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Exploring CYP2B6 activity by measuring the presence of nevirapine hydroxy metabolites in plasma

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Background/aim: Nevirapine is a reverse-transcriptase inhibitor widely used in combination therapy to treat HIV infection. Nevirapine is extensively metabolized in the liver and CYP2B6 is mainly responsible for oxidation of 3-hydroxynevirapine (3-OH NVP). This study aims to explore CYP2B6 activity by measuring 2-hydroxynevirapine (2-OH NVP) and 3-OH NVP in plasma and to identify factors associated with nevirapine pharmacokinetic parameters.

Materials and methods: A total of 112 patients were recruited and treated with nevirapine-based antiretroviral therapy. Plasma nevirapine and metabolite concentrations were assayed using high-performance liquid chromatography via liquid–liquid extraction.

Results: Thirty-nine (34.8%) of the patients had no 3-OH NVP detected in their plasma while 2-OH NVP was detected in all patients. Metabolite concentrations were low compared to nevirapine. Positive correlations were observed between nevirapine and its metabolites, 2-OH NVP ($P < 0.01$) and 3-OH NVP ($P = 0.012$). Nevirapine concentration was decreased when concomitantly administered with methadone. Univariate analysis showed that ALT level, AST level, and detection of 3-OH NVP were associated with nevirapine pharmacokinetic parameters.

Conclusion: The variability of nevirapine pharmacokinetic parameters was caused by liver enzymes and the presence of 3-OH NVP metabolites. The presence of 3-OH NVP can probably be used to distinguished CYP2B6 activity and efficacy of nevirapine in patients with HIV infection.

Key words: Nevirapine, 2-hydroxynevirapine, 3-hydroxynevirapine

1. Introduction

Nevirapine is the first nonnucleoside reverse-transcriptase inhibitor approved and is widely used in combination therapy to treat HIV infection. In Malaysia, the defined daily dose of nevirapine was 0.0145 per 1000 population per day in 2010, which increased from 0.0144 in 2009 (1).

Malaysia is a country that has a concentrated HIV epidemic based on the WHO classification with a cumulative total of 79,855 people living with HIV at the end of 2011 (2). In Malaysia, the government provides first-line highly active antiretroviral therapy free of charge to all eligible patients who are treated in government hospitals. Apart from that, the Malaysian government also implemented a harm reduction program in 2006. The government's approach to reducing HIV infection has been successful because the notification rate of HIV decreased from 28.4 in 2002 to 23.4 in 2005 and to 12.2 cases per 100,000 of population in 2011 (2). In 2013, Malaysia reported an average of 9 cases per day or 3393 new cases, given a cumulative total of 101,672 HIV cases with 20,235 AIDS cases and 16,340 deaths related to HIV/AIDS, thus giving a reported total of 85,332 people living with HIV (3).

Nevirapine acts by binding directly to the reverse transcriptase and blocks the RNA- and DNA-dependent polymerase activities by causing a disruption of the enzyme's catalytic site. Nevirapine is extensively metabolized in the liver mainly by 2-, 3-, 8-, and 12-hydroxylation followed by glucuronidation of these hydroxyl metabolites. Studies of the biotransformation of nevirapine by human liver microsomes showed that CYP2B6 is mainly responsible for oxidation of

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3-hydroxynevirapine (3-OH NVP) and CYP3A4 for 2- and 12-hydroxynevirapine (2- and 12-OH NVP), while 2D6 mediates oxidation for 8- and 12-hydroxynevirapine and CYP3A5 is a minor contributor in the oxidation of 2- and 12-OH NVP (4). A single nucleotide polymorphism (SNP) in exon 4 (G516T) is associated with a significant reduction in CYP2B6 catalytic activity (5). Researchers also associated nevirapine concentrations with CYP2B6 516 in several populations, such as Ugandans, HIV-infected Thai women, and the Swiss population (6–8). However, the correlation of nevirapine metabolites with CYP2B6 activity is unknown. Thus, this study explored CYP2B6 activity by measuring 2-OH and 3-OH NVP in plasma and identified the associations between the presence of 3-OH NVP and nevirapine pharmacokinetic parameters.

2. Materials and methods

2.1. Subjects

The study was conducted following approval from the Human Research Ethics Committee, University Sains Malaysia, and the Malaysia Ministry of Health Research Ethical Committee. One hundred and twelve HIV patients from three state hospitals in Malaysia were recruited. To be included in the study, candidates had to be HIV-positive, more than 18 years old, and started on nevirapine (200 mg twice daily) at least 2 months prior to the pharmacokinetic sampling in order to achieve steady-state conditions. All participants provided written informed consent before participation.

