Influence of *CYP2B6* and *ABCB1* SNPs on nevirapine plasma concentrations in Burundese HIV-positive patients using dried sample spot devices

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Nevirapine pharmacokinetics are affected by several factors including *CYP2B6* single nucleotide polymorphisms (SNPs). These genetic profiles are more common in African patients and they affect the drug clearance being associated with higher trough concentrations. Pharmacokinetic/pharmacogenetic (PK/PG) studies are difficult to perform in remote areas where refrigeration is not available, although dried plasma and dried blood methods have been validated.

WHAT THIS STUDY ADDS

- Dried plasma spots are useful tools for studying nevirapine PK with a good association to plasma concentrations and they can be used in rural areas since a cold chain is not necessary. Dried blood spots can be used to store and analyze patients' DNA for PG polymorphisms. Nevirapine trough concentrations in Burundese patients, not studied so far, are above the target concentration (3000 ng ml⁻¹) in 84% of patients. *CYP2B6* (both at position 516 and 983) but not *ABCB1* (3435 and 1236) SNPs as well as age correlate with higher nevirapine exposure.

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AIMS

The pharmacokinetics (PK) and pharmacogenetics (PG) of nevirapine have been studied in rich and limited-resource countries. *CYP2B6* single nucleotide polymorphisms (SNPs) have been associated with decreased drug clearance. We evaluated the PG determinants of nevirapine trough concentrations in a rural cohort in Burundi using easy to store and transport dried sample spot devices.

METHODS

A cross-sectional analysis in HIV-positive nevirapine-treated patients in Kiremba, north of Burundi, was performed in 2009. After blood withdrawal whole blood was stored on dried blood spots and plasma (after centrifugation) was placed on dried plasma spot devices and stored at room temperature. Nevirapine plasma and dried sample spot concentrations were compared to test the clinical usefulness of this method. SNPs in *CYP2B6* and *ABCB1* (using a real time PCR technique) were analyzed and associated with nevirapine plasma trough concentrations.

RESULTS

Nevirapine concentrations measured on dried plasma spot devices were highly related to plasma concentrations in 60 patients, although a negative bias was observed (−18%). Nevirapine trough concentrations were above the target concentration (3000 ng ml⁻¹) in 84% of patients and they were associated with *CYP2B6* SNPs (both at position 516 and 983). No effect of *ABCB1* SNPs was noted.

CONCLUSIONS

Dried plasma spot devices are accurate tools for measuring nevirapine concentrations in rural settings where refrigeration is not available, despite a moderate underestimation bias. They allowed the evaluation of nevirapine concentrations in a cohort of HIV-infected people in rural Burundi, confirming very good exposure and correlation with PG polymorphisms in the *CYP2B6* encoding gene.

Introduction

Highly active antiretroviral treatment (HAART) has changed the natural history of HIV infection both in rich and limited-resource countries (LRCs). While in the former resources allow multiple therapeutic options and diagnostic procedures, in the latter two lines of treatment are widely available and almost no test, apart from the CD4 cell count, is performed for monitoring treatment outcome. Therapy is therefore guided by a public health approach with the aim of reducing HIV-related deaths and new infections. In 2009 almost 5.2 million people were on HAART, representing approximately 32% of those in need [1]. While several studies highlighted the efficacy of generic fixed-dose nevirapine-based regimens in LRCs [2], some pharmacological data showed that in the vast majority of patients the exposure obtained with generic fixed-dose drugs is comparable with that obtained with trade compounds [3, 4].

Burundi is a east-African country in which HIV prevalence in adults is estimated to be 4.1–8.8%, with great differences among rural, semi urban and urban settings (respectively 2–4%, 8–9% and 6–7%). First-line treatment is weight-based Triomune® (stavudine, lamivudine, nevirapine), but no evaluation has yet been published on the treatment outcome, viral genotypes or pharmacological and pharmacogenetic analysis in the Burundese population.

