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Phenotyping and genotyping of CYP2C19 using comparative metabolism of proguanil in sickle-cell disease patients and healthy controls in Nigeria

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Abstract

Polymorphic expression of metabolic enzymes have been identified as one of the key factors responsible for the interindividual/ethnic/racial variability in drug metabolism and effect. In Nigeria, there is a disproportionately high incidence of sickle-cell disease (SCD), a condition characterized by painful crisis frequently triggered by malaria. Proguanil, a substrate of the polymorphic CYP2C19, is a chemoprophylactic antimalarial drug widely used among SCD patients in Nigeria. This study aimed to conduct a comparative CYP2C19 phenotyping among SCD patients and healthy controls and to compare the results with those previously reported. One hundred seventy-seven unrelated subjects comprising 131 SCD patients and 46 non-SCD volunteers were phenotyped. This was carried out by collecting pooled urine samples over 8 h following PG administration. Proguanil and its major CYP2C19-dependent metabolites were measured by high-performance liquid chromatography. Metabolic ratios (MRs) were computed and employed in classifying subjects into poor or extensive metabolizers. Among SCD group, 130 (99.2%) were extensive metabolizers (EMs) and 1 (0.8%) was poor metabolizer (PM) of PG, while 95.7 and 4.3% non-SCDs were EMs and PMs, respectively. MRs ranged from 0.02 to 8.70 for SCD EMs and from 0.22 to 8.33 for non-SCD EMs. Two non-SCDs with MRs of 18.18 and 25.76 and the SCD with MR of 16.77 regarded as PMs had earlier been genotyped as CYP2C19*2/*2. Poor metabolizers of proguanil in SCD patients are reported for the first time. Regardless of clinical significance, a difference in metabolic disposition of proguanil and CYP2C19 by SCDs and non-SCDs was established.

Abbreviations

CG, cycloguanil; CPB, chlorophenylbiguanide; EM, extensive metabolizers; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; MR, metabolic ratio; PG, proguanil; PLC, performance liquid chromatography; PM, poor metabolizer; SCD, sickle-cell disease.

Introduction

Interindividual, ethnic, and racial variability in drug metabolism has been largely attributed to polymorphic expression of metabolizing enzymes, especially cytochrome P450 (CYP) (Mise et al. 2004; Shirasaka et al. 2015). Certain disease conditions, apart from exerting profound effects on drug metabolism, have been reported to influence enzyme expressions (Brooks et al. 2007; Morgan 2009; Yeung et al. 2014). Thus, the complex interplay between genetic, pathologic, and environmental factors on drug disposition has been the subject of several research efforts and findings which have impacted therapeutic outcomes.

Africa accounts for about 89% of global sickle-cell carriers with Nigeria disproportionately contributing over 25% of the global pool. According to a 1989 study, about 2–3% of the Nigerian population have sickle-cell disease (SCD) (Fleming 1989). Malaria, a mosquito-borne parasitic infectious disease, endemic to the tropical regions of the world, is a frequent precipitating cause of the life-threatening sickle-cell crises in SCD patients. Thus, the prophylaxis against malaria has been an important component of the prevention of sickle-cell crisis among this population (Oniyangi and Omari 2006).

Proguanil (PG) is an antimalarial drug widely used in the tropics for prophylaxis against *Plasmodium falciparum* malaria. In Nigeria, it is also the drug of choice for malaria suppression in patients with SCD (Bolaji et al. 2002). In fact, most SCD patients in Nigeria are placed on a lifetime antimalarial chemoprophylaxis with proguanil (Nwokolo 1960; Bhatt 1994; Oniyangi and Omari 2006). Proguanil exerts its antimalarial effect through the activity of its active metabolite–cycloguanil (CG) – which inhibits plasmodial dihydrofolate reductase, impeding DNA synthesis and cell multiplication in the parasite (Ward et al. 1991; Fidock et al. 1998).

The metabolism of PG to CG is mediated in the liver predominantly by CYP2C19, which is known to exhibit genetic polymorphism, and on the basis of which a population may be divided into two groups – extensive metabolizers (EMs) and poor metabolizers (PMs) (Wright et al. 1995; Desta et al. 2002). CYP2C19 is one of the important CYP in drug biotransformation, responsible for the primary metabolism of approximately 10% of commonly used drugs (Gardiner and Begg 2006), including the proton pump inhibitors, tricyclic antidepressants, some selective serotonin reuptake inhibitors, benzodiazepines (diazepam, flunitrazepam, quazepam, clobazam), S-mephenytoin, bortezomib, voriconazole, selegiline, proguanil, and nelfinavir (Zhou et al. 2009).

