GEOGRAPHIC VARIABILITY OF CYP 450 MEDIATED METABOLISM IN CYNOMOLGUS MACAQUES (MACACA FASCICULARIS)

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INTRODUCTION

The cynomolgus macaque is an important non-human primate model utilized to develop safety and efficacy profiles for new therapeutics entities. These profiles are closely linked to their metabolism and elimination.

Cynomolgus macaques are also used extensively in pharmacokinetic / pharmacotoxic studies. The rate and the pattern of biotransformation is critical to understanding the safety and efficacy profiles and how they relate to man. Blood levels of the parent compound and the metabolites resulting from biotransformation are directly related to their efficacy and toxicity.

Group sizes in a typical non-human primate study are relatively small and when combined with unknown genetic heterogeneity can produce results with a large variance around test parameters. Previous genetic studies of the cynomolgus macaque clearly indicate population differences depending upon their geographical source (Bonhomme et al. (2005, 2008)) and possible hybridization with other macaque species.

Genetic profiles provide clear evidence that significant genetic background differences may occur between animals within a study group (See Diagram 1). This could lead to variable responses with high standard deviations which will obscure potential important effects and prevent accurate assessments of effects.

The genetic similarity of the non-human primate model to humans, including CYP 450 isoforms, results in similar metabolic profiles for many xenobiotics. However, Cynomolgus monkeys used in research derive from several geographical sources with little information on their genetic diversity.

DNA analysis of targeted genetic loci can be used to confirm geographic sourcing and compare genetic backgrounds, including diversity, relatedness and heterozygocity. Genotypes are clearly different for different geographic sources of animals. Such genotypic differences may also lead to differences in the rate and pattern of metabolism in addition to the normal inter-animal variations which occur.

Non-human primates are considered as a good surrogate model for human CYP450 medication metabolism since they share similar CYPs as humans with a high percentage of amino acid sequence homology. Major human CYPs and homolgous Cynomolgus monkey CYPs (Cyno CYP, % amino acid sequence homology) are as follows: 1A2 (1A2, 93), 2C9 (2C43, 93), 2D6 (2D17, 93), 2E1 (2E1, 94), and 3A4 (3A8, 93). Probes for the primary human CYPs have been identified for most of the common CYP450's. We previously demonstrated that these probes could be used as a phenotyping assay to compare microsomes from the cynomolgus monkey to the human. A UHPLC/MS/MS CYP 450 phenotyping assay was used to characterize cynomolgus monkey metabolism using in-vitro microsomal incubations. The following probes with the corresponding CYP 450 in parenthesis were selected for comparisons between individual cynomolgus monkey microsomal evaluations: caffeine (1A2), dextromethorphan (2D6 and 3A4), testosterone (3A4), chlorzoxazone (2E1), and diclofenac (2C9) along with their respective metabolites paraxanthine, dextrorphan, methoxymorphinan, 6-beta hydroxytestosterone, 6-hydroxychlorzoxazone, and 4/5-hydroxydiclofenac.

Human CYP 450 phenotyping probes can be used to characterize metabolic variability between animals and provide more confidence in the data generated. The use of these probes to evaluate liver microsome patterns and rates of metabolism for cynomolgus monkeys from various geographic sources was evaluated.

METHODS

Human CYP 450 phenotyping probes were used to characterize metabolic variability and DNA analysis of targeted genetic loci was used to confirm geographic sourcing and compare genetic backgrounds.

Genotyping Analysis

DNA Extraction: DNA from all samples was extracted using the Qiagen Puregene Core Kit A for DNA extraction from liver samples.

