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In-vitro synergy and enhanced murine brain penetration of saquinavir co-administered with mefloquine.

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Running Title: Antiretroviral synergy between saquinavir and mefloquine

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Nonstandard Abbreviations: HAART, highly active antiretroviral therapy; CQ, chloroquine; MQ, mefloquine; MC, mepacrine; AQ, amadiaquine; HF, hyfantrin; SQV, saquinavir; RTV, ritonavir; NFV, nelfinavir; IDV, indinavir; APV, amprenavir; PI, protease inhibitor; Pgp, p-glycoprotein; MRP, multidrug resistance associated protein; MTT, methyl tetrazolium; FIC, fractional inhibitory concentration; CAR, cellular accumulation ratio; GAPDH, glyceraldehydes phosphate dehydrogenase;

MDR, multidrug resistance; ECL, enhanced chemiluminescence; NISBC, National Institute for Biological Standards and Control; EC_{50}, effective concentration causing 50% of the maximal response; IC_{50}, inhibitory concentration causing 50% of the maximal response; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; EDANS, 5-(aminoethyl)aminonaphthalene sulfonate; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulphoxide; FITC, fluorescein isothiocyanate.

ABSTRACT

Highly active antiretroviral therapy has substantially improved prognosis in HIV. However, the integration of proviral DNA, development of viral resistance and lack of permeability of drugs into sanctuary sites (brain, lymphocyte etc) 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 4 other 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 MQ was also shown to displace SQV from orosomucoid (AAG) in vitro. Finally, co-administration 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 HIV positive patients.

INTRODUCTION

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 its analogue 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 plays a major role in determining inter-individual variation 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; Jones et al., 2001b; 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 co-therapy 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 over-express these transporters (Riffkin et al., 1996; Vezmar and Georges, 1998; Fujita et al., 2000; Vezmar and Georges, 2000).

In this study, we screened 5 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 post-integration 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 utilised 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 increase free drug concentration in serum was investigated. Finally, we investigated the tissue distribution of SQV in CD-1 mice when co-administered with MQ.

METHODS

i) Cells and virus

U937, CEM (parental), CEM_{VBL}(overexpressing Pgp; (Owen et al., 2003b)), CEM_{E1000} (overexpressing MRP1; (Owen et al., 2003b)) and MT4 were grown in RPMI 1640, supplemented with 10% (v/v) foetal calf serum (FCS) and 2mM 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 $TCID_{50}$ calculated. Viral production was measured immunoenzymatically using commercially available p24 antigen enzyme-linked immunosorbent assay (ELISA) 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 manuscript) was assessed using the standard methyl-tetrazolium (MTT) assay as previously validated within our laboratory (Sim et al., 1998). For viral quantitation, an aliquot (100µL) was centrifuged at 13 000 x g for 10min and the supernatant taken for p24 antigen assay as described above.

ii) **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 37ºC for 1h in the presence of SQV, CQ, MQ, MC, HF or AQ (0.1-100µM in third logs). Cells were then washed three times before re-suspension at 2.5×10^5 cells in 2mL fresh culture medium in the presence

and absence of SQV, CQ, MQ, MC, HF or AQ (0.1-100μM in third logs). Persistently infected U937 cells (to assess the effects on the post-integration 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 (0.1-100μM 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 and the selectivity indices calculated as the IC_{50}/EC_{50} ratio.

iii) Isobolograms and modulation of PI activity by CQ and MQ

In order 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. Following 7-days incubation, viral replication and cellular toxicity were assessed as described above. This allowed calculation of the fractional inhibitory concentrations (FIC) of the resulting EC_{50} values for each drug which were plotted as isobolograms as previously described (Berenbaum, 1978). These were interpreted according to recently published guidelines (Odds, 2003) i.e. FIC interpretations of ‘synergy’ (FIC ≤ 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 (10μM) were also assessed against HIV-IIIB 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, 5min) and the supernatant fraction discarded. Cells were resuspended in RPMI 1640 containing 10% FCS, counted using a haemocytometer and the concentration adjusted to 1 x 10^6/mL. HIVIIIB cell free supernatant was added to the cell suspension (1 x 10^{-2} $TCID_{50}$ per cell). Cell suspension was added to all wells (50mL, final cell concentration 5 x 10^5/mL containing PI in doubling dilutions (100 - 0.015nM) with or without MQ or CQ (10μM) except cell free negative controls, which contained media only. Drug free positive controls containing cells alone were also included. Following incubation (37°C, 5% CO_2, 7 days) plates were assayed for cytotoxicity as described above.