Several mutations for the gene that codes for CYP2C19, and resulting in the production of inactive CYP2C19, have been identified (De Morais et al. 1994a,b). Specific base substitution mutations in the gene are responsible for the PM phenotype which is inherited as a recessive autosomal trait, while EMs are either heterozygous or homozygous for the wild-type allele(s) (*1/*2 or *1/*1). Variability in individual's response to CYP substrates can, therefore, be largely attributed to inherited genetic differences in the drug targets (e.g., receptors and enzymes), individual's age, race, organ function, drug interactions, and concomitant illnesses (Meyer and Zanger 1997; McLeod and Evans 2001; Evans and McLeod 2003; Weinshilboum 2003). Earlier, CYP2D6 polymorphisms has been reported to exert significant effect of on the response to pain treatment in pediatric patients experiencing sickle-cell pain crisis (Brousseau et al. 2007).

The prevalence of PMs varies among races and ranged from 1% to 7.5% in Black Americans and Black Africans, 3–10% in Caucasians, 19% in Asian populations, and as high as 70.6% in subjects from Vanuatu (Edstein et al. 1994; Kaneko et al. 1997; Mizutani 2003).

The conversion of S-mephenytoin to its 4′-hydroxylated derivative has been used as a marker for CYP2C19 activity. The polymorphic expression of CYP2C19 is known to be responsible for the polymorphism in S-mephenytoin metabolism. Diminished formation of CG from proguanil has been shown in poor metabolizers of S-mephenytoin, which demonstrates the dependence of CG formation on CYP2C19 (Ward et al. 1991; Brøsen et al. 1993). Consequently, urinary recovery of CG has been employed as a phenotypic probe of CYP2C19 activity assessment (Brøsen et al. 1993; Wanwimolruk et al. 1995a,b).

Previous studies have reported 4.8% and 4.3% poor metabolizers of proguanil and S-mephenytoin, respectively, in healthy Nigerian population (Iyun et al. 1990; Bolaji et al. 2002). With the high SCD burden and chronic use of proguanil among SCD patients in Nigeria, this study was aimed at phenotyping SCD patients and non-SCD controls for CYP2C19 using proguanil metabolism measured through urinary elimination and to compare the results with previously reported genotyping study in this population.

Materials and Methods

Subjects

This study is a continuation of the study on CYP2C19 genotype earlier reported in Nigerian SCD and healthy controls (Babalola et al. 2010). For the current study, 193 unrelated Nigerian (143 SCD patients and 50 controls, aged 6–61 years) volunteers were recruited after written informed consent was provided. However, phenotype data

were successfully computed for 177 volunteers (131 SCD [63 males, 68 females] and 46 healthy controls [21 males, 25 females]). For pediatric subjects, informed consent was obtained from the parents. SCD patients were recruited from those attending State Hospital, Ibadan, General Hospital, Ijebu Ode, General Hospital, Abeokuta and Oni Memorial Children's Hospital, Ibadan, all located in the Southwest of Nigeria. Excluded from the study were individuals with history of current/recent alcohol consumption and/or tobacco smoking and subjects with comorbidities and/or on medications including over-the-counter and herbal drugs. SCD volunteers were only on routine hematinics specifically folic acid, Vit B Co, Vit C, and paracetamol; drugs that have not been found to affect the CYP2C19 phenotype. Control subjects included individuals with HbAA or HbAS who were healthy as determined by their medical history and who were not taking any medications. The study protocol (UI/IRC/05/0067) was approved by the Joint University of Ibadan/University College Hospital Institutional Review Committee. Blood samples (5 mL) were collected from every participant for hematology screening where RBC count, hematocrit, Hb, blood group, bilirubin, G6PD status, platelet counts, reticulocyte counts, HbF and leukocyte counts were determined and recorded.

Drug administration and sample collection

After emptying their bladder, each subject received proguanil (2.5 mg/kg) based on body weight. Total urine voided was collected over 8 h (Bolaji et al. 2002). Urine volume and pH were measured and recorded, and aliquots of the urine samples immediately stored at −20°C until analyzed. Samples were collected by phlebotomists/health workers assisted by nurses.

Drug analysis

Proguanil, cycloguanil, and 4-chlorophenylbiguanide were analyzed in urine using slightly modified reversed phase high-performance liquid chromatography (RP-HPLC) method earlier developed and applied in our laboratory (Onyeji et al. 1989; Bolaji et al. 2002; Ebeshi et al. 2005).

