Background: Concerns have been raised about the possibility of subtherapeutic efavirenz (EFV) plasma levels in children with the current dosing guideline. Single nucleotide polymorphisms of the hepatic cytochrome P450 isoenzyme 2B6 (CYP2B6) gene have been associated with high inter-individual variations in EFV plasma concentrations. Our objective was to determine the adequacy of EFV dosing and explore the influence of CYP2B6-516G>T polymorphisms on EFV plasma concentrations in Thai HIV-infected children.

Methods: A total of 63 HIV-infected children receiving EFV for ≥4 weeks were assessed. Children received EFV daily doses on the basis of body weight bands. Between 12 to 16 h after EFV intake, a blood sample was drawn to measure the EFV plasma concentration and to determine the CYP2B6-516G>T polymorphism using HPLC and direct gene sequencing, respectively.

Results: The median age (range) was 12.3 years (3.1–18.7). The mean (±sd) EFV plasma concentration was 3,138 ng/ml (3,313). Eight (13%), 45 (71%) and 10 (16%) children had an EFV concentration <1,000 ng/ml, 1,000–4,000 ng/ml and >4,000 ng/ml, respectively. CYP2B6-516 G/G, G/T and T/T genotypes were found in 48%, 41% and 11% children, respectively. The CYP2B6-516 G>T allele frequency was 31.75%. The mean (±sd) EFV concentration for children with G/G, G/T and T/T genotypes were 1,604 ng/ml (729), 2,635 ng/ml (1,199) and 11,582 ng/ml (2,972), respectively (P<0.001). A correlation between EFV concentrations >4,000 ng/ml and psychiatric side effects was observed (P=0.02), but there was no association with rash, hepatotoxicity or central nervous system disturbances.

Conclusions: Current EFV dosing guidelines provide adequate plasma drug concentrations in Thai HIV-infected children. CYP2B6-516G>T polymorphisms significantly affect the drug metabolism of EFV in children.

Introduction

Efavirenz (EFV) plus two nucleoside reverse transcriptase inhibitors (NRTIs) is currently the preferred choice of highly active antiretroviral therapy for antiretroviral-naïve HIV-infected children aged 3 years and older [1,2]. EFV plasma concentrations have been reported to be a predictor of virological response and drug adverse events. Virological failure was observed in 50% of adult patients with an EFV level <1,000 ng/ml (measured at an average of 14 h after intake) versus 22% of patients with an EFV level 1,000–4,000 ng/ml. Central nervous system (CNS) toxicity was approximately 3× more frequent in patients with an EFV level >4,000 ng/ml [3]. Thus, although the role of routine therapeutic drug monitoring of antiretroviral drugs is unclear, it has been proposed that mid-interval EFV drug levels of 1,000–4,000 ng/ml would be preferable to ensure efficacy and reduce the risk of drug toxicity.

EFV dosing in children is once daily and is calculated on the basis of body weight. The World Health Organization (WHO) currently recommends an EFV dosage of 15 mg/kg/day [1] and the US pediatric antiretroviral treatment guidelines recommend EFV dosing on the basis of body weight bands [2]. Few data are available on EFV plasma concentrations in children. In a US adolescent...
cohort, 95% of children had an adequate EFV plasma level [4]. By contrast, data from 15 South African children reported that only 60% had adequate plasma levels [5] and a report from Germany reported that only 36% of children had adequate plasma levels with the dose currently recommended by the US paediatric antiretroviral treatment guidelines [6]. Currently, there are no published EFV plasma concentration data from Thai or Asian children.

EFV is primarily metabolized by the cytochrome P450 isoenzyme 2B6 (CYP2B). A CYP2B6-516G>T gene polymorphism has been reported to influence EFV plasma concentrations with the homozygous variant allele (T/T) being associated with higher EFV plasma concentrations. In a study of 100 HIV-infected adults, 19% of patients with the wild-type allele (G/G) had subtherapeutic EFV levels (<1,000 ng/ml) compared with 2% of patients with CYP2B6-516G>T polymorphisms (G/T or T/T) [7]. The frequency of polymorphisms in CYP2B6 varies among different ethnicities. Allele frequencies of CYP2B6-516G>T polymorphisms are 14% 15%, 21%, 26% and 49% in Japanese, Korean, Chinese, Caucasian and West African populations, respectively [8,9]. Currently, there are no data on the frequency of the CYP2B6-516G>T polymorphisms in the Thai population.

The aim of the study was to determine the influence of CYP2B6-516G>T polymorphisms on EFV plasma concentrations in Thai HIV-infected children and to investigate the association between EFV plasma concentrations and clinical outcomes.