iv) 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; NISBC) was combined with SQV (0.3nM to 10μM) with or without MQ or CQ (10μM) in protease buffer (0.1M NaAc; 1M NaCl; 1mM EDTA; 1mM DTT; 10% DMSO; 1mg/mL BSA; pH 4.7). To start the reaction a final concentration of 0.1μM HIV protease substrate 1 (Molecular Probes, Leiden, NL) 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, EDANS) and the other to an acceptor chromophore (4-dimethylaminoazobenzene-4-carboxylate, dabcyl), resulting in quenching of the nearby fluorophore through resonance energy transfer. Excitation was therefore performed at 340nm and the

emission simultaneously measured at 490nm for 10min. The mean velocity for formation of the cleaved substrate was calculated for each drug concentration and normalised to controls. Comparisons were then made between SQV inhibition in the presence and absence of MQ or CQ (10μM).

v) Cellular accumulation studies

In order 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. Following preincubation for 10min with 0, 1, 3, 10, 30 or 50μM MQ, cells were incubated in the presence of 1μM [^{3}H]-SQV (specific activity 26μCi/mg) for 30min at 37°C in serum-free media.

At the end of the incubation period, the samples were removed and centrifuged (15,000*g* for 2min) in a chilled microcentrifuge. An aliquot (50μl) of the supernatant was taken for scintillation counting and the cell pellets were washed three times in ice-cold PBS before the cells were solubilised by adding 100μl of a cocktail containing five parts tissue solubiliser, two parts H_2O_2, and two parts glacial acetic acid. The samples were then analysed 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.

vi) Transporter expression

Real-time reverse transcriptase polymerase chain reaction: Quantification of mRNA transcripts for MDR1, MRP1 and MRP2 was achieved by real-time PCR using an Opticon2 Sequence Detection System. GAPDH was used as the housekeeping

gene. 40ng of cDNA was combined with Universal master mix, sense and antisense primers (0.4μM each) and oligonucleotide probe (0.2μM) in a final volume of 25μl. 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 website.

Western blot analysis: Infected and non-infected U937 cells (typically $5x10^6$ cells/mL) were incubated in the presence or absence of SQV (10nM), CQ (10μM), MQ (10μM), CQ/SQV or MQ/SQV for three 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 in order to enrich the transporter-containing fraction of the cells. Breifly, U937 cells were homogenised in ice-cold homogenization buffer (250 mM sucrose, 10 mM 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 1 mM ethylenediaminetetraacetic acid [EDTA] and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and the nuclei pelleted at 500 x g for 10min. The supernatant fraction was then further centrifuged at 100,000 x g for 30min in order to pellet the membrane fraction.

Briefly, 50μg total protein from each cell line was electrophoresed on 3-12% Tris-Acetate gels. Following transfer, membranes were blocked with 10% non-fat 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 (1:10000) and transporters were detected using enhanced chemiluminescence (ECL) reagent.

Flow cytometry: flow cytometric analysis was carried out for Pgp and MRP1 in U937 cells in order to characterise these cells with respect to efflux transporters known to transport SQV. MRP2 flow cytometry was also attempted but the antibody was found to be non-compatible with this technique (data not shown). Pgp flow cytometry was carried out using monoclonal antibody UIC2 as previously validated in our laboratory (Chandler et al., 2003). Briefly, cells (1×10^6) were fixed with CellFIX (4°C; 30min) and incubated with UIC2 (RT; 60min). Following 3 washes with HBSS, cells were stained with PE-conjugated secondary antibody (RT; 60min). After another 3 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 (30min, 4°C) and permeablised with saponin (0.1 mg/mL in HBSS). Cells were then labelled with the MRP1 specific mouse anti human primary antibody, QCRL-1 (200µL, 0.12 µg/mL) and detection was achieved with FITC conjugated-IgG secondary antibody (200µL, 0.2 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 in order to calculate the antibody-specific fluorescence.

vii) Assessment of SQV protein binding in the presence and absence of MQ.

In order to determine any effects of MQ on SQV protein binding, ^{3}H-SQV (10μM) was incubated with recombinant orosomucoid (150mg/dL; median physiological concentration), recombinant albumin (4.5g/dL; median physiological concentration) or FCS (10%) in RPMI. In addition, the ability of MQ to increase SQV free drug concentration in human serum was assessed (n=6). A range of MQ concentrations (0-100μM) were pre-incubated with protein-containing RPMI or serum for 10min prior to the addition of SQV. Following 30min incubation at 37°C, the samples (500μL) were applied to an Amicon Centrifree Filter System (molecular weight cutoff 30,000 kDa; Millipore Corporation, Bedford, MA), and centrifuged (1500 g, 60 minutes; 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.

viii) Murine tissue distribution of SQV in the presence and absence of MQ.