HPLC instrumentation and chromatographic conditions

Chromatography was performed with HPLC System (Agilent technologies 1100 Series) equipped with G1379A degasser, G1311A quaternary pump, syringe loading injector with a 20-μL loop size and variable wavelength detector. Chromatographic separations of the compounds were achieved on Zorbax SB C18 Column (250 × 4.6 mm, 5 μm) at 25°C, and the absorbance was monitored at 254 nm. The mobile phase consisted of methanol/acetonitrile/ammonium acetate in the ratio 40:5:55 and pH was adjusted to 2.2 with 0.5% 75 mmol/L perchloric acid ($HClO_4$). The flow rate of the mobile phase was set at 1.0 mL/min, and pyrimethamine was used as internal standard.

The limit of detection determined for proguanil and CG and 4-chlorophenylbiguanide (4CPB) were 5, 9, and 7.5 ng/mL, respectively, while the limit of quantitation was 15, 26, and 23 ng/mL for proguanil, CG, and 4CPB respectively.

Chemicals and reagents for HPLC analysis

Proguanil hydrochloride (CAS No 637-32-1; EC No 211-283-7), cycloguanil (CAS No 516-21-2), and 4-chlorophenylbiguanide (4CPB; Ref No. UC0705108) were received as gifts from AstraZeneca, England. Paludrine tablets 100 mg (proguanil B.P.) LOT CT790 manufactured by Boots Contract Manufacturing, Nottingham, United Kingdom, for AstraZeneca UK Limited (Macclesfield, Cheshire, United Kingdom) was donated by Reals Pharmaceuticals PLC, Nigeria. Pyrimethamine powder (BDH) was a kind donation from Malaria Research Laboratories, IMRAT, University College Hospital, Ibadan. Ammonium acetate, perchloric acid 60% w/w, diethylether, orthophosphoric acid, hydrochloric acid, sodium hydroxide, chloroform, triethylamine, acetone, HPLC-grade methanol, and acetonitrile were obtained from Sigma Aldrich (St Louis, MO).

Data analysis

The total concentration of PG and its metabolites in urine was calculated, and the metabolic ratio (MR) – the measure of enzyme activity – was calculated based on PG/CG ratios. Subjects were classified phenotypically as PM or EM types based on MR values greater or less than 10, respectively, as previously established (Ward et al. 1989). For correlation analysis, nonparametric Spearman's rank correlation coefficient was employed to determine the association between selected variables.

Statistical analysis

Z-test was used to compare mean MRs for EMs within the controls and SCD groups. Chi-square test was used to compare PM frequency among controls for both phenotype and genotype studies (4.3% vs. 4.7%; $\chi^2 = 0.0047$, $P > 0.05$). *P*-values for the comparison of MRs between *1/*1 and *1/*2 for the groups were obtained by SPSS version 20, 2014. Mean MRs, ranges, and median for each population were computed using the Microsoft excel program.

Results

One hundred seventy-seven (131 SCD patients and 46 healthy controls) human volunteers were administered with a single-dose proguanil followed by urinary collection and analysis for parent drug and the major CYP2C19-dependent metabolites. None of the subjects who participated in the study reported any adverse effect.

There was complete resolution and separation of the parent PG and its two major metabolites – CG and 4CPB. The retention times were 10.13, 4.22 and 3.27 min, respectively, while the internal standard eluted at 8.99 min. The frequency distribution histograms of the urinary PG/CG ratios (MR) for the SCD patients and the healthy controls are shown in Figure 1.

Tables 1 and 2 show the percent dose recoveries of PG, CG, and 4-CPB in healthy controls and SCD patients, respectively. MRs for the total EM subpopulation within the SCD patients ranged from 0.02 to 8.7 (mean ± SD = 2.04 ± 1.93; median = 1.27). Using an antimode of 10 as earlier established for the allotment of MR in Caucasians (Ward et al. 1989), only one (0.8%) patient out of the 131 SCD volunteers fell into the PM phenotype group with an MR of 16.77. All the other 130 SCD subjects, constituting 99.2% were EMs of PG.

The MR for the EMs in the control group ranged from 0.22 to 8.33 (mean ± SD = 5.46 ± 2.3; median = 5.49). Forty-four (44) control subjects phenotyped with MR values <10 EMs of PG, and this corresponds to EM frequency of 95.7%. Only two control subjects (4.3%) were identified as PMs, each having a PG/CG ratio >10 (MR = 18.18 and 25.76), respectively. Higher mean MRs were observed for EMs within the control group (mean ± SD = 5.46 ± 2.3; median = 5.49) than in the SCD group (mean ± SD = 2.04 ± 1.93; median = 1.27) ($P < 0.05$). In total, only three subjects of the 177 phenotyped corresponding to 1.7% were PMs of PG.