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that is widely used in combination with nucleoside reverse transcriptase inhibitors (NRTIs) for the treatment of HIV infection and the prevention of mother-to-child transmission (MTCT). The relationship between NVP plasma concentrations and virological response has been evaluated in several studies, and a minimum trough concentration (C_{trough}) of 3000 ng ml⁻¹ has been proposed as a target to guarantee a considerable prevalence of virological success [5]. Several factors can influence NVP exposure, including gender, concomitant drugs and genetic polymorphisms in the gene encoding for the cytochrome P450 2B6 enzyme (CYP2B6), which is involved in the drug's main metabolic pathway [6, 7]. Single nucleotide polymorphisms (SNPs) in exon 4 (516 G > T) and in exon 7 (983 T > C) are associated with a significant reduction in CYP2B6 catalytic activity [8, 9]. Both SNPs have been shown to be more frequent in patients of African origin and to be associated with decreased NVP clearance and higher plasma C_{trough} . This association between CYP2B6 SNPs and NVP plasma concentrations has been confirmed in several middle and low-income countries; these data suggest a possible reason for the observed higher exposure obtained in non-Caucasian subjects. Data on the effect of genetic variants in genes encoding for transporters (such as ABCB1) on NVP exposure are conflicting with a suggested minor effect [10].

Several logistic and economic barriers are challenging the opportunity to collect and analyze samples both for virological and pharmacological tests. New tools have been developed to increase the feasibility and decrease the cost of such analysis. A thin layer chromatography for semi-quantitative detection of saliva NVP concentrations was developed and validated in Tanzania, showing a high sensitivity (100%) and sensibility (99%) when compared with plasma concentrations measured by standard high performance liquid chromatography (HPLC) methods [11]. Dried blood spots have been evaluated for collection and transport, showing a strong correlation with plasma concentrations for several drugs and an increased feasibility due to the avoidance of venipuncture and cold chain conservation [12, 13]. We previously validated the use of dried sample spot devices (DSSDs) for the determination of plasma concentrations of different antiretrovirals, showing stability in different temperature conditions: NVP samples showed excellent stability (>90 days) with a very good recovery (>90days to lose around 5% of the drug concentration) after 3 months at room temperature [14].

The objective of this study was to analyze the influence of CYP2B6 (516 G > T and 983T>C) and ABCB1 (3435 C > T and 1236 C > T) SNPs on NVP plasma concentrations in a cohort of Burundese HIV-positive patients using DSSD. The influence of ABCB1 SNPs was tested given the controversial results, the previous limited analyzed sample sizes and the high expected frequency of less-functioning CYP2B6 in the African population. The association between DSSD and plasma concentrations was tested in the first 60 patients.

Methods

Study participants

The study was conducted as a cross-sectional analysis on HIV-positive patients attending the outpatient clinic at the Kiremba Hospital, Kiremba, in the north of Burundi during 2009. To be considered for this study, candidates had to be HIV positive, above 18 years of age, in good general health, on NVP-containing HAART for at least 6 months and currently not on any other treatment containing interacting drugs (such as rifampicin or fluconazole).

The first 60 enrolled patients participated in the DSSD and plasma substudy. This sample size was chosen given the previous published results, the time needed to enrol patients (and therefore the days frozen samples had to remain *in situ* with the risk of power shortage) and transportation duration and cost. The study was approved by the Local Review Board and written informed consent was obtained from all participants.

Pharmacokinetics and pharmacogenetic analysis

Venous blood samples were obtained from all subjects in 7 ml lithium heparin tubes and whole blood (50 µl per

spot) was spotted on Whatman 903 protein saver cards (VWR International, Milan Italy) for PG analysis. After centrifugation (3000 rev min⁻¹ at 4°C for 10 min) 100 µl plasma was spotted onto each DSSD for therapeutic drug monitoring (TDM) (Dried Sample Spots Device, purchased from Laboratori Biomicron srl, Italy). Samples of dry plasma spots (DPS) were allowed to dry at room temperature for 10 min and then heated at 58°C for 30 min, to inactivate fully HIV and to aid drying of the DSSD. DBS and DPS on DSSD were separately inserted in a foil bag with desiccant (Foil Bag and Dessiccant Packs, purchased from Laboratori Biomicron srl, Italy) and then stored at room temperature. The remaining plasma was frozen at -20°C in a subset of 60 patients.

Dried samples were transported at room temperature while frozen plasma samples were transported at -4°C and then stored at -20°C at the Pharmacokinetics and Pharmacogenetics Laboratory, University of Torino, Torino, Italy and analyzed within 3 months. NVP concentrations were determined using a HPLC with a SPE extraction method for frozen plasma samples, and using a mass spectrometry coupled HPLC (HPLC-MS) for DPS on DSSD according to methods described and fully validated in previous studies [14, 15]. Comparing DPS (HPLC-MS) and plasma (HPLC-PDA) assays the former showed comparable intra and inter-day precision, a higher accuracy (-2.5% vs. 6.3%) and a lower LLOQ (31.2 ng ml⁻¹ vs. 62.5 ng ml⁻¹). The limit of detection was 15.6 ng ml⁻¹ for both assays.