Male CD-1 mice were administered a bolus intravenous dose of MQ (10mg/Kg) 10 minutes prior to a bolus intraperitoneal dose of SQV (10mg/Kg). Following 1 hour, the animals were sacrificed with a rising concentration of CO_2 and a cardiac puncture performed in order to obtain blood samples. The brain, testis, liver and kidneys were then removed and frozen in liquid nitrogen. On the following day, tissues were

homogenised and SQV concentrations assessed by LC/MS/MS as previously reported (Khoo et al., 2002).

ix) 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 employed.

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) >>> MQ (2.5) > CQ (2.2) > MC (1.6). Neither HF nor AQ exhibited any selectivity towards 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 selectivity against HIV (selectivity index <1 for CQ, MQ, MC, HF and AQ).

Isobolograms for MQ/CQ in combination with SQV

In order 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 (Figure 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 (Figure 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

In order to investigate whether the synergy observed between MQ and SQV were a drug specific or a class specific occurrence, the effect of MQ and CQ on RTV, SQV, NFV, IDV and APV were investigated in HIVIIIB infected MT4 cells (Figure 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 (Figure 2B). Toxicity was not observed in non-infected 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 (10μM) 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

In order to examine the effects of CQ and MQ on the intracellular accumulation, [^{3}H]-SQV (1μM) was incubated with increasing concentrations of MQ and CQ (0-50μM) in U937 cells (Figure 3).

CQ decreased cell associated SQV in a dose dependant manner from a cellular accumulation ratio of 101 ± 13 in controls to 80 ± 21 at 1μM (p = 0.08), 71 ± 13 at 3μM (p = 0.02; 95% CI = 5.8, 50.9), 65 ± 12 at 10μM (p = 0.005; 95% CI = 12.1,

57.1), 61 ± 5 at 30μM (p = 0.003; 95% CI = 15.5, 60.5) and 62 ± 4 at 50μM (p = 0.003; 95% CI = 14.8, 59.8). Conversely, MQ increased cell associated SQV in a dose dependant manner from a cellular accumulation ratio of 101 ± 13 in controls to 121 ± 12 at 10μM (p = 0.01; 95% CI = -37, -5), 124 ± 5 at 30μM MQ (p = 0.004; 95% CI = -41, -9) and 135 ± 4 at 50μM MQ (p = 0.0002; 95% CI = -52, -20).

Drug transporter expression in U937 cells

In order to determine whether transporters known to transport SQV were expressed in U937 cells, real-time RT-PCR was carried out for MDR1, MRP1 and MRP2 (Figure 4A). Comparisons were made to pooled human liver and pooled human PBMC cDNAs. MDR1 mRNA was ~230 and ~460 fold lower in U937 cells than PBMC and liver respectively. MRP1 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 (Figure 4B). Although a band of approximately 170kDa for Pgp (using C219 monoclonal antibody), 190kDa for MRP1 (using MRPm5 monoclonal antibody) and MRP2 (using M2I-4 monoclonal antibody) were observed in appropriate controls, no Pgp, MRP1 or MRP2 were stained in either non-infected U937 cells or U937 cells treated with CQ (10μM), MQ (10μM), SQV (10nM), CQ/SQV or MQ/SQV (protein analyses were carried out on infected cells treated with drugs and combinations of drugs in order 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 utilised for Western blotting (to enrich the transporter-containing fraction of the cells), MRP1 and MRP2 but not P-gp 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, -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, -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

In order to assess selective reversal of P-gp 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 10μM (53.6 ± 8.0; p = 0.17) and 100μM (64.5 ± 10.5; p < 0.03; 95% CI for the difference = -31.3, -1.3) relative to no MQ controls (48.2 ± 6.6; Figure 5). Similarly, in CEM_{VBL} cells MQ enhanced accumulation of SQV at both 10μM (21.0 ± 2.3; p < 0.0001; 95% CI = -17.9, -11.6) and 100μM (54.9 ± 7.9; p < 0.0001; 95% CI = -58.5, -38.8) relative to controls (6.2 ± 1.2; Figure 5). Taken 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 10μM (p = 0.04; 95% CI = 0.55, 17.76) and 68.5 ± 7.8 at 100μM (p < 0.0001; 95% CI = 20.1, 39.3).

Displacement of SQV protein binding by MQ.

