Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study

Summary

Background HIV-1-infected patients vary considerably by their response to antiretroviral treatment, drug concentrations in plasma, toxic events, and rate of immune recovery. This variability could have a genetic basis. We did a pharmacogenetics study to analyse the association between response to antiretroviral treatment and allelic variants of several genes.

Methods In 123 patients, we did PCR analyses of the gene for the multidrug-resistance transporter (MDR1), which codes for P-glycoprotein, of genes coding for isoenzymes of cytochrome P450, CYP3A4, CYP3A5, CYP2D6, and CYP2C19, and of the gene for the chemokine receptor CCR5. We measured concentrations in plasma of the antiretroviral agents efavirenz and nelfinavir by high-performance liquid-chromatography, and measured levels of P-glycoprotein expression, CD4-cell count, and HIV-1 viraemia.

Findings Median drug concentrations in patients with the MDR1 TT genotype 6 months after starting treatment had a greater rise in CD4-cell count (257 cells/µL) than patients with the CT (165 cells/µL) and CC (121 cells/µL) genotype (p=0.0048), and the best recovery of naive CD4-cells.

Interpretation The polymorphism MDR1 3435 C/T predicts immune recovery after initiation of antiretroviral treatment. This finding suggests that P-glycoprotein has an important role in admittance of antiretroviral drugs to restricted compartments in vivo.

Lancet 2002; 359: 30–36

Introduction

The multidrug-resistance transporter gene MDR1 (HUGO nomenclature: ATP-binding cassette transporter gene, ABCB1) codes for P-glycoprotein. This protein has an important role in transportation of many different substrates—including some antiretroviral drugs—at compartmental and cellular levels. In the intestine, P-glycoprotein restricts drug entry into the body. This protein is also abundant in the apical membrane of many other epithelial barriers, such as the blood-brain, blood-nerve, blood-testis, and maternal-fetal barriers. Furthermore, it is expressed in haemopoietic progenitor cells, lymphocytes, and macrophages in a development-specific and differentiation-specific manner.

We do not know whether variation in expression of MDR1 will lead to in-vivo differences in drug exposure, which could affect virological and immunological response to antiretroviral treatment. In the mdr1-knockout mouse in which expression of P-glycoprotein is absent, concentrations in brain tissue of HIV-1 protease inhibitors are many times higher than in syngeneic animals with wild-type genes. By contrast, overexpression of P-glycoprotein lowers intracellular concentrations of protease inhibitors. Thus, differences in expression of this protein should lead—in various compartments and cells—to modifications in accumulation of different antiretroviral drugs.

We aimed to investigate the role of P-glycoprotein in response to antiretroviral treatment in a well characterised population of HIV-1-infected patients. To investigate possible compensatory mechanisms of transporter function, we looked at the genes coding for CYP3A4, CYP3A5, CYP2D6, and CYP2C19. These isoenzymes of cytochrome P450 have been identified as important factors in the metabolism of HIV-1 protease inhibitors and non-nucleoside reverse-transcriptase inhibitors. Function of CYP2D6, CYP2C19, and CYP3A5 can be predicted from genotype.

Patients and methods

Patients

Three populations of patients were included in the study. The first group included 67 white patients who were selected on the basis of: long-term viral suppression; treatment with nelfinavir (1250 mg twice daily) or efavirenz (600 mg per day); antiretroviral treatment that suppressed viral load to less than 400 RNA copies/mL; stable plasma drug concentrations (two or more measurements with <25% variation obtained at 3-month intervals during follow-up); and no potentially interacting co-medications. These factors indicated that patients were fully adherent to treatment—a prerequisite in the assessment of the contribution of various genes and genetic variants to treatment response.