Microsatellite Analysis: All samples were genotyped for 12 human microsatellite loci following conditions in Bonhomme et al. (2005, 2008) and include D1S548, D2S1326, D3S1768, D4S2365, D5S820, D7S2204, D8S1106, D10S1432, D14S306, D16S402, D17S791, and D18S536. PCR reactions used 1 X PCR buffer; 1.5 mM MgCl2; 0.2 mM dNTPs; 4 pmol of each primer; 0.5 U GoTaq Flexi DNA polymerase (Promega, Corp.) and 40 to 90 ng genomic DNA in a total volume of 12.5 µl. PCR products were separated using the Beckman/Coulter CEQ8000 capillary electrophoresis system. The DNA Analysis System Software, version 4.3.9 (Beckman Coulter, Inc.) was used to visualize and size all fragments. Forward primers were fluorescently labeled with WellRed™ Beckman/Coulter dyes. Genotype scoring errors were monitored by re-amplification of approximately 10 individuals at all loci and allele binning by plotting fragment size distribution per locus for each genotype by hand (Amos et al. 2007). Possible scoring errors and null alleles were also checked using Microchecker V.2.2.3 (van Oosterhout et al. 2004).

Statistical Analysis: MStools: Microsatellite Toolkit v 3.1 for PC Microsoft Excel (Park 2001) was used to calculate allele frequencies observed and expected heterozygosities and to convert data files for other applications. Observed and expected heterozygosities over all loci per population were also calculated using GENEPOP v 3.3 (Raymond and Rousset 1995). GENEPOP was used to test for linkage disequilibrium between all pairs of loci and significance was determined using Fisher's Exact test with 1000 permutations.

Structure Diagram: A burn-in of 50,000 iterations followed by 100,000 repetitions for analysis was used.

Phenotyping Analysis

Cynomolgus macaques were studied with a UHPLC/MS/MS CYP 450 phenotyping assay using in-vitro microsomal incubations. Liver microsomes were prepared from cynomolgus macaques attributed to several geographic sources, including Mauritius, Cambodia and China. The UHPLC separation was carried out on a Waters Acquity HSS T3 1.8mcm (2.1x100mm) column with an Acquity HSS T3 1.8mcM Vanguard Pre-Column. Flow rate was 0.8mL/min with an analysis time of 11 minutes utilizing a Dionex RSLC 3000 UHPLC. Compounds were eluted with a gradient of Acetonitrile/ THF/ Formic Acid (20/ 4/ 0.1) in methanol. Mass spectrometry detection was carried out with an AB Sciex 4000 Qtrap triple quadrupole mass spectrometer equipped with a Turbo V IonSpray as an LC/MS interface. Positive and negative ion mode. ESI mass spectra were acquired via multiple reaction monitoring in positive ion mode for the following probes (corresponding CYP 450 in parenthesis): caffeine (1A2), dextromethorphan (2D6 and 3A4), and testosterone (3A4) along with their metabolites paraxanthine, dextrorphan, hydroxymorphinan methoxymorphinan, and 6-beta hydroxytestosterone. Negative ion mode was used to identify probes (CYP 450): chlorzoxazone (2E1) and diclofenac (2C9) along with their metabolites 6-hydroxychlorzoxazone and 4-hydroxydiclofenac.

Incubations were performed using a combined substrate solution, NADPH regeneration solution, potassium phosphate buffer q.s. to 300mcl. Three substrate mixture concentrations were used to obtain near km concentrations for each substrate probe. Concentrations of substrate probes in incubations were caffeine (150mcM), dextromethorphan (10, 40 mcM), chlorzoxazone (60mcM), testosterone (69.3mcM), and diclofenac (6mcM). Incubation time was 10 minutes at 37oC. Samples were centrifuged, the supernatant eluted with acetonitrile through an Ostro plate, reconstituted w/ 10/90% (MEOH/water), and loaded onto the HPLC MS/MS.

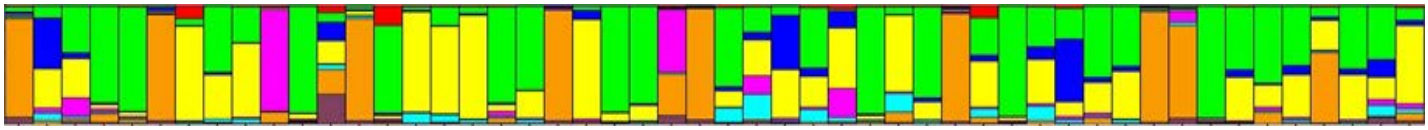
RESULTS

A structure diagram was used to compare the specimens (Diagram 1). Mauritian animals were expectedly homogeneous in regards to genetics. Mauritius homogeneity was also strongly observed for metabolic phenotype. In fact, an animal initially identified as a female Mauritian displayed differential metabolic phenotype compared to the other Mauritian females tested. After genetic determination the animal was found to be Indonesian and not Mauritian.

