Effect of α_1 -acid glycoprotein on the intracellular accumulation of the HIV protease inhibitors saquinavir, ritonavir and indinavir *in vitro*

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Aims Since α_1 -acid glycoprotein (AGP) levels may be raised during HIV infection, we have examined *in vitro* the effect of increasing the concentration of AGP on the intracellular accumulation of the HIV protease inhibitors saquinavir (SQV), ritonavir (RTV) and indinavir (IDV).

Methods U937 cells (5×10^6 cells in 5 ml RPMI growth medium) were incubated at 37°C for 18 h with [14 C]-SQV (0.1 μ Ci), [3 H]-RTV and [3 H]-IDV (0.135 μ Ci) to a final concentration of 1 μ M in the presence of 0, 0.5 and 2.0 mg ml $^{-1}$ AGP. Following extraction in 60% methanol the intracellular drug concentration was determined by liquid scintillation counting.

Results Binding to AGP (2.0 mg ml $^{-1}$) reduced the mean intracellular concentration of SQV from 31.5 μ M to 7.4 μ M ($P < 0.0001$; 95% CI 19.4–28.8). RTV concentration was also reduced (8.8 μ M to 1.6 μ M; $P < 0.0001$; 95% CI 5.4–9.0) as was the concentration of IDV (3.0 μ M to 1.5 μ M; $P < 0.0001$; 95% CI 1.1–1.9).

Conclusions Reduced intracellular protease inhibitor concentrations in the presence of increasing concentrations of AGP will certainly impact on the antiviral activity *in vitro*. However, since protease inhibitors are high clearance drugs, free drug concentration will likely remain unaffected in the presence of elevated AGP during chronic oral dosing although there will be an increase in total plasma drug concentration.

Keywords: α_1 -acid glycoprotein, HIV, intracellular accumulation, protease inhibitors, U937 cells

Introduction

Treatment of HIV infection involves the use of HIV protease inhibitors in combination with other antiretroviral agents. However, patients exhibit wide variability in virological response to these regimens, due, in part, to interindividual variability in plasma concentration of the protease inhibitors. Differences in drug sensitivity of viral strains are also important since treatment response is inferior in previously treated compared with treatment naïve patients.

The most important determinants of drug activity *in vivo* are the drug-sensitivity of the virus and the concentration

of free (unbound) drug at the site of action. The precise site of action of the protease inhibitors has yet to be defined. Inhibition of HIV protease is likely to take place intracellularly during assembly and budding of new virions, although it is possible that protease inhibitors may act extracellularly as well. Protease inhibitors are bound predominantly to α_1 -acid glycoprotein (AGP). Binding is >95% for SQV and RTV [1, 2] but lower for IDV (>60% [3]). The unbound drug not only represents the amount of drug available to exert a pharmacological effect, but may also influence the penetration of drugs into tissue compartments and cells. AGP is an acute phase protein that is elevated in many acute and chronic infections, including HIV. AGP concentrations may vary by as much as 4-fold between healthy volunteers and patients [4]. An increase in AGP concentration may affect the unbound fraction of drug with little appreciable change in plasma (free) drug concentrations [5]. However

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the total plasma concentration will be increased during chronic oral administration.

Zhang *et al.* [6] demonstrated that a four fold increase in AGP concentration resulted in a corresponding increase in the IC_{50} of the HIV protease inhibitors. Addition of AGP at 0.5 and 2.0 mg ml⁻¹ altered the antiviral activity against two wild type HIV-1 isolates, and with a partial and highly resistant HIV-1 strain, a concentration dependent loss in activity was observed. This effect was most marked with RTV and amprenavir with the least effect on IDV.

In the present study we have examined *in vitro* the effect of increasing the concentration of AGP on the intracellular accumulation of SQV, RTV and IDV.

Methods

Cells

U937 cells were acquired from Porton Down, Salisbury, UK and propagated in RPMI 1640 medium (Sigma Chemical Company, Poole, UK) supplemented with 10% foetal calf serum and 2 mM L-glutamine in a humidified 5% CO₂ gassed incubator at 37°C. U937 cells are a human promonocytic cell line with lymphocyte characteristics previously described as a good model for peripheral blood mononuclear cells (PBMCs) [7].

Drugs

RTV (Abbot laboratories, IL, USA), SQV and [¹⁴C]-SQV (Roche Pharmaceuticals, Welwyn Garden City, Herts., UK [41.3 µCi mg⁻¹; >99% purity]), IDV (Merck, West Point, PA, USA), [³H]-RTV and [³H]-IDV (Moravek Biochemicals, Brea, CA, USA [1.1 Ci mmol⁻¹; 99.9% purity]) were dissolved in 60% methanol. Human AGP was purchased from Sigma.

Experimental protocol

Drug concentrations of HIV protease inhibitors were selected to reflect concentrations of the drugs *in vivo* with standard dosing. U937 cells (5×10^6 cells in 5 ml RPMI growth medium) were incubated at 37°C for 18 h with [¹⁴C]-SQV (0.1 µCi), [³H]-RTV (0.135 µCi) and [³H]-IDV (0.135 µCi) to a final concentration of 1 µM in the presence of 0, 0.5 and 2.0 mg ml⁻¹ AGP. Four experiments were performed on separate days. Following incubation, the cell suspensions were transferred to 30 ml universal tubes, an aliquot removed (8.5 µl) to determine the cell number and then centrifuged (2772 g; 4 min; 4°C). Four aliquots (100 µl) of the supernatant fraction were removed to determine the extracellular concentration. The resulting cell pellet was then resuspended in PBS and washed twice to remove excess radioactivity. Cell

pellets were then extracted for at least 3 h in 60% methanol. Following centrifugation (2772 g; 4 min; 4°C) the methanol fraction was decanted off and evaporated to dryness. The resulting residue was then reconstituted in 150 µl of 60% methanol and an aliquot (100 µl) was taken to determine the intracellular concentration by liquid scintillation counting.