The second study group included 56 white patients who were prospectively recruited between March, 2000,
values for the sample with the lowest expression (arbitrary
by division of the amplification threshold cycle by the
negative and positive controls, respectively. To control
MDR1 transcripts (one of the two primers was placed at the
avoid amplification of genomic DNA was used to quantify
measurements. Real-time PCR with primers designed to
MiniKit (Qiagen, Hilden, Germany) from viable
transcripts, total RNA was extracted with RNeasy
procedures
We analysed, by PCR amplification and sequencing, MDR1 exon 26 and its 5’ flanking region, the CYP3A4 promoter and exon 7, and CYP2C19 exons 4 and 5. Analysis of CYP2D6 was done by an allele-specific PCR strategy that identifies alleles *3, *4, and *6. These alleles are the three most frequent inactivating mutations in white people, which result in a poor-metaboliser phenotype. Analysis of CYP2D6 gene amplification by a long-range PCR strategy allowed prediction of the ultrarapid-metaboliser phenotype.7 We analysed CYP3A5 intron 3 and heterozygosity for the CCR5 Δ32 mutation by real-time PCR with TaqMan (Applied Biosystems, Foster City, CA, USA). With the exception of CYP2D6 analysis, all genotyping strategies were developed for our study, and we validated the methods by sequence analysis and creation of a panel of reference alleles. Primers and conditions for PCR analysis are available on request from the authors.

Plasma drug concentrations were measured by high-
performance liquid chromatography, which allows simultaneous separation and quantification of nelfinavir and efavirenz. For comparison between drugs, results were expressed as percentile distribution on the basis of our database, which includes measurements of more than 4000 drug concentrations.

For quantification of MDR1, ABCG1, and ABCB2 transcripts, total RNA was extracted with RNeasy MiniKit (Qiagen, Hilden, Germany) from viable peripheral-blood mononuclear cells conserved in liquid nitrogen. We quantified RNA by optical density measurements. Real-time PCR with primers designed to avoid amplification of genomic DNA was used to quantify transcripts (one of the two primers was placed at the junction between two exons). For quantification of MDR1, we used the LLC-PK1 cell line (derived from porcine kidney, very low P-glycoprotein expression) and the L-MDR1 cell line (which was transfected with human MDR1, very high expression of P-glycoprotein) as negative and positive controls, respectively. To control for differences in amount of total RNA added to reactions, quantification results of the target gene were normalised to β-actin. Expression levels were calculated by division of the amplification threshold cycle by the value for the sample with the lowest expression (arbitrary units).

For analysis of expression of P-glycoprotein, we isolated peripheral-blood mononuclear cells and incubated them with either an antibody against human P-glycoprotein (UIC2; Immunotech, Marseille, France) or mouse IgG2a isotype control (Serotec, Oxford, UK) for 30 min on ice; unstained cells were used as a negative control. Cells were washed twice with cold phosphate-buffered saline, centrifuged, and then incubated with R-phycocerythrin-

bind IgG secondary antibody (Sigma Chemicals, Dorset, UK) for 30 min on ice in the dark. We washed the cells twice with cold phosphate-buffered saline and fixed them in paraformaldehyde. All samples were analysed by flow cytometry, on a Coulter Epics XL-MCL cytometer (Beckman-Coulter, Fullerton, CA, USA). P-glycoprotein expression was defined as a one-fold increase in fluorescence, which is calculated by division of the median fluorescence intensity seen with UIC2 antibody by that seen with the IgG2a isotype control (arbitrary units).

Midazolam is a short-acting benzodiazepine that undergoes oxidative metabolism to one major metabolite, 1’-hydroxymidazolam. This metabolic pathway is mediated almost exclusively by CYP3A isoforms. We quantified midazolam and 1’-hydroxymidazolam concentrations after administration of one-hundredth (0·075 mg) of the therapeutic dose of midazolam by gas chromatography, mass spectrometry, and negative-

chemical ionisation. 30 min after oral administration of the drug, we obtained 9-mL blood samples in Monovettes (Sarstedt, Nümbrecht, Germany) containing lithium heparinate. The tubes were centrifuged within 30 min, and the plasma was frozen until analysis. We did analyses at baseline and 1 month after initiation of treatment.

Statistical analysis
Continuous data were analysed by non-parametric (Kruskal-Wallis) or parametric (ANOVA) tests. We used logistic regression to analyse variables associated with drug concentrations in plasma or with increases in CD4-cell count. Stata software version 7.0 (College Station, TX, USA) was used for statistical analyses.