2.2. Nevirapine quantification

Plasma concentrations of 2-OH NVP, 3-OH NVP, and nevirapine were quantified using HPLC-UV analysis (Agilent 1200 series HPLC system with DAD detector) via a method previously described (9). The nevirapine and metabolites were extracted using the liquid–liquid extraction method with 4 mL of diethyl ether and 1 mL of hexane. The limits of detection were 0.5 μg/mL, 0.02 μg/mL, and 0.015 μg/mL for nevirapine, 2-OH NVP, and 3-OH NVP, respectively. Linear regression analysis data for the calibration plot showed good linear relationships between response and concentration for nevirapine and the oxidative metabolites with regression coefficient values of $r^2 > 0.99$. Briefly explained, each plasma aliquot of 1 mL was heated at 56 °C for 90 min to inactivate the HIV-1 virus and was spiked with carbamazepine as an internal standard. Chromatographic separation of the compounds was accomplished using a Zorbax SB-C8 column and a mobile phase consisting of ammonium acetate and acetonitrile (8:2 v/v). Detection was performed at 280 nm and peaks were quantified at 5.87 min for 2-OH NVP, 7.42 min for 3-OH NVP, 12.88 min for nevirapine, and 26 min for the internal standard.

2.3. Blood sampling

Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 3, 4, and 8 h after the morning dose. We assumed that all patients had identical predose and 12-h postdose levels. The AUC of nevirapine was obtained using the trapezoid rule with linear interpolation with a noncompartmental model with PK-Solver. The minimum plasma concentration (Cmin) and maximum plasma concentration (Cmax) of nevirapine were obtained from visual inspections of the concentration–time curves.

2.4. Statistical methods

The target variables in our study were Cmax, Cmin, and AUC. All statistical tests were performed using SPSS 22 (IBM Corp., Armonk, NY, USA). Normality distribution and equality of variance were determined prior to statistical analysis. Univariate analyses were done using simple linear regression, the independent t-test, or the Mann–Whitney U test. Correlations between the concentrations of nevirapine and its metabolites were tested using the Pearson correlation. From the results of univariate analyses, independent variables that had a P-value of <0.25 were included in the multiple linear regression analysis. $P < 0.05$ was accepted as significant.

3. Results

Patients' characteristics at the time of sampling are summarized in Table 1. Among the 112 patients recruited, 39 of them had no 3-OH NVP detected in their plasma while 2-OH NVP was detected in all patients. Nearly an equal number of male and female patients were recruited

Table 1. Patients' characteristics at the time of sampling.

Patients' characteristics	n [%]
Sex, n [%] Male Female	 63 [56.3%] 49 [43.8%]
Age (years), median [range]	40 [19–65]
BMI (kg/m^2), median [range]	21.2 [15.41–39.06]
Weight (kg), median [range]	58.1 [37.5–102.5]
CD4 count (cell/mm^3), median [range]	373 [90–1376]
AST, mean [SD]	37.71 [17.95]
ALT, mean [SD]	35.52 [28.21]
Viral load (copies/mL), n [%] Not detected <34 ≥34	 60 [53.6%] 30 [26.8%] 22 [19.6%]

and median BMI was 21.2 kg/m^2. Eighty percent of the patients had undetectable viral loads or had less than 34 copies/mL at the time of study, and CD4 T cell counts ranged from 90 to 1376 cell/mm^3. Seventeen of the patients were on methadone replacement therapy and seven patients were on isoniazid preventive therapy. Five of the patients were coinfected with the hepatitis B/C virus.

Correlations between nevirapine and its metabolites and the metabolite ratio are shown in Table 2. Factors associated with nevirapine pharmacokinetic parameters are shown in Tables 3–5. There was considerable interindividual variability in the nevirapine plasma concentrations at Cmax, ranging from a minimum value of 2.01 μg/mL to a maximum value of 24.32 μg/mL.

Table 2. Metabolic ratios and correlation of plasma hydroxyl nevirapine with nevirapine in HIV-infected patients.

	Mean concentration, μmol (SD)	Metabolic ratio	r value[#]	P-value[a]
Nevirapine	31.332 (12.82)			
2-OH NVP	1.364 (0.64)	0.04	0.789	<0.01
3-OH NVP	0.125 (0.067)	0.004	0.294	0.012

[#] Correlation between nevirapine hydroxyl metabolites and nevirapine.
[a] Pearson correlation was applied.

Table 3. Predictors of nevirapine Cmin analysis with multiple linear regression with the stepwise method.