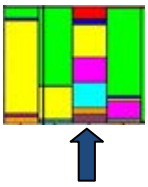
DIAGRAM 1

Chinese

Reference

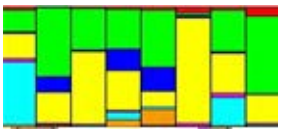


Study Animals

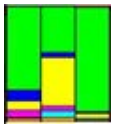


Cambodian

Reference

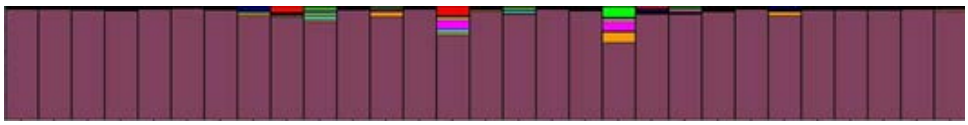


Study Animals

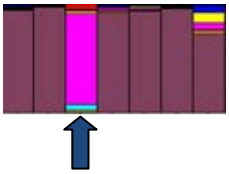


Mauritian

Reference



Study Animals



Structure Diagram: STRUCTURE version 2.2 (Pritchard et al. 2000) with a burn-in of 50,000 iterations followed by 100,000 repetitions for analysis was used to assign individuals to their geographic origin.

Population Statistics - Study

Population	Sample size	Loci typed	Unbiased Hz	Unbiased Hz SD	Obs Hz	Obs Hz SD	No Alleles	No Alleles SD
Chinese	3	12	0.8500	0.0234	0.8333	0.0621	4.17	0.83
Cambodian	3	12	0.7167	0.0813	0.7778	0.0693	3.75	1.54
Mauritius	7	12	0.6493	0.0589	0.5714	0.0540	4.25	1.54

Population Statistics - Reference

Population	Sample size	Loci typed	Unbiased Hz	Unbiased Hz SD	Obs Hz	Obs Hz SD	No Alleles	No Alleles SD
Chinese	54	12	0.8189	0.0259	0.7500	0.0170	12.17	3.51
Cambodian	11	12	0.7821	0.0487	0.7803	0.0360	7.75	2.80
Mauritius	68	12	0.6701	0.0432	0.6078	0.0171	6.25	1.66

Table 1 provides the means and the standard deviations for the groups evaluated.

Table 1. Mean Metabolic Phenotype Values of Cynomolgus Macaques from Different Geographic Sources

CYP 450	Probe Metabolite	Mauritian-Males n=3* Mean Rate (pmol/min/mg)		Mauritian-Females n=3 Mean Rate (pmol/min/mg)		Chinese-Females n=4 Mean Rate (pmol/min/mg)		Cambodian-Females n=4 Mean Rate (pmol/min/mg)
1A2	Paraxanthine	1.0		1.1		1.3		1.4
2C8/9	4/5-OH Diclofenac	655.0		46.0		343.6		421.7
2D6	Dextrorphan	116.4		27.3		48.1		40.4
2E1	6-OH Chlorzoxazone	3794.9		821.3		1829.4		2005.5
3A4	6-OH Testosterone	110.4		20.4		52.2		58.1
3A4	Methoxymorphinan	52.6		6.6		20.7		25.8
CYP 450	**Probe Metabolite**	**%SD**		**%SD**		**%SD**		**%SD**
1A2	Paraxanthine	2.4		27.4		17.7		22.6
2C8/9	4/5-OH Diclofenac	16.9		18.5		75.6		27.3
2D6	Dextrorphan	6.9		51.1		54.3		41.9
2E1	6-OH Chlorzoxazone	12.8		41.5		68.9		25.1
3A4	6-OH Testosterone	11.2		34.7		72.6		22.5
3A4	Methoxymorphinan	10.5		50