Role of the funding source
The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
On the basis of previous analyses of functional polymorphisms in MDR1,13 we ascertained the genotype of the 5’ flanking region and exon 26 of this gene for 123 patients receiving antiretroviral treatment. Although we did not identify any mutation in the 5’ flanking region, a particular polymorphism at exon 26 (3435C→T) was associated with differences in plasma drug concentrations. Patients with the TT genotype had median drug concentrations at the 50th percentile (IQR 15–50). By contrast, patients with the CT genotype had median drug concentrations at the 50th percentile (25–75) and those with the CC genotype had concentrations at the 75th percentile (60–85; p=0·0001, figure 1). For the different MDR1 genotypes, the 69 patients receiving efavirenz (p=0·02), which is not a substrate of P-glycoprotein, had an in-vivo distribution pattern similar to that of the 54 patients receiving nelfinavir (p=0·03), which is a well defined substrate of P-glycoprotein.14 The association between MDR1 3435 TT genotype and lower drug concentrations in plasma was also confirmed in 107 patients who did not receive additional drugs (other than nelfinavir or efavirenz) that could interact with
CYP450 (p=0.01), in 56 patients starting antiretroviral treatment, and in 67 patients on stable chronic treatment. Quantification of MDR1 transcripts in peripheral-blood mononuclear cells from 59 patients showed an association between MDR1 3435 TT genotype and a lower level of MDR1 expression (median 1.87, IQR 1.74–2.11) compared with CT genotype (2.36, 1.89–2.44) and CC genotype (2.79, 2.07–3.15; p=0.02). This association was confirmed by fluorescence-activated cell-sorter analysis of P-glycoprotein expression in peripheral-blood mononuclear cells. The correlation coefficient between transcript and protein expression for the same set of samples was r=0.58 (p=0.0006). There was a weak correlation between MDR1 expression and drug concentrations in plasma (r=0.22, p=0.10) and between P-glycoprotein expression and drug concentrations in plasma (r=0.25, p=0.06).

Expression levels of two other transporters, ABCG1 and ABCG2, were measured in peripheral-blood mononuclear cells from the same subset of patients in whom MDR1 expression was analysed. There was no relation between MDR1 and ABCG1 expression (r=0.03, p=0.2), MDR1 and ABCG2 expression (r=–0.05, p=0.2), or ABCG1 or ABCG2 expression and drug concentrations in plasma. Patients who were homozygous or heterozygous for a CYP2D6 allele associated with a poor-metaboliser phenotype had higher median concentrations in plasma of both nelfinavir and efavirenz (percentile 62.5, IQR 47.5–77.5) than patients with a CYP2D6 extensive-metaboliser genotype (45, 25–75; p=0.04; figure 1). By contrast, we did not note a contribution of CYP2C19 genotype to nelfinavir or efavirenz concentrations in plasma, despite the fact that in-vitro data identified CYP2C19 as the main cytochrome in the metabolism of nelfinavir.14 Patients who were homozygous or heterozygous for a CYP2C19 allele associated with a poor-metaboliser phenotype had median concentrations of both nelfinavir and efavirenz in plasma at the 60th percentile (IQR 30–75) compared with those with a CYP2C19 extensive-metaboliser genotype (50th percentile, 25–75; p=0.007; figure 1).

CYP3A4 function cannot be predicted from genotype. We did however assess two recognised polymorphic regions: promoter CYP3A4*1B and exon 7 CYP3A4*2. Exon 7 is thought to be functionally relevant because it is associated with reduced CYP3A4 function.15 Analysis of CYP3A was completed by assessment of the CYP3A5 region in which mutation leads to aberrant splicing and changes in CYP3A5 expression.16 We did not identify any association between allele CYP3A4*1B (present at a frequency of 4.0%), allele CYP3A4*2 (0.6%), or allele CYP3A5*1 (11.3%) and drug concentrations in plasma (figure 1).
To investigate whether 

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*Complete genotype analysis was unsuccessful in ten patients. †wt= wild type, pm= poor metaboliser (alleles 2D6*4, *3, *6), um= ultra-rapid metaboliser (gene duplication). ‡wt= wild type, pm= poor metaboliser (allele 2C19*2). §When more than three patients per genotype, data are median value (IQR). When three patients or less, every percentile is shown.