In order 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 pre-incubated with 1μM (10.1 ± 0.2 % unbound; p = 0.004; 95% CI = -5.22, -1.21), 10μM (13.3 ± 0.3 % unbound; p < 0.0001; 95% CI = -8.38, -4.38) or 100μM (26.4 ± 2.6 % unbound; p < 0.0001; 95% CI = -21.5, -17.5) MQ as compared to the control (6.9 ± 0.12 % unbound). MQ did not displace SQV when incubated in recombinant albumin, FCS or human serum (data not shown).

Murine tissue distribution of SQV in the presence and absence of MQ.

In order 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 as compared to the SQV alone animals (Figure 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, -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-co-administered 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 pg/g versus 7.1 ± 2.4 pg/g; p = 0.01; 95% CI = -5.2, -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) was observed for MQ and MC. However, these effects were extremely modest in comparison to SQV, and only observed at high concentrations and in acutely infected cells (suggesting a pre-integration site of action). No antiviral effects were observed with HF and AQ. The narrow therapeutic index of these drugs combined with the high concentrations 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 naïve 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 uncharacterised 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 recognise that there are a number of differences between the studies by Savarino et al (Savarino et al., 2001a; Savarino et al., 2001b; Savarino et al., 2004) and our present study. Firstly, 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). Secondly, the synergy described between CQ and PIs in the previous study were conducted in cells expressing P-gp (Savarino et al., 2004). In our study, we have shown that P-gp 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 P-gp expression since MT4 cells cultured in our lab 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 utilised 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 P-gp 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: 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 over 10 years ago and as such phenotypic differences may have arisen during this time. 3) The antibodies utilised in previous studies are different to those utilised here and as such there may be differences in reactivity.

In the CEM_{VBL} and CEM_{E1000} models of P-gp and MRP1 overexpression, dose dependant 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 sub-lines 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.

In order 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. In order 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 co-incubated 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 co-administered group compared to control, suggesting that the bioavailability and tissue permeation of SQV was enhanced by MQ. Indeed, the ratio of tissue to blood concentrations were increased in every tissue assayed. Although this was partly explicable by the observation that the MQ-treated animals had lower blood concentrations (as you 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 co-administered 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 in order to define this drug-drug interaction more clearly with the use of a clinically relevant co-dosing regimen.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1 A) Isobologram illustrating the effect of chloroquine (CQ) in combination with saquinavir (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 mefloquine (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 is expressed as the mean and standard deviation of n = 4 experiments. The dashed lines illustrate the theoretical line of additivity. FIC = Fractional inhibitory concentration.

Figure 2 The effects of MQ (A; 10μM) and CQ (B; 10μM) on antiretroviral activity (EC_{50}) of RTV, NFV, SQV, 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.

Figure 3 Effect of mefloquine (MQ) on the intracellular accumulation of saquinavir (SQV) in U937 cells. SQV (1μM) was incubated with U937 for 30min in the presence and absence of MQ (0-50μM). 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. CAR = Cellular accumulation ratio.

Figure 4 (A) MDR1, MRP1 and MRP2 transcripts in U937 cells relative to pooled cDNA from peripheral blood mononuclear cells (PBMC) and liver. (B) Western blot analysis of Pgp, MRP1 and MRP2 in U937 cells. Separate lanes include non-infected cells (NV) as well as HIVIIIB infected controls (CTL) and infected cells incubated with MQ (10μM), CQ (10μM), SQV (10μM) 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 non-infected cells.

Figure 5 Cellular accumulation of SQV (1μM) in CEM_{VBL} (Pgp-overexpressing) and CEM_{E1000} (MRP1-overexpressing) cells and the effect of MQ (10 and 100μM) 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.

Figure 6 Tissue distribution of SQV in the presence and absence of MQ. CD-1 mice received a intra-venous (tail-vein) dose of MQ (10mg/kg) or vehicle alone 10min prior to an intra-peritoneal dose of SQV (1mg/kg). Tissues were isolated 1h 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.