Methods

Study participants
From January to February 2008, patients were recruited at the HIV clinic at the Department of Pediatrics, Chiang Mai University Hospital, Chiang Mai, Thailand. HIV-infected children >3 years of age who had received an EFV-based antiretroviral therapy (ART) for ≥4 weeks were enrolled. EFV dosing was in accordance with the dose by body weight band recommended by the US paediatric antiretroviral treatment guidelines [2]: for children weighing 10–<15 kg, 15–<20 kg, 20–<25 kg, 25–≤32.5 kg, 32.5–40 kg and ≥40 kg the EFV dose was 200, 250, 300, 350, 400 and 600 mg daily, respectively. Children screened within our cohort who met the inclusion criteria were sequentially enrolled. The day prior to the scheduled study visit, a research nurse contacted the children (or caregivers) to remind them to take their EFV dose as normal and to document the exact time of drug intake. At the study visit the next day, a blood sample was drawn between 12 and 16 h after their last dose of EFV to measure the EFV plasma concentration and to determine their CYP2B6-516G>T genotype.

Drug adherence during the 3–7 days prior to the visit was assessed in an interview. Any concomitant medication taken within 24 h prior to the time of blood sample collection was also recorded.

Clinical information related to ART, including clinical symptoms, CD4+ T-cell count, plasma HIV RNA viral load and drug adverse events were extracted from medical records. The drug adverse events were categorized into rash, hepatitis, CNS-related symptoms (for example, headache, dizziness, insomnia, nightmares, anxiety and difficulty concentrating) and psychiatric problems (for example, depression, suicidal thoughts, aggressive behaviour and psychosis).

The study was approved by the research ethics committee of Chiang Mai University. Written informed consent was obtained from the parent of each child or guardian before enrolment.

Identification of CYP2B6-516G>T gene polymorphisms DNA samples were extracted from peripheral blood specimens obtained from the study participants with NucleoSpin® Blood (Macherey–Nagel, Düren, Germany). The DNA product was amplified and directly sequenced. Partial genomic and complementary DNA sequences of the CYP2B6 gene were obtained from public databases (GenBank accession numbers NC_000019 and NM_000019). Targeted CYP2B6-516G>T polymorphisms in exon 4 were amplified by PCR using a forward primer (5′-GGTCTGCCCATCTATAAC-3′) and a reverse primer (5′-TCATCCTTTTCTGTGTGT-TCT-3′) [10,11]. The conditions that the amplifications were performed with were denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min for 35 cycles. These were preceded by denaturation at 95°C for 10 min to activate the AmpliTaq Gold® enzyme (Gene Systems Co., Ltd., Bangkok, Thailand) followed by 10 min at 72°C for the final extension step. The amplicon was then directly sequenced with a forward primer (5′-GGTCTGCCCATCTATAAC-3′) using a fluorescent DNA sequencing BigDye® Terminator Reaction Kit (Gene Systems Co., Ltd.) on an automated laser fluorescent sequencer ABI Prism® 3130xl (Gene Systems Co., Ltd.). Because the CYP2B6 nucleotide sequence is 95% homologous to CYP2B7 [12], the sequence homology to CYP2B6 exon 4 was checked and verified by BLASTN programme [13]. Genetic laboratory tests were performed at the Faculty of Medicine, Chiang Mai University.

Measurement of efavirenz plasma concentrations Blood samples were centrifuged and the plasma was aliquoted and frozen within 4 h of collection at -70°C. EFV plasma drug concentrations were measured at the Faculty of Associated Medical Sciences (Chiang Mai University) using an isocratic reversed-phase HPLC
method with ultraviolet detection at 245 nm. Briefly, patient plasma samples (300 µl) and all calibration and control samples were heat-inactivated in a water bath at 56°C for 30 min prior to assay. Sample pretreatment involved protein precipitation with acetonitrile (360 µl) followed by centrifugation. The sample supernatant was then injected into the HPLC machine. Chromatography was performed using an Agilent 1100 HPLC machine (Varian, Inc., Lake Forest, CA, USA), with an Omnispher C18 (150×4.6 mm ID/particle size 5 µm) analytical column, a Chromquard RP guard column and a mobile phase consisting of 10 mM KH2PO4 pH 3.1 acetonitrile (50:50 v/v). This method was validated using the AIDS Clinical Trials Group (ACTG) method validation guidelines over the concentration range of 78–10,000 ng/ml. The average accuracy was 102–103% and precision (inter- and intra-assay) was <3% of the coefficient of variation. Overall extraction recovery was 106% and EFV was stable under various storage conditions. Plasma samples with EFV concentrations >10,000 ng/ml were diluted and the assay was repeated. This laboratory participates in the international external quality Pharmacology Quality Control (Precision Testing) programme of the ACTG [14].