**Distribution of MDR1, CYP2D6, CYP2C19, CYP3A4, and CYP3A5 genotypes in 113 study participants**

However, we recorded linkage disequilibrium between CYP3A4*1B and CYP3A5*1 (correlation coefficient ρ=0.37, p=0.0002). To further assess CYP3A function, we analysed the ratio of 1'-hydroxymidazolam to midazolam in blood 30 min after oral administration of midazolam, which is a probe of CYP3A activity.11 We did this test in 50 patients at baseline and at steady-state, after at least 1 month of antiretroviral treatment. We did not identify any relation between baseline CYP3A activity and MDR1 3435 genotype (p=0.9) or drug concentrations in plasma during subsequent treatment (r=–0.05, p=0.9). Efavirenz induced CYP3A function, whereas nelfinavir limited conversion of midazolam to its metabolite. There was no correlation between efavirenz or nefarnivir concentrations in plasma and rate of midazolam metabolism (r=0.12, p=0.37; and r=–0.17, p=0.8, respectively). However, the fact that midazolam hydroxylation was almost completely inhibited by nefarnivir does not necessarily prove that metabolism of nefarnivir by CYP3A would also be completely inhibited.

Drug concentrations in plasma could be predicted to a certain extent by the noted genetic variation between MDR1 and CYP2D6 (table). This possibility was further explored by logistic-regression analysis including age, sex, weight, MDR1 3435, CYP2D6, CYP2C19, and CYP3A4*1B. Drug concentrations in plasma above the 50th percentile were associated with both MDR1 3435 CC genotype (odds ratio 7·9, 95% CI 2.1–31.3–20) and CYP2D6 poor-metaboliser genotype (4·5, 1.5–13.8).

To investigate whether MDR1 3435 allelic variants predicted not only drug concentration in plasma but also response to treatment, we assessed viraemia and rise in CD4-cell count after initiation of antiretroviral treatment. Patients with the various MDR1 3435 genotypes had similar CD4-cell counts before initiation of treatment. Mean (SD) CD4 count at baseline was 163 cells/µL (139) in patients with the MDR1 3435 TT genotype, 194 cells/µL (156) in those with the CT genotype, and 175 cells/µL (162) in those with the CC genotype (p=0.6). Mean (SD) viral load at baseline in patients with the MDR1 3435 TT genotype was 5·37 log₁₀ copies/mL (3·54), 5·01 log₁₀ copies/mL (0·60) in patients with the CT genotype, and 5·04 log₁₀ copies/mL (0·72) in those with the CC genotype (p=0·07). On initiation of antiretroviral treatment, all groups of patients had a similar rate of viraemia decay (figure 2). However, patients with the MDR1 3435 TT genotype had a higher mean (SE) CD4-cell count at 6 months (257 cells/µL [20]) than patients with the CT and CC genotypes (192 cells/µL [15] in patients with the CYP2D6 extensive-metaboliser genotype (p=0.25). None of the other genes analysed were associated with treatment response. Furthermore, no correlation was noted between drug concentrations in plasma and CD4-cell recovery (r=0.01, p=0.89), which underscores the specific relevance of the MDR1 genotype.
Male sex
Hepatitis C
(100 cells/CD4 at baseline (per log copies/mL)
HIV-1 RNA at baseline
CCR5
MDR1
panel) after initiation of treatment. Data are mean (SE).

Figure 2: Response to antiretroviral treatment according to MDR1 3435 genotype
CD4-cell count increase (upper panel) and suppression of viraemia (lower panel) after initiation of treatment. Data are mean (SE).