Variables	b (95% CI)	Univariate analysis P-value[a]	Multiple linear regression analysis		
			Adjusted b (95% CI)	t-stat	P-value
Age	0.041 (–0.014, 0.097)	0.145			
Weight	–0.002 (–0.038, 0.033)	0.894			
BMI	–0.011 (–0.105, 0.083)	0.821			
CD4 count	–0.001 (–0.004, 0.001)	0.298			
ALT	0.021 (0.005, 0.038)	0.01*	0.021 (0.005, 0.037)	2.614	0.01*
AST	0.03 (0.005, 0.056)	0.021*			
3-OH metabolic ratio	–29.60 (–204.85, 145.65)	0.738			
2-OH metabolic ratio	–23.12 (–55.96, 9.72)	0.166			
	Median (IQR) μg/mL	P-value[b]			
Sex Male Female	5.63 (2.63)[#] 5.12 (2.35)	0.295			
Viral load detection Yes No	5.49 (2.43) 5.48 (2.66)	0.993			
3-OH detection Yes No	5.26 (2.33) 4.88 (2.89)	0.088[c]	0.971 (0.021, 1.92)	2.026	0.045*
Methadone Yes No	4.46 (3.68) 5.26 (2.45)	0.096[c]			

[a] Simple linear regression. [b] Mann–Whitney test. [c] Independent t-test. *Significant. [#] Presented as mean (SD). ALT = alanine aminotransferase, AST = aspartate aminotransferase, BMI = body mass. index

Table 4. Predictors of nevirapine Cmax analysis with multiple linear regression with the stepwise method.

Variables	b (95% CI)	Univariate analysis P-value[a]	Multiple linear regression analysis		
			Adjusted b (95% CI)	t-stat	P-value
Age	0.056 (–0.02, 0.132)	0.147			
Weight	–0.023 (–0.071, 0.025)	0.351			
BMI	–0.002 (–0.005, 0.001)	0.226			
CD4 count	–0.091 (–0.218, 0.035)	0.155			
ALT	0.037 (0.016, 0.059)	0.01*	0.037 (0.015, 0.058)	3.313	0.001*
AST	0.046 (0.011, 0.081)	0.01*			
3-OH metabolic ratio	–131.35 (–368.07, 105.38)	0.274			
2-OH metabolic ratio	–36.31 (–80.76, 8.13)	0.108			
	Median (IQR) µg/mL	P-value[b]			
Sex Male Female	 8.73 (3.55)[#] 7.83 (3.19)	0.167[c]			
Viral load detection Yes No	 8.42 (3.23) 8.49 (3.64)	0.919			
3-OH detection Yes No	 8.21 (3.42) 7.09 (4.46)	0.196			
Methadone Yes No	6.53 (4.87) 8.11 (3.46)	0.086			

[a] Simple linear regression. [b] Mann–Whitney test. [c] Independent t-test. *Significant. [#] Presented as mean (SD).

4. Discussion

CYP2B6 variants have been associated with 4-fold higher nevirapine concentrations in plasma (8). As mentioned earlier, nevirapine is metabolized in the liver primarily by CYP3A4 and CYP2B6 for 2-OH and 3-OH NVP, respectively. In this study, we measured nevirapine, 2-OH NVP, and 3-OH NVP plasma concentrations and justified the CYP2B6 activity by using the 3-OH NVP plasma concentration as a marker. Since the limits of detection for 2-OH and 3-OH NVP were 0.02 µg/mL and 0.015 µg/mL, respectively, concentrations detected below the limit were considered to be undetected. 2-OH NVP was detected in all patients; however, in only 73 of them was it detected together with 3-OH NVP. For the metabolite ratio, concentrations of 2-OH and 3-OH NVP at the Tmax of nevirapine were compared with the nevirapine concentration. Concentrations were converted to micromolar units by dividing the 2-OH NVP, 3-OH NVP, and nevirapine concentrations (µg/mL) into a molecular weight of 282 for hydroxynevirapine and 266 for nevirapine.

Fan-Havard et al. (10) demonstrated lower concentrations of nevirapine metabolites when comparing pharmacokinetic parameters of nevirapine between a single dose of nevirapine in HIV-negative African Americans and nevirapine at steady state in HIV-infected Cambodian patients. Our study showed similar findings. The metabolite ratios of 2-OH and 3-OH NVP examined were 0.04 and 0.004, respectively, which are 25 times and 250 times lower than the parent drug. The predominant metabolite was 2-OH NVP, which is contrary to the Cambodian population, where the 3-OH NVP plasma concentration was higher than that of 2-OH NVP. Further analysis showed positive correlations between concentrations of nevirapine and its metabolites. Strong correlations were observed between 2-OH NVP and nevirapine concentrations while 3-OH NVP and nevirapine demonstrated weak correlations. In a study to

Table 5. Predictors of nevirapine AUC analysis with multiple linear regression with the stepwise method.