Statistical analyses
Deviation of genotype frequencies from Hardy–Weinberg equilibrium expectations were assessed with a χ² test at 1 degree of freedom. Allelic frequency was estimated from the total copies of patient alleles divided by all alleles in the population [10,15]. The comparison of EFV plasma concentrations between groups were examined using a one-way ANOVA or Kruskal–Wallis test as appropriate. A P-value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA).

Results
Patient characteristics
A total of 63 children were enrolled. The baseline characteristics are shown in Table 1. The median (range) age and body weight was 12.3 years (3.1–18.7) and 33.0 kg (12.0–58.5), respectively. Full adherence to ART during the 7 days prior to blood sample collection was reported by all children. Concomitant medication was reported by 11 children. These included antihistamines, cough syrup, prednisolone, an antipyretic and one child reported using an unknown antibiotic. The mean (s.d) CD4⁺ T-cell percentage at the time of study was 28.4% (5.6) and 94% of the children had a plasma HIV RNA level <50 copies/ml.

From the review of medical records, 10 children (16%) had a rash during the first few weeks after ART initiation, 14 (22%) had CNS disturbance (8 with dizziness, 2 with headaches, 2 with bad dreams and 2 with drowsiness) and 5 (8%) had psychiatric problems (4 with depression and 1 with delusions). Among children who had CNS disturbance, all symptoms developed within the first 4 weeks; however, all children developed tolerance to these side effects and none required treatment modification. Five children (8%) had increased alanine aminotransferase level grade 1, 3 (5%) had increased aspartate aminotransferase level grade 1, 4 (6%) had increased alkaline phosphatase during the first 6 months of treatment and no children had clinical hepatitis.

Efavirenz plasma concentrations
The mean (±s.d) EFV concentration was 3,138 ng/ml (3,313) at 14.8 h (0.8) after drug intake. A total of 45 children (71%) had an EFV plasma concentration 1,000–4,000 ng/ml, 8 children (13%) had a level <1,000 ng/ml and 10 children (16%) had a level >4,000 ng/ml. Mean EFV plasma concentrations did not differ among the different dosing weight bands (P=0.15).

CYP2B6-516G>T polymorphisms
The CYP2B6-516G/G genotype was found in 48% (30/63) of the children, the G/T genotype (heterozygous) in 41% (26/63) and the T/T genotype

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (n=63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>12.3 (3.1–18.7)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>25 (40)</td>
</tr>
<tr>
<td>Median body weight, kg (range)</td>
<td>33.0 (12.0–58.5)</td>
</tr>
<tr>
<td>Mean height, cm (±s.d)</td>
<td>138 (14.3)</td>
</tr>
<tr>
<td>Current antiretroviral regimen</td>
<td></td>
</tr>
<tr>
<td>AZT+3TC+EFV, n (%)</td>
<td>60 (95)</td>
</tr>
<tr>
<td>d4T+3TC+EFV, n (%)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>TDF+3TC+EFV, n (%)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Mean dose of EFV per kg (±s.d)</td>
<td>12.0 (1.6)</td>
</tr>
<tr>
<td>Body weight band</td>
<td></td>
</tr>
<tr>
<td>10–&lt;15 kg, n (%)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>15–&lt;20 kg, n (%)</td>
<td>4 (6)</td>
</tr>
<tr>
<td>20–&lt;25 kg, n (%)</td>
<td>14 (22)</td>
</tr>
<tr>
<td>25–&lt;32.5 kg, n (%)</td>
<td>16 (25)</td>
</tr>
<tr>
<td>32.5–40 kg, n (%)</td>
<td>13 (21)</td>
</tr>
<tr>
<td>&gt;40 kg, n (%)</td>
<td>15 (24)</td>
</tr>
<tr>
<td>Mean duration between blood sampling and last dose, h (±s.d)</td>
<td>14.8 (0.8)</td>
</tr>
<tr>
<td>Mean current CD4⁺ T-cell percentage, % (±s.d)</td>
<td>28.4 (5.6)</td>
</tr>
<tr>
<td>Patients with VL&lt;50 copies/ml, n (%)</td>
<td>58 (94)*</td>
</tr>
<tr>
<td>Mean duration of antiretroviral therapy, weeks (±s.d)</td>
<td>236 (41)</td>
</tr>
</tbody>
</table>

n=63. AZT, zidovudine; d4T, stavudine; EFV, efavirenz; TDF, tenofovir disoproxil fumarate; VL, viral load; 3TC, lamivudine.
(homozygous) in 11% (7/63) children. The allelic frequency was 31.75% and the frequency of all genotypes was within the Hardy–Weinberg equilibrium (P=0.75).