TABLES

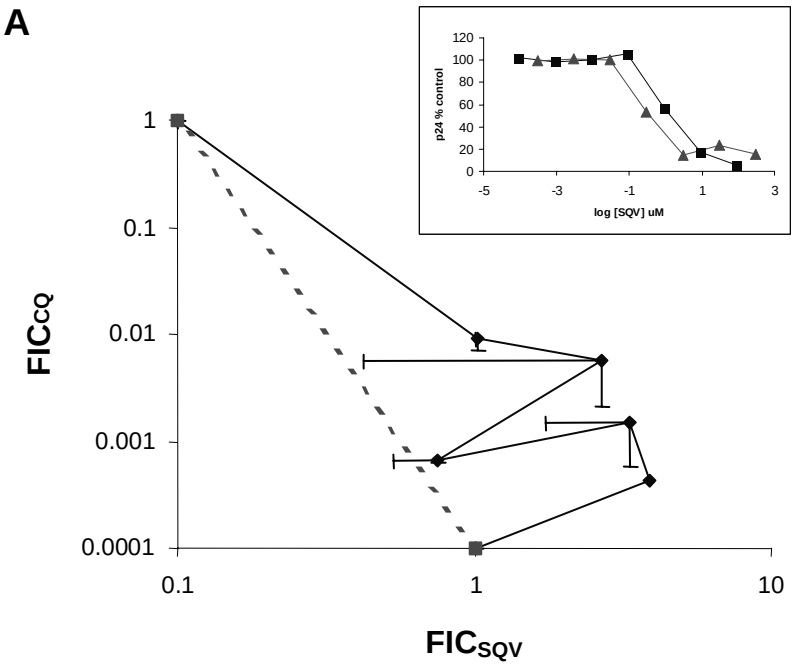
Table 1

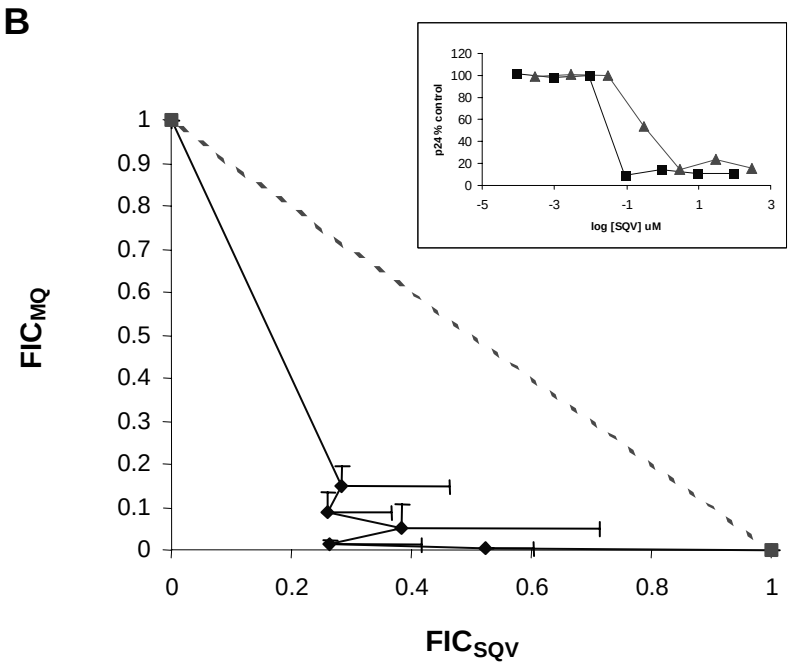
The effects of saquinavir (SQV), chloroquine (CQ), mefloquine (MQ), mepacrine (MC), halofantrin (HF) and amadioquine (AQ) (0.1-100μM) on viral p24 (EC_{50}) and U937 MTT (toxicity; IC_{50}) in cells acutely and persistently infected with HIV-1 following incubation for 7 days. Data are calculated from the mean of n=4 experiments.