Because no data on CD4-cell subpopulations were available from the study participants, we analysed MDR1 genotype for 80 patients in two clinical studies—one in which 47 patients were given abacavir and amprenavir and the other in which 33 received abacavir, nelfinavir, and saquinavir or amprenavir. The pattern of CD4-cell recovery was available for all patients. Despite the negligible degree of immunosuppression that characterised participants in these trials (mean 710 cells/µL [SE 32]), patients with the MDR1 3435 TT genotype 6 months after initiation of antiretroviral therapy had a greater rise in CD4-cell count (196 cells/µL [50]) and more effective recovery of naïve T cells (89 cells/µL [53]) than patients with the CT genotype (161 cells/µL [63] and 46 cells/µL [55], respectively) or the CC genotype (142 cells/µL [53] and 50 cells/µL [50], respectively). Analysis of trend over 24 weeks after initiation of antiretroviral treatment showed that naïve CD4-cell recovery was greater in patients with the TT genotype than in those with CT and CC genotypes (p=0·05).

We assessed all recognised factors associated with differences in rate of recovery of CD4-cell count: age, sex, baseline CD4-cell count and viral load, and hepatitis C serostatus.17 The only genetic marker that has been reported to be associated with differences in treatment response (the chemokine receptor CCR5 Δ32/Δ32) affects viral decay after initiation of treatment.16 When these variables and the MDR1 3435 genotype were included in a logistic-regression model, TT genotype was a strong independent predictor of rise in CD4-cell count by 200 CD4 cells/µL at 1 month (odds ratio 5·4, 95% CI 1·6–18·8), 3 months (2·3, 1·0–5·5), and 6 months (3·0, 1·3–7·1; figure 3).

Discussion
This study addresses the contribution of seven genes—which encode isoenzymes of cytochrome P450 and multidrug transporters—to drug concentrations and treatment effectiveness of two antiretroviral drugs (nelfinavir and efavirenz) in vivo. The most relevant findings of our study relate to the multidrug transporter P-glycoprotein. MDR1 allelic variants are a potential molecular basis for interindividual differences in pace of CD4-cell immune recovery that is seen after initiation of treatment. Furthermore, genetic variation in MDR1—and to a lesser degree in CYP2D6—predicts the noted interindividual variation in plasma concentrations of the two study drugs. However, this work should also emphasise the complexity of pharmacological monitoring of antiretroviral treatment, at the same time underscoring the interest to explore P-glycoprotein inhibition as a strategy in management of HIV-1-infected individuals.19

How could levels of P-glycoprotein expression affect recovery of CD4 cells and the naïve cell subset? This protein is expressed in haemopoietic progenitor cells, with the highest levels of expression being noted in cells with characteristics of pluripotent stem cells.1 P-glycoprotein could have a role in protection of these cells from toxic substances, and potentially, in reduction of accumulation of regulatory substances that could affect differentiation or proliferation.1 Furthermore, lymphocytes and macrophages express MDR1 in a development-specific and differentiation-specific manner.1 In thymus tissue, 30% of cells are reported to be P-glycoprotein-positive, including CD3, CD4, and CD8 progenitor cells and mature CD4 and CD8 thymocytes.26 Mature T cells in blood include a large proportion (50–60%) of cells with P-glycoprotein activity, particularly in the subset of naïve cells.30 Thus, the immunological benefit noted in...
individuals with MDRI 3435 TT genotype and low expression of P-glycoprotein could suggest enhanced penetration of antiretroviral drugs in cell populations susceptible to HIV-1 infection, in infected lymphocytes, and in pharmacological sanctuaries.21–23 Although we did not note differences in rate of viral suppression between the different MDRI 3435 genotypes, the design of our study did not allow precise analysis of decay rates and, in particular, effect of P-glycoprotein expression in a multiple-compartment model of viral decay kinetics.23

Controversy remains about the association between MDRI genetic variation, P-glycoprotein expression and function, and concentrations in plasma of various substrates of P-glycoprotein. The MDRI 3435 C/T polymorphism is non-coding, and it could be in linkage disequilibrium with a polymorphism elsewhere in the genome that modifies MDRI expression or function.13,24,25

In the study by Hoffmeyer and colleagues,22 the MDRI 3435 TT genotype was associated with low P-glycoprotein expression in enterocytes and high concentrations in plasma of digoxin. However, in a study by Kim and colleagues,27 the MDRI 3435 TT genotype (in the context of a C1236T, G2677T haplotype) was associated with high P-glycoprotein expression in vitro and low concentrations in plasma of the drug fexofenadine. In our study, the MDRI 3435 TT genotype was associated with low expression of the MDRI transcript and P-glycoprotein in peripheral blood mononuclear cells and with low concentrations in plasma of nelfinavir and efavirenz.