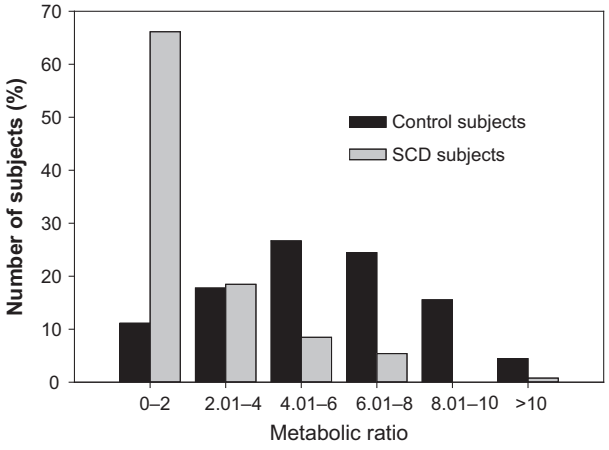

Figure 1. Frequency distribution of proguanil metabolic ratios in 131 SCD patients and 46 healthy control subjects.

The differences in the mean urinary dose recovery of CG between the PMs and EMs was significant. In the SCD patients, the PM excreted only about 7% of the average quantity of CG excreted by the EMs (Table 2). While for the healthy control group, PMs, on the average, excreted about 23.1% of the quantity of CG excreted by the EMs. The correlation of phenotyping result with earlier genotyping result is shown in Table 3.

Discussion

PG metabolism has been used as a probe to phenotype 126 unrelated healthy Nigerian subjects for polymorphic drug oxidation (Bolaji et al. 2002) in a study that did not include SCD patients, the predominant users of the drug for malaria prophylaxis. The therapeutic efficacy of an antimalarial drug depends on its bioavailability, the susceptibility of the parasites, and the antimalarial immune status of the human host (Kaneko et al. 1999). In the case

Table 1. Mean (±SD), urinary recoveries of proguanil and its two metabolites in 8-h urine and the urinary metabolic ratios in the control group.

	Extensive metabolizers	Poor metabolizers[1]
Number of subjects	44	2
Age (years)	25.31 ± 6.48	21, 23
Weight (kg)	57.29 ± 10.36	68, 60.5
Proguanil (% dose)	0.57 ± 0.51	0.73, 0.58
Cycloguanil (% dose)	0.13 ± 0.14	0.04, 0.02
4-Chlorophenylbiguanide (% dose)	0.05 ± 0.08	0.09, 0.04
Metabolic ratio (PG/CG) (range)	5.46 ± 1.3 (0.22–8.33)	18.18, 25.76 (18.18–25.76)

[1]Values provided in the column are the individual values for the two subjects (PM1, PM2).

Table 2. Mean (±SD), urinary recoveries of proguanil, and its two metabolites in 8-h urine and the urinary metabolic ratios (MRs) in the sickle-cell disease group.

	Extensive metabolizers	Poor metabolizers
Number of subjects	130	1
Age (years)	18.19 ± 8.37	20
Weight (kg)	38.23 ± 13.18	42
Proguanil (% dose)	0.50 ± 0.63	0.64
Cycloguanil (% dose)	0.44 ± 0.73	0.04
4-Chlorophenylbiguanide (% dose)	0.17 ± 0.29	0.05
MR (PG/CG) (range)	2.04 ± 1.93 (0.02–8.70)	16.77

Table 3. Correlation of phenotyping results with reported genotyping in this population and comparison of this study with other studies carried out in Nigerian subjects.

	Phenotyping (current study)		Genotyping[1]		Phenotyping[2]	Phenotyping[3]
TP (*n*)	177		158		126	92
SP (*n*)	SCD (130)	Controls (46)	SCD (115)	Controls (43)	Healthy volunteers	Healthy volunteers
Extensive metabolizers (%)	130 (99.2)	44 (95.7)	114 (99.1)	41 (95.3)	120 (95.2)	88 (95.7)
PM (%)	1 (0.8)	2 (4.3)	1 (0.9)	2 (4.7)	6 (4.8)	4 (4.3)
TP Ems (%)	130 + 44 = 174 (98.3)		114 + 41 = 155 (98.1)		n/a	n/a
TP PMs (%)	1 + 2 = 3 (1.7)		1 + 2 = 3 (1.9)		n/a	n/a