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

FIGURES

Figure 1





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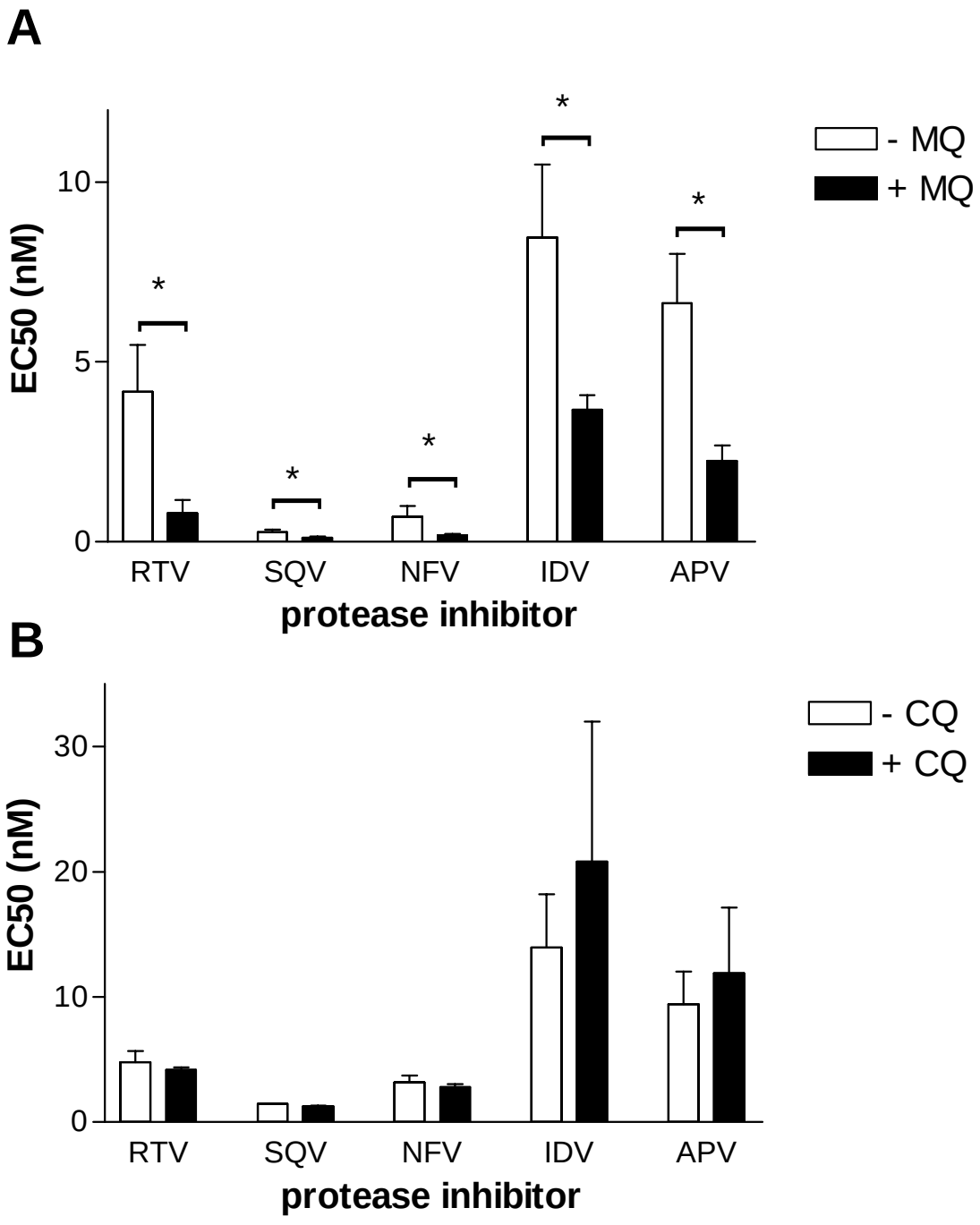
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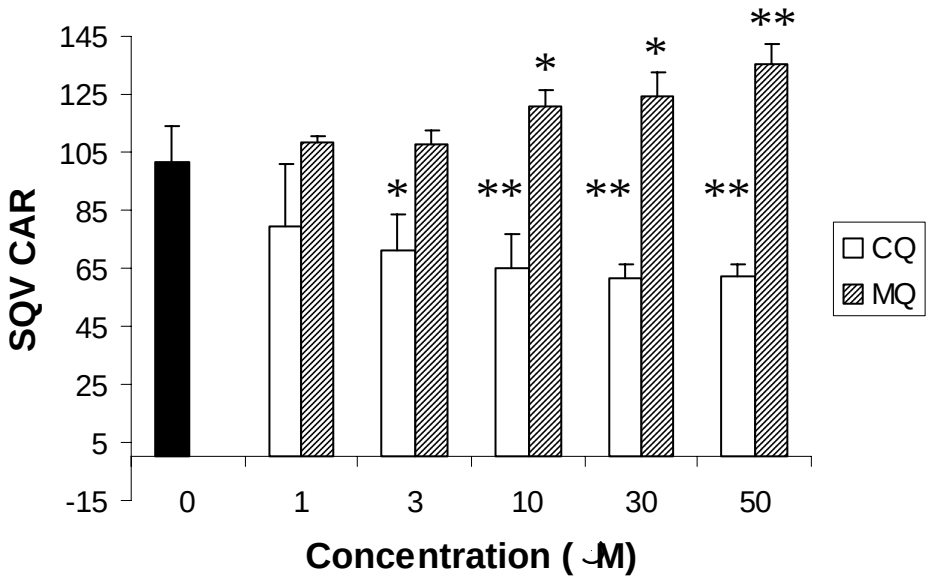
Figure 3