Statistical analysis

Intracellular concentrations were standardized to µM using cell number and the volume of one U937 cell being 1 pl (based on previous studies [7]). The lower limit of detection was 0.5 µM for all protease inhibitors. Coefficient of variation for the analysis of intracellular protease inhibitor ranged from 6 to 11%. Data were analysed using ANOVA followed by a *t*-test (Bonferroni).

Results

The total recovery of drug from the supernatant fraction, cell pellet and washes (in AGP free media) was $80 \pm 10\%$ (mean $\pm$ s.d.; $n=4$). The recovery of drug from the supernatant was $72 \pm 6\%$, with $5 \pm 1\%$ being recovered from the cell pellet.

There was significant accumulation of all three protease inhibitors SQV, RTV and IDV inside U937 cells compared with the extracellular concentration of 1 µM (Table 1). SQV showed the greatest accumulation with a 30-fold difference between intracellular and extracellular concentrations in the absence of AGP. The accumulation of RTV was 3.5-fold less than SQV with IDV showing the least accumulation (10-fold less than SQV).

Addition of AGP (0.5 and 2.0 mg ml⁻¹) resulted in a concentration dependent reduction in the intracellular accumulation of SQV and RTV (Table 1). Although addition of AGP resulted in a significant decrease in the intracellular accumulation of IDV no incremental effect was observed at the higher concentration of AGP (2.0 mg ml⁻¹).

The reduction in intracellular accumulation associated with AGP was most marked with RTV. Addition of 2.0 mg ml⁻¹ AGP resulted in an 82% reduction of intracellular RTV compared with control (Table 1). SQV accumulation was also greatly reduced (76%) with IDV being the least affected (50%).

Discussion

In an individual patient, the prevailing strains of virus will lie somewhere between being completely sensitive and completely resistant to drug. Treatment success is a reflection of the relationship between the IC_{95} (drug concentration required to inhibit 95% of viral replication)

Table 1 Effect of α_1 -acid glycoprotein on the intracellular accumulation of saquinavir, zidovudine and didanosine

Concentration of AGP	Intracellular concentration of protease inhibitor (μM)		
	Saquinavir [95% CI]	Ritonavir [95% CI]	Indinavir [95% CI]
0 mg ml ⁻¹	31.5 $\pm$ 4.5 (100%)	8.8 $\pm$ 1.1 (100%)	3.0 $\pm$ 0.3 (100%)
0.5 mg ml ⁻¹	11.8 $\pm$ 0.9 ^a (37.5%) [15.0–24.4]	3.2 $\pm$ 0.7 ^a (36.4%) [3, 4, 7, 9]	1.3 $\pm$ 0.1 ^a (43.3%) [1, 1–3]
2.0 mg ml ⁻¹	7.4 $\pm$ 1.8 ^{a,b} (23.5%) [19.4–28.8]	1.6 $\pm$ 0.7 ^{a,b} (18.2%) [5.4–9.0]	1.5 $\pm$ 0.1 ^a (50%) [1, 1, 1, 9]

Data are expressed as mean $\pm$ s.d., $n=4$ (four separate experiments on different days). Statistical analysis performed by ANOVA followed by a *t*-test (Bonferroni) accepting $P<0.05$ as being significant. ^a $P<0.0001$ compared with control (0 mg ml⁻¹ AGP), ^b $P<0.05$ compared with 0.5 mg ml⁻¹ AGP. Values in brackets are the percentage of control. AGP— α_1 -acid glycoprotein. CI—95% confidence interval for mean differences compared with control.

for that virus and the concentration of free drug present at the site of action, and neither can be considered in isolation. We have previously reported differences in intracellular accumulation between the protease inhibitors SQV, RTV and IDV [8]. In this study we observed that intracellular accumulation of the drugs was affected by the amount of AGP. IDV was least affected, followed by SQV and then RTV. These differences are probably related to the extent of protein binding of these drugs, which is less for IDV compared with SQV and RTV.

Zhang *et al.* [6] demonstrated that the antiviral activity of protease inhibitors was reduced in wild type and resistant isolates in the presence of AGP. The greatest decrease in antiviral activity was seen with RTV and amprenavir with a comparatively small effect on IDV activity. These results are consistent with our data showing reduced accumulation of the protease inhibitors in the intracellular compartment where HIV replication takes place. The largest percentage decrease upon addition of AGP was seen with RTV. *In vitro* studies have consistently shown that human serum AGP reduces the antiviral activity of protease inhibitors both in primary lymphocytes and immortal cell lines [9–13].

Both HIV infection itself and the resulting susceptibility to other infections may be associated with fluctuating concentrations of AGP. We have shown *in vitro* that this affects the accumulation of protease inhibitors within cells. Increases in AGP leading to a decreased fraction unbound may, depending on the clearance mechanism of the protease inhibitor, have an effect on drug efficacy. However, since we are dealing with high clearance drugs it is most likely that there will be little change in free drug concentration in plasma but an increase in total plasma concentration during chronic oral dosing. Although considerable data have now accrued to suggest that virological responses correlate with protease inhibitor concentrations in plasma [14], our results suggest that the effect of AGP binding may need to be taken into account

when advocating the use of therapeutic drug monitoring, e.g. during an acute intercurrent illness.

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