TP, total population; SP, study population; SCD, sickle-cell disease; n/a, not applicable; *n*, number; PM, poor metabolizer.
[1]Babalola et al. (2010).
[2]Bolaji et al. (2002).
[3]Iyun et al. (1990).

of proguanil, CYP2C19-dependent metabolism is necessary for activity. Thus, the understanding of the polymorphic expression of CYP2C19 in patients who take proguanil is clinically important. Patients phenotypic group (PM or EMs) may, therefore, play important role in determining the dosage regimens of proguanil. This study is not just the first to phenotype CYP2C19 in Nigerian SCD patients in comparison with healthy controls, and its use of proguanil is important as it reflects applicable clinical situations.

Total 8-h period of pooled urine has earlier been validated for sufficient proguanil metabolism for useful MR computation (Bolaji et al. 2002). Pharmacokinetic studies of proguanil, as available in literature, reported that the peak concentrations of proguanil and cycloguanil are achieved within 2–4 and 4–7 h post dose, respectively (Wattanagoon et al. 1987). This further supports the appropriateness of 8-h urinary analysis for proguanil and its metabolites.

From the results, 4.3% of the healthy control subjects were PMs and this was comparable to 4.8% PMs reported in a previous study involving healthy subjects (Bolaji et al. 2002). Table 3 shows the comparisons of the results from this study with previously reported values from different studies in Nigerian population. From Table 3, it could be seen that PM phenotype frequencies for healthy subjects from the different studies were similar, with values of 4.3% (this study), 4.3% (Iyun et al. 1990), 4.8% (Bolaji et al. 2002), and 4.7% (Babalola et al. 2010). These results were also comparable to the values reported for other Black African populations which ranged from 1.0% to 7.5% (Xie et al. 1999) and 4% in Zimbabweans (Masimirembwa et al. 1995). The 0.8% PMs in SCD patients, within the statistical limits, may signify significant difference, worthy of further investigation in this group. Lower number of PMs in SCD patients may reduce the incidence of subtherapeutic exposure to CG after proguanil administration. The other possibility is the increased risk of accumulation of CYP2C19 substrate drugs in PM phenotype group. This may be important because the majority of subjects are EMs, a factor that might have influenced the decision of current dosage guidelines.

Generally, however, these observations were not significantly different ($P > 0.05$) from what was observed from the genotyping study in this same population which reported 99.1% EMs and 0.9% PMs (Babalola et al. 2010). Also, the phenotypic PM frequency for the healthy controls in this study was not different from the previously reported genotypic frequency (phenotype vs. genotype; 4.3% vs. 4.7%, $P > 0.05$) (Babalola et al. 2010). This implies a good correlation between the phenotype frequencies obtained in this study and the previously reported genotype frequencies. For example, two volunteers with MRs of 18.18 and 25.76 among the non-SCD and one SCD patient with MR of 16.77 were genotyped as CYP2C19*2/*2. They were also phenotyped as PM showing complete concordance between genotyping and phenotyping of CYP2C19. The PM frequency in the total population phenotyped was 1.7% and 98.3% EMs, and this also correlated with 1.9% PMs and 98.1% EMs previously reported from the genotype study (Babalola et al. 2010).

Overall, the results revealed that the frequency of PMs was lower in SCD patients (phenotype vs. genotype: 0.8% vs. 0.9%) than in healthy volunteers (phenotype vs. genotype: 4.3% vs. 4.7%). This result suggests that SCD patients who use proguanil daily may have lesser risk of treatment failure when compared to healthy controls. However, further studies with more substrates of CYP2C19 may be required to investigate this.

Furthermore, the lower quantity of CG (Tables 1 and 2) excreted by the PMs may be clinically relevant when compared to the desired therapeutic range. A previous report has shown undetectable levels of CG in whole-blood samples of PMs following proguanil administration

(Watkins et al. 1990). For therapeutic success in preventing malaria-induced sickle-cell crisis, it may be important to tailor proguanil dosage based on the phenotype group of the patient. The findings from this study suggest that there may be variation in the occurrence of PM and EM phenotypes in SCD patients compared with other groups.

Conclusion

The prevalence of PM phenotype frequencies was evaluated in Nigerian SCD patients together with healthy controls for the first time. The phenotypic frequencies obtained in this study were found to correlate with the previously reported genotypic frequencies in this same population. Difference in metabolic disposition of proguanil in SCDs and non-SCDs was observed.

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Disclosure

The authors declare no competing financial interest in relation to this work.

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