SQV CAR
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105
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Concentration (μM)
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MQ
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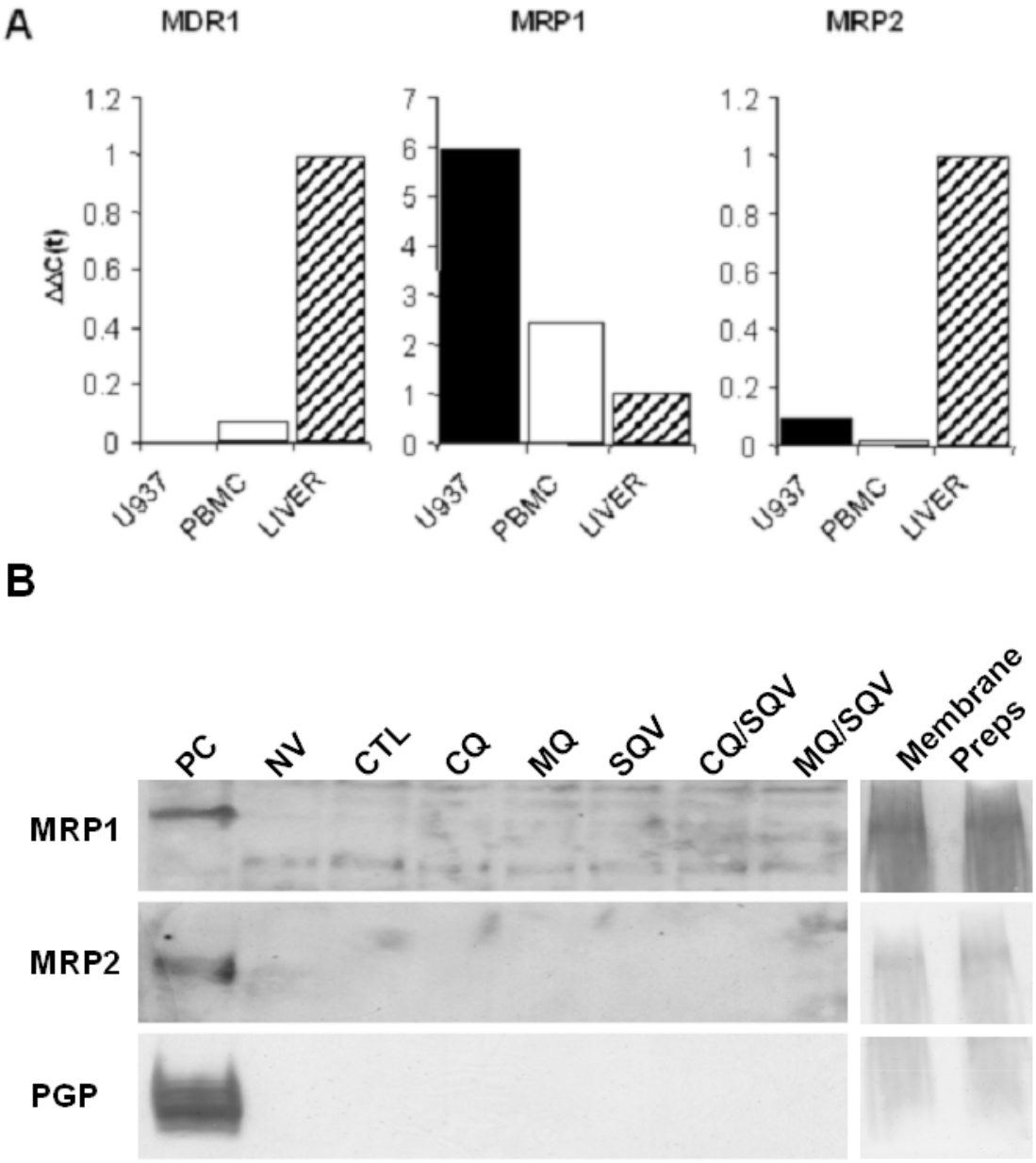
Figure 4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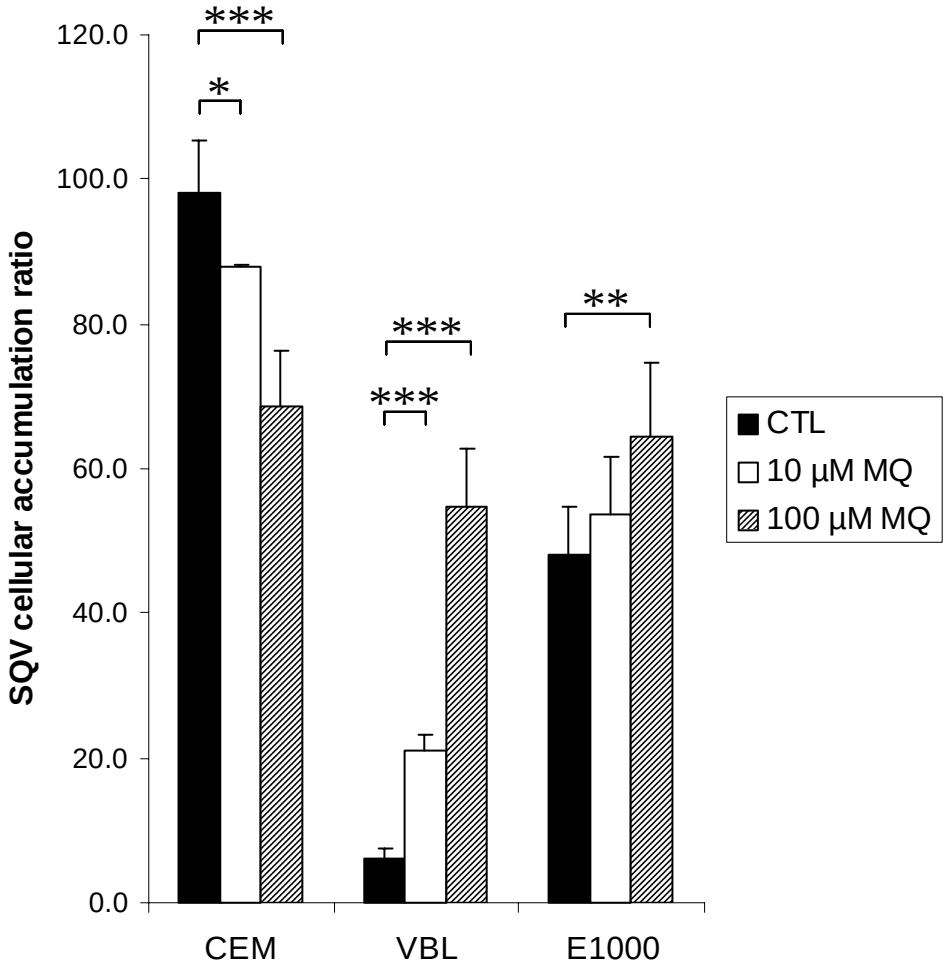
Membrane Preps
MRP1
MRP2
PGP