DNA was isolated from each DBS following the protocol of FTA Kit Whatman (VWR International, Milan Italy). *CYP2B6* and *ABCB1* genotypes were determined using the ABI TaqMan allelic discrimination kits by real time PCR using standard methodology (Applied Biosystem, UK).

Adherence

Adherence in the last month was measured with a visual scale (from 0 to 100%). The last 12 months of visits were recorded and they were considered to be not on time if later than 2 days from the planned appointments (every 30 days).

Statistical analysis

Normally distributed variables were described with means ($\pm$ SD) and analyzed with parametric testing while not normally distributed ones were described with medians (inter-quartile ranges) and analyzed with non-parametric tests. Pearson's correlation was used to test the association between DSSD and plasma concentrations. Analysis of variance was used to test for differences in NVP C_{trough} among the genotypes. A logistic regression analysis was performed to identify factors associated to C_{trough}. A *P* value of <0.05 was accepted as significant. SPSS version 18.0 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

Results

Study population

Two hundred and four patients were enrolled. Patients' characteristics are described in Table 1. No significant difference emerged between the substudy group and the total study population.

Concomitant antiretroviral drugs were mainly stavudine and lamivudine (co formulated with NVP in Triomune®) in 194 patients (95.6%). Ten patients (4.9%) were receiving zidovudine and lamivudine. Concomitant drugs were recorded: 27.5% of patients were taking prophylaxis for *Pneumocystis carini* pneumonia with cotrimoxazole, one was on theophylline and one received carbamazepine.

Mean adherence to medications during the previous 30 days was 97% ($\pm$ 3) and most of the patients (86.3%) reported adherence to above 95% of the doses. Seventy-four percent of the visits during the previous year were considered on time.

DSSD and plasma concentrations

Sixty patients were included in the DSSD/plasma analysis. Samples were withdrawn 2 to 16 h after drug intake. NVP mean C_{trough} was 4525 ng ml⁻¹ ($\pm$ 2213) on DSSD and 5552 ng ml⁻¹ ($\pm$ 2511) on plasma samples. The mean DSSD: plasma ratio was 0.82 ($\pm$ 0.1, 95% CI 0.55, 1.17)

Table 1

Demographic and immunovirologic characteristics of study population; data are expressed as mean ($\pm$ SD) or number (percentage)

		Baseline characteristics Study population <i>n</i> = 204		Substudy group <i>n</i> = 60	
Gender: female	<i>n</i> (%)	143	70.1%	48	80%
Age (years)	Median (IQR)	39	(32–47)	38.5	(34–47)
BMI (kg m ⁻²)	Median (IQR)	20.7	(19.2–22.4)	20.7	(18.8–22.3)
WHO stage 3	<i>n</i> (%)	157	77%	26	43.3%
CD4 cell count	Median (IQR)	429	(239–692)	291	(189–530)
Months of treatment with antiretrovirals	Median (IQR)	25	(16.8–38)	32.2	(13.9–42.9)

Figure 1

Scatter-dot plot of nevirapine DSSD and plasma concentrations (ng ml^{-1}). Trend line is represented as a dotted line with 95% confidence intervals (continuous lines)

indicating that the DSSD method had a moderate negative bias. Using the target value of 3000 ng ml^{-1} , only five patients were judged to have suboptimal concentrations by DSSD but normal ones by plasma evaluation: in these subjects DSSD concentrations ranged from 2447 to 2880 ng ml^{-1} and plasma ones ranged from 3479 to 4816 ng ml^{-1} . A significant correlation was noted between DSSD concentrations and plasma concentrations (Pearson's $r^2 = 0.79$, $P \leq 0.001$) as shown in Figure 1.

DSSD nevirapine concentrations

DSSD concentrations over time are shown in Figure 2. Only seven samples (3.8%) were below the limit of detection.

One hundred and eighty-two samples obtained 10 to 18 h after drug intake were considered C_{trough} . Mean NVP C_{trough} was 6590 ng ml^{-1} (± 3851). NVP C_{trough} concentrations were below the limit of detection, below 3000 ng ml^{-1} , between 3000 and 4300 ng ml^{-1} and above 4300 ng ml^{-1} respectively in 3.8%, 12.1%, 11.5% and 72.5% of patients.

Effect of CYP2B6 and ABCB1 SNPs on trough concentrations

The overall frequencies of CYP2B6 genotypes were as follows: 50% GG (102 patients), 36.8% GT (75 patients) and 13.2% TT (27 patients) at position 516 and 87.6% TT (177 patients), 10.9% TC (22 patients) and 1.5% CC (three patients) at position 983. ABCB1 genotype prevalence was as follows: 78.4% CC (160 patients), 20.1% CT (41 patients) and 1.5% TT (three patients) at position 3435 and 80% CC (160 patients), 16% CT (32 patients) and 4% TT (eight patients) at position 1236. In six different subjects amplification was not successful (two at position 983 in the CYP2B6 gene and four at position 1236 in the ABCB1 gene).

Figure 2

Scatter-dot plot of nevirapine DSSD concentrations (ng ml^{-1}) and time after drug intake (h)

All the reported polymorphisms but ABCB1 1236CT were in Hardy–Weinburg equilibrium.

CYP2B6 genotypes were associated with NVP DSSD C_{trough} concentrations. Patients with GG, GT and TT at position 516 showed respectively NVP C_{trough} of 6624 ng ml^{-1} (± 3921), 6485 ng ml^{-1} (± 3022) and 8769 ng ml^{-1} (± 4098). A significant association emerged at the ANOVA test ($P = 0.014$) (Figure 3A). Patients with TT, TC and CC 983 phenotypes showed respectively mean NVP C_{trough} concentrations of 6544 ng ml^{-1} (± 3485), 8743 ng ml^{-1} (± 4243) and a single sample showing 7354 ng ml^{-1} (Figure 3B). A significant difference emerged in NVP C_{trough} concentrations between TT and TC genotypes at position 983 (t -test, $t = -2.53$, $P = 0.012$).

ABCB1 3435C>T and 1236C>T genotypes were not associated with NVP C_{trough} concentrations (respectively $P = 0.73$ and $P = 0.95$).

Determinants of nevirapine trough concentrations

After univariate analysis the only factors showing a significant correlation to NVP C_{trough} concentrations were age and CYP2B6 SNPs. Age was very weakly but significantly correlated with NVP C_{trough} concentrations (Pearson's $r = 0.03$, $P = 0.019$). After linear regression analysis NVP C_{trough} concentrations were significantly and independently associated with age ($P = 0.04$, 95% CI 2.82, 122.5), CYP2B6 516G>T SNPs ($P = 0.007$, 95% CI 292, 1807) and CYP2B6 983T>C SNPs ($P = 0.005$, 95% CI 671, 3774).

Discussion

In rich-resource countries guidelines suggest the use of TDM in selected clinical scenarios [16]. In restrained-

Figure 3

Box plot of the influence of *CYP2B6* SNPs on nevirapine DSSD trough concentrations; *CYP2B6* 516 G > T (A) and *CYP2B6* 983 T > C (B). Black lines and grey boxes represent respectively median values and interquartile ranges; open dots represent outlier values

resource countries economical and technical limitations make such a tool almost unavailable. Therefore antiretroviral plasma exposure is seldom checked in such settings. Alternative means of collecting, storing and analyzing samples have been developed and TDM has been performed on saliva or hair (easily obtainable and transportable) or through simplified methods (such as single layer chromatography) in small studies [11–13].

Our group has tested the use of glass filter papers to collect and store plasma for PK analysis. For most of the drugs the DSSD method was accurate and stable even at room temperature [14]. Therefore we performed a preliminary analysis through which we compared DSSDNVP

C_{trough} concentrations with the plasma ones collected in 60 patients from our study population. DPS performed quite well in this clinical scenario showing a very good correlation with plasma samples revealing approximately 18% lower concentrations. This moderate underestimation would probably have minor impact on clinical decisions since the misjudged samples (5/60) were near the clinical threshold (2447 to 2880 ng ml⁻¹). Since NVP stability has been demonstrated, factors accounting for the discrepancy with tests performed in the laboratory) could be technical (un-calibrated or defective pipettes) or human (technicians' skills) sampling errors and/or instrumental analysis variability (HPLC-MS has a higher standard error as compared with HPLC-UV/PDA). Furthermore, since the use of HPLC-MS is widely unaffordable due to high costs and technical issues, a HPLC-UV method is currently under development, envisaging the potential implementation of cheaper and simpler methods at a national level. In this cohort of adult HIV-positive Burundese patients the mean NVP C_{trough} concentration was 6590 ng ml⁻¹, which were comparable with the ones from other studies from sub-Saharan Africa [17, 18] and slightly higher than the ones reported from rich-resource countries [7]. Undetectable and low concentrations (below 3000 ng ml⁻¹) were uncommon, being found respectively in 3.8% and 12.1% of patients. Nevertheless 50 patients (27.5%) were at risk of selecting resistance associated mutations showing NVP C_{trough} concentrations below 4300 ng ml⁻¹ [19]. In limited-resource countries, in which antiretroviral regimens are limited and the price of failure is almost unbearable the optimization of drug exposure should be pursued. Concomitant interacting drugs were excluded in this cohort, although unreported traditional and herbal compounds are rarely declared. The influence of unknown interacting drugs cannot be therefore excluded.

Adherence was measured both by a visual scale and by counting the number of visits in the last year performed on time. Both evaluations suggested a fairly high level of adherence. None of the factors was associated with either DSSD concentrations or with the infrequent discovery of undetectable NVP concentrations (7/204). A possible discrepancy between the reported and the real compliance should be taken into account.

CYP2B6 genetic variants were not uncommon with 50% and 12.4% of patients carrying the 516 GT or TT genotype and the 983 TC or CC genotype respectively. Both SNPs were significantly associated with higher NVP C_{trough} concentrations as previously reported [6, 8, 9]. 983CC variants were confirmed to be quite rare with a single patient showing this genetic profile. More patients carrying uncommon variants in *CYP2B6* showed NVP C_{trough} concentrations above 4300 ng ml⁻¹, both for 516GT and TT as compared with GG (91.3%, 74.2% and 72.1% respectively) and 983 TC vs. TT (84.2% vs. 73.9). SNPs in *ABCB1* (both 3435 C > T and 1234 C > T) were not associated with NVP C_{trough}

concentrations. These data suggest that no more than a minor influence could be attributed to ABCB1 in affecting NVP PK profile.

CYP2B6 SNPs seem to explain some of the variability in NVP PK. Nevertheless some outliers can still be identified as shown in Figure 2. This apparently separated small group of patients shows very high NVP $C_{\max}$ ($n = 2$) or C_{trough} concentrations ($n = 8$) with values above 15000 ng ml⁻¹. Though sampling errors cannot be excluded we think the influence of uncommon CYP2B6 SNPs (such as 82 T > C) [20] should be studied.

NVP C_{trough} concentrations, apart from the impact of genetic variants, were associated with age (increasing concentrations with older age). This association, although very weak, seems to be significant on multivariate analysis. This effect has not been so far reported even in population pharmacokinetic approaches and it could be peculiar to our population [18, 21]. A decrement of metabolism activity of CYPs in the elderly could be the mechanism explaining higher NVP C_{trough} concentrations in older patients. The higher prevalence of (unreported) co-medications in our elderly patients has to be taken into account.

A major limitation of our study was the cross-sectional design, where a possible selection bias was the enrolment of stable patients receiving a NVP-based HAART for a median of 2 years; patients showing intolerance, toxicity and early virological failure were therefore not included. Minor limitations were the small sample size of trough concentrations and the lack of laboratory data (for instance liver enzyme concentrations).

In conclusion our study shows nevirapine C_{trough} concentrations similar to those so far published, but with a noticeable percentage of patients with suboptimal exposure, in a previously unstudied population in East Africa. Confirmation of the influence of CYP2B6 SNPs on NVP C_{trough} concentrations has been provided as well as an unexpected age effect. Finally, this current study provides clinical validation for the use of dried sample spot devices for measuring NVP concentrations, and suggests a possible easy to store and transport tool for performing TDM in limited-resource countries, in which the cost of failures is extremely high.

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Competing Interests

A.C., S.B and G.D.P. received speaker honoraria and travel grants from Boeringher-Ingelheim, the manufacturer of Viramune (nevirapine). Other authors have no potential conflict of interest to declare.

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