflux transporters could mediate these observed effects? Here, we demonstrate by flow cytometric and Western blot analyses that Pgp was not detectable in U937 cells. Similarly, mRNA for MDR1 was undetectable in these cells. Conversely, MRP1 and MRP2 mRNA were detected, along with the corresponding proteins when Western blotting was conducted on membrane preparations. This indicates that inhibition of multiple drug efflux transporters or membrane effects may have contributed to the enhanced accumulation of SQV and MQ-SQV or MQ-PI synergy in these cells. The observation that Pgp is absent from the U937 cells cultured in our laboratory is contradictory to previous reports (Gollapudi and Gupta, 1990; Andreana et al., 1994; Bailly et al., 1995). There are a number of possible explanations for this contradiction. 1) We have previously reported other phenotypic differences between cell lines cultured in different laboratories (Speck et al., 2002; Owen et al., 2003a). 2) The previous studies were conducted more than 10 years ago, and as such, phenotypic differences may have arisen during this time. 3) The antibodies used in previous studies are different to those used here, and as such, there may be differences in reactivity.

In the CEM_{VBL} and CEM_{E1000} models of Pgp and MRP1 overexpression, dose-dependent reversal of altered accumulation of SQV by MQ was observed, indicating that MQ is able to inhibit drug efflux of SQV by these transporters. Curiously, in the parental cell line, the opposite of what was noted in U937 and the CEM sublines was observed. Again, one can only speculate that this phenomenon may be due to inhibition of an as yet unidentified influx transporter within these cells.

To assess any potential interactions of MQ with SQV protein binding, we assessed the potential for displacement from orosomucoid, albumin, FCS, and human serum. We conclude from these experiments that protein-binding effects are unlikely to contribute to the in vitro synergy observed, since MQ did not displace SQV from FCS. However, we observed a dose-dependent displacement of SQV from orosomucoid but not albumin by MQ. To assess whether SQV-free drug concentrations could be modulated in vivo, we carried out similar experiments using human serum. Although data indicated that free drug concentrations were not altered in serum, one cannot rule out the possibility that the high-affinity binding to orosomucoid was displaced, and albumin simply "mopped up" the excess. This has important implications since, theoretically, the shift from high-affinity to low-affinity binding may influence the ability of compounds to cross biological membranes. Indeed, differences in orosomucoid binding have been shown to alter hepatic extraction ratio of quinidine (Mansor et al., 1991), a drug that is bound to both orosomucoid and albumin (Mihaly et al., 1987).

Given the potent enhancement in antiviral activities of SQV and other PIs and the displacement of SQV from orosomucoid when coincubated with MQ, we investigated the potential for MQ to increase the tissue penetration of SQV in CD-1 mice. Interestingly, we observed an increased tissue distribution of SQV in the MQ coadministered group compared with control, suggesting that the bioavailability and tissue permeation of SQV were enhanced by MQ. Indeed, the ratio of tissue to blood concentrations was increased in every tissue assayed. Although this was partly explicable by the observation that the MQ-treated animals had lower blood concentrations (as one would expect if more of the drug is free to infiltrate surrounding tissue), the concentration in brain was doubled, even when not corrected for blood concentration. A potential question that arises from the presented data

relates to the location of the SQV in brain. For example, whether MQ increases *trans*-endothelial transport of SQV or simply increases sequestration in the choroid plexus. Microdialysis experiments would clarify these issues.

We show here improvements in the in vitro antiviral effects of SQV (and other PIs) when coadministered with MQ and potentiation of the retention of SQV in sanctuary sites of CD-1 mice. Given these exciting observations, more preclinical studies are now warranted to define this drug-drug interaction more clearly with the use of a clinically relevant codosing regimen.

Acknowledgments

We thank Ross Davey (Department of Clinical Oncology, Royal North Shore Hospital, St Leonards, NSW, Australia) for supplying the CEM sublines used in these experiments and the National Institute for Biological Standards and Control for the supply of many of the reagents necessary for the virological analyses.

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