**CYP2B6 and efavirenz plasma concentrations**

There was a strong correlation between CYP2B-516-G>T polymorphisms and EFV plasma concentrations (Figure 1). The mean (±sd) plasma EFV concentrations of children who had the G/G, G/T and T/T genotype were 1,604 ng/ml (729), 2,635 ng/ml (1,199) and 11,582 ng/ml (2,972), respectively (P<0.001). All children with the T/T genotype had EFV concentrations >6,000 ng/ml. Of the children with the G/G genotype, 20% had subtherapeutic EFV levels compared with 8% of children with the G/T genotype and no children with the T/T genotype (P<0.001).

**Efavirenz plasma concentrations and drug adverse events**

There was no statistical association between EFV plasma concentrations and the appearance of rash, hepatotoxicity or CNS disturbance (data not shown); however, there was a strong correlation between EFV plasma concentrations and psychiatric side effects. Five children experienced psychiatric issues. None of 8 (0%), 2 of 45 (4%) and 3 of 10 (30%) patients with plasma EFV levels <1,000 ng/ml, 1,000–4,000 ng/ml and >4,000 ng/ml, respectively, had psychiatric problems (P=0.02).

**Association between efavirenz plasma concentrations or CYP2B6-G516T gene polymorphisms and virological response**

No correlation between virological response (defined as plasma HIV RNA level <50 copies/ml) and EFV plasma concentrations was observed. At the time of study, 89%, 96% and 100% of children with EFV plasma concentrations <1,000 ng/ml, 1,000–4,000 ng/ml and >4,000 ng/ml had virological suppression (P=0.55). Similarly, no correlation was observed between virological response and the different CYP2B6-G516T gene polymorphisms (P=0.47).

**Discussion**

To our knowledge, this is the first study to evaluate EFV plasma concentrations and its association with the CYP2B6-516G>T polymorphism in Asian HIV-infected children. We found that 87% of children receiving the EFV dose currently recommended by the US paediatric antiretroviral treatment guidelines had an EFV plasma concentration above the recommended efficacy threshold. The CYP2B6 allele frequency at position 516 was 31.75%. There was a strong correlation between the CYP2B6-516G>T polymorphisms and EFV concentrations, which is consistent with previously published data in adult study populations and American children.

The mean (±sd) EFV plasma concentration in our study was 3,138 ng/ml (3,313), similar to that reported in adults receiving the standard 600 mg once daily dose [3]. In a cohort of 33 HIV-infected children in Germany, the median EFV concentration reported was 2,800 ng/ml and 8.8% of the samples had an EFV concentration <1,000 ng/ml [16]. Data from 21 US adolescents who received EFV showed that 95% of them had an adequate drug exposure (0–24 h area under the curve) [4]. By contrast, a study in 15 South African children reported a median EFV plasma concentration of 1,580 ng/ml [5]. The lower EFV levels observed in the South African cohort might be explained by their younger age (median age 6.8 years compared with 12.5 years in our study). Hepatic cytochrome enzyme activities are known to significantly change during the development of children, with higher catalytic activity than adult levels between 1 and 4 years of age [17]. Differences in genetic polymorphisms could also help explain the disparity between cohorts, but no genetic polymorphism data was reported in the South African cohort. The results of our study support the current US paediatric antiretroviral treatment guidelines [2] for EFV dosing. Moreover, the result of EFV plasma concentrations are consistent with the reported long-term efficacy data of EFV-based ART in Thai HIV-infected children, which has shown virological suppression rates of 85% and 83% at 1 and 4 years, respectively [18,19]. We offered to repeat the EFV blood level for children who had a plasma level outside the recommended 1,000–4,000 ng/ml therapeutic interval. Of the children who had EFV level >8,000 ng/ml, six

**Figure 1. Efavirenz plasma concentrations of 63 children divided into the CYP2B6-516 polymorphisms G/G, G/T or T/T.**
had their dose reduced to two-thirds of the original dose. Five out of six children had a significant reduction in their EFV plasma level, but the level was still >4,000 ng/ml. One child had a higher EFV level after dose reduction. Among the eight children who had an EFV level <1,000 ng/ml, two children continued to have an EFV level <1,000 ng/ml and two had EFV levels within the 1,000–4,000 ng/ml range. A second measurement of EFV level was not done in the remaining four children.