Figure 5


SQV cellular accumulation ratio
120.0
100.0
80.0
60.0
40.0
20.0
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*
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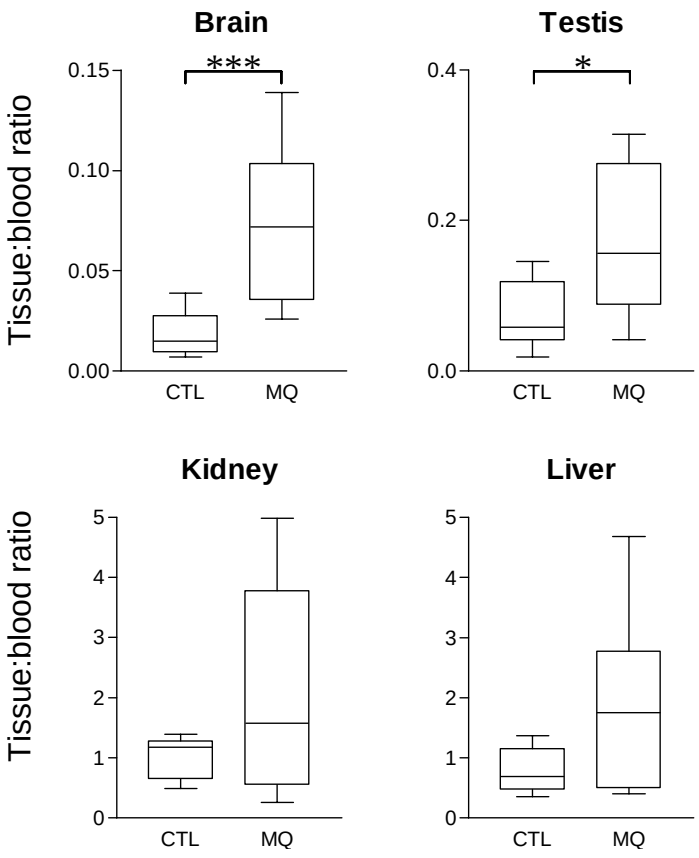
CEM
VBL
E1000
CTL
10 µM MQ
100 µM MQ

Figure 6


Brain
Testis
Kidney
Liver
Tissue:blood ratio
Tissue:blood ratio
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3
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CTL
MQ