The MDRI 3435 TT genotype has also been described in mdr1−/− knockout mice,28 low P-glycoprotein expression in CD56 natural-killer cells28 and in human placental tissue.24 Blockage of transport by the P-glycoprotein inhibitor LY-335979, leading to raised concentrations of nelfinavir in brain and testicular tissue, resulted in small changes in plasma nelfinavir concentrations in mice.27 Differences in study strategy and substrates could explain the discrepancies; however, there is urgent need for additional work to reconcile these opposing results.

The fact that concentrations of nelfinavir and efavirenz in plasma are low when P-glycoprotein expression is also low could suggest an indirect effect of the MDRI 3435 TT genotype. We explored two hypotheses that address this paradox. First, low concentrations of P-glycoprotein might be compensated for by overexpression of other transporters with affinity for antiretroviral drugs,1,2,8 leading to a reduction of plasma drug concentrations. For example, in mdr1−/− knockout mice, the cumulative renal output of voriconazole, a substrate of this protein, was amplified several-fold compared with that in wild-type mice, which was attributed to upregulation of other renal transporters.20

Second, intestinal CYP3A and P-glycoprotein act together to limit drug absorption.26 Schuetz and colleagues10 reported that mdr1-knockout mice could, by induction of CYP3A, compensate for loss of P-glycoprotein function, thus leading to diminished concentrations of common substrates in plasma. There is additional complexity, since some antiretroviral agents are both inhibitors and inducers of cytochrome P450 and P-glycoprotein.21 However, we could not identify such compensatory mechanisms, as suggested by the results of analysis of transcription levels of ABCG1 and ABCG2, which encode for multidrug-resistant proteins with affinity for antiretroviral agents,13,15 or by assessment of CYP3A activity.

Further to the role of P-glycoprotein in response to treatment through pharmacokinetic modulation, this protein could affect the natural history of HIV-1 disease. Reports suggest that overexpression of P-glycoprotein leads to a reduction in infectiveness and cellular permissiveness in vitro by interference with viral fusion and, possibly, viral release.28 These effects are partly reversed by selective inhibition of the transporter. Because this protein has been proposed to localise in lipid rafts,26 which are important sites for viral fusion and assembly, these findings are useful for the understanding of these stages in the viral lifecycle.

The potential effect of P-glycoprotein expression on progression of HIV-1 disease before initiation of antiretroviral treatment should be investigated by longitudinal analysis of large cohorts of patients. Analyses should pay particular attention to ethnic origin, in view of the differences noted in MDRI 3435 genotype distribution in different human populations. In white people, and also in participants of our study, MDRI 3435 polymorphism shows a 25% TT, 50% CT, and 25% CC distribution. By contrast, frequency of CC genotype in African black people is 67–83%, and frequency of TT genotype is very low.21,23 This variation could lead to different patterns of HIV-1 disease evolution and responses to antiretroviral treatment in human populations.

Contributors
J Fellay, C B Eap, and A Talenti designed the study. C Marzolini and L A Decosterd measured drug concentrations in plasma. J Fellay, E R Meaden, D J Back, D Retelska, and C B Eap did the genetic and expression analyses. G Pantaleo did the immunological analyses. T Buchin and A H Schinkel had a major intellectual input. J-P Chave, H Furrer, M Opravil, L Ruizi, and P Vernazza were responsible for recruitment of patients and clinical assessment. The report was written by J Fellay and A Talenti, with contributions from all co-authors.

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Conflict of interest statement
None declared.

Acknowledgments
Support for this work was provided by the Swiss National Science Foundation (Grant 3344E-62092.99) and by the Swiss HIV Cohort Study (Swiss National Science Foundation, Grant 3345-06204). We thank P Taffe for statistical input.

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