Allele frequencies of CYP2B6 at position 516 have been reported in different ethnic groups. To our knowledge, this is the first study reporting the CYP2B6-516G>T frequency in the Thai population. The CYP2B6-516G>T frequency in Thai children was 31.75%, higher than that observed in other populations, for example, Korean (15%), Japanese (14%), Taiwanese (14%) and Caucasian (21%). However, the frequency is similar to that found in an African-American (38%) study population [9,20]. The T/T genotype frequency in Thais (11%) was similar to that in African-Americans (20%) and higher than those in Caucasian (3.4%) and Hispanic (6.7%) populations.

We observed a strong correlation between the CYP2B6-516G>T polymorphisms and EFV plasma concentrations, which is consistent with previous reports from adults in Spain [7] and in children in the US [10] and the Netherlands [21]. In our cohort, approximately one-fifth of patients with the G/T genotype had subtherapeutic EFV concentrations, whereas 8% of patients with the G/G genotype and no patients with the T/T genotype had subtherapeutic levels. A previous study in adults has reported that only 2% of patients with the CYP2B6-516G>T polymorphisms (G/T or T/T) had subtherapeutic EFV concentrations [7]. The T/T genotype was associated with significantly lower oral clearance compared with the G/T or G/G genotypes in children from the US [10]. Interestingly, all children in our cohort with the T/T genotype had high EFV concentrations >6,000 ng/ml. A report in 35 Japanese adult patients also found that the CYP2B6-516 T/T genotype was consistently associated with high EFV concentrations [22,23]. Other CYP2B6 variant alleles as well as polymorphisms in other genes, such as CYP3A4, CYP3A5 and MDR1, which were not analysed in this study, have also been linked to lower EFV metabolism [15,22–26]. It would be expected that other polymorphisms influence EFV pharmacokinetics, but the CYP2B6-516G>T polymorphisms plays a major role.

Data from HIV-infected adults showed that the risk of virological failure significantly increased if the EFV trough concentration was <1,100 ng/ml with the sensitivity to predict virological failure of 64% and a negative predictive value of 89%. However, in this study we found no association between EFV plasma concentrations and virological response. The incidence of CNS and psychiatric side effects in this study was similar to data (24–36%) previously reported [16,27]. Psychiatric side effects (such as depression or delusion), which have been reported in adults [28] but rarely in paediatric studies [16,27], were reported in 8% of our patients. To date, only one case report of a 12-year-old girl with psychosis related to EFV has been published. This girl had an EFV level of 19,013 ng/ml and was heterozygous for the CYP2B6-516G>T gene polymorphism [29].

The high incidence of psychiatric side effects in our report might have been related to the long-term use of EFV with our cohort compared with other studies, which only report CNS disturbance that primarily occurs within the first 4 weeks of treatment. The strong association between the T/T genotype and high EFV plasma concentrations, together with higher frequency of psychiatric problems in children, suggests that performing a CYP2B6 genotype test before EFV use might be an option. One approach has been to initiate lower EFV doses in patients with the T/T genotype [23]. This strategy might increase the safety and tolerability of EFV in clinical practice. This pharmacogenomic approach has been used in abacavir treatment where there is a strong correlation between the HLA-B*5701 allele and the risk of hypersensitivity, and thus abacavir should not be used in patients with HLA-B*5701 [2].

This study is the first study reporting the EFV plasma concentrations in a large cohort of Thai HIV-infected children using the dose by body weight bands recommended in the US paediatric antiretroviral treatment guidelines [2] and the allelic frequency of CYP2B6 gene polymorphisms; however, there are some limitations. Firstly, the majority of children in our cohort had been on a stable EFV-based regimen for several years and had undetectable HIV RNA level at the time of enrolment, perhaps leading to a selection bias towards patients with adequate EFV plasma concentrations. Secondly, there was only one child younger than 5 years of age; therefore, we could not explore the association of age and EFV concentrations. Thirdly, the assessment of side effects of EFV was performed from a retrospective review of the medical records.

In conclusion, the results of the present study suggest that the current recommended dose of EFV provides adequate blood levels in Thai HIV-infected children. CYP2B6-516G>T polymorphisms significantly affect EFV metabolism in Thai children and psychiatric side effects of EFV are associated with plasma concentrations >4,000 ng/ml. For patients who developed EFV-related toxicity, EFV dosage adaptation guided by EFV plasma levels should be performed. Given the high
incidence of CYP2B6 polymorphisms in the Thai population single nucleotide polymorphism analysis might be an option to help identify patients who might be at risk of drug toxicity.

Acknowledgements
This study was supported by a grant from the Research Institute for Health Sciences (Chiang Mai University).

Disclosure statement
The authors declare no competing interests.

Reference