Variables	b (95% CI)	Univariate analysis P-value[a]	Multiple linear regression analysis		
			Adjusted b (95% CI)	t-stat	P-value
Age	0.571 (–0.163, 1.305)	0.126			
Weight	–0.147 (–0.615, 0.322)	0.537			
BMI	–0.654 (–1.885, 0.577)	0.295			
CD4 count	–0.016 (–0.047, 0.015)	0.302			
ALT	0.362 (0.151, 0.572)	0.001*	0.518 (0.254, 0.782)	3.893	<0.001*
AST	0.507 (0.173, 0.842)	0.003*			
3-OH metabolic ratio	–1179.23 (–3476.60, 1118.144)	0.311			
2-OH metabolic ratio	–391.318 (–821.105, 38.469)	0.074			
	Median (IQR) mg h/L	P-value[b]			
Sex Male Female	 74.69 (42.19) 71.01 (37.21)	0.093			
Viral load detection Yes No	 74.69 (38.63) 71.30 (32.38)	0.464			
3-OH detection Yes No	 72.79 (33.75) 67.41 (45.67)	0.131			
Methadone Yes No	 62.47 (60.44) 74.57 (32.42)	0.051			

[a] Simple linear regression. [b] Mann–Whitney test. *Significant.

assess the pharmacokinetics of nevirapine and metabolites in patients with hepatic fibrosis, the metabolite profiles were comparable across the stages of the disease (11).

Risk of virological failure increased 5-fold when nevirapine concentrations were below 3 mg/L and dose adjustment has been suggested if the nevirapine Cmin is lower than 3 mg/L (12,13). Our results show that higher trends of nevirapine plasma concentration were demonstrated in patients with 3-OH NVP for both Cmax and Cmin. However, a significant difference was only observed in Cmin. Although not statistically significant, a higher trend was also observed in the area under the plasma drug concentration–time curve (AUC 0–12 h) in patients with 3-OH NVP. The AUC reflects the actual body exposure to drugs after the administration of a dose of the drug. The comparison of nevirapine concentrations provides insight into nevirapine disposition, which may be related to variants in the *CYP2B6* gene involved in the formation of 3-OH NVP.

Comparing the concentrations and AUC 0–12 h of nevirapine, no difference was observed between groups of patients with detected and undetected viral load. However, the majority of them (71%) had a viral load detected of less than 34 copies/mL.

Nevirapine was previously reported to decrease the concentration and efficacy of methadone (14). To our knowledge, no study has yet reported the effects of concomitant administration of methadone on the nevirapine concentration. In the current study, the nevirapine concentrations and AUC following twice daily doses of nevirapine at 200 mg were lower when it was taken concomitantly with methadone (Tables 3–5). Although not statistically significant, the percentage lowered was 16% in the AUC, 15% in Cmin, and up to 19.5% in Cmax. A previous study demonstrated that CYP2B6 is also responsible in the metabolism of methadone and this might explain the drug–drug interaction between nevirapine and methadone, particularly in concentrations

and AUC, as observed in the current study (15). It is suggested to perform nevirapine plasma concentration monitoring as methadone may influence the efficacy of treatment because it causes a decrease in nevirapine concentrations.

Elevated AST levels, age, and weight are identified factors contributing to a high interindividual variance of nevirapine (12,14,16–19). In our study, univariate analysis showed that the AST and ALT levels were significantly associated with nevirapine concentrations and AUC 0–12 h. Furthermore, multivariate analyses confirmed that increased ALT levels significantly increased nevirapine concentrations and AUC 0–12 h. A recent study reported that hepatotoxicity was significantly associated with higher nevirapine trough concentrations (20). An abnormally high ALT level was also reported to be an independent risk factor for the development of hepatotoxicity during nevirapine-containing treatments (21). Therefore, frequent liver enzyme monitoring is suggested to avoid nevirapine-induced toxicity.

CYP2B6 polymorphisms also reportedly influenced nevirapine pharmacokinetic parameters (15,16,22). In Malaysia, CYP2B6*6 has been found to occur in 13% to 26% of Malays, Chinese, Indians, and opiate-dependent individuals; the CYP2B6*9 allele occurs in 4.6% to 10.2% of the group; and the CYP2B6*2 allele was found in 0.8% to 3.2% of the group (23). The consequences of these variations are numerous, from no detectable function to partially reduced function and also increased expression, and, therefore, they may affect nevirapine metabolism to 3-OH NVP. Note that our study has limitations because the study was not assessing the effect of polymorphisms. Furthermore, we only assessed concentrations of 2-OH and 3-OH metabolites; therefore, we cannot fully characterize the disposition of nevirapine.

In conclusion, this study demonstrates that AST level, ALT level, and detection of 3-OH NVP are significantly associated with nevirapine pharmacokinetic parameters. The detection of the 3-OH metabolite may provide a useful indicator for distinguishing CYP2B6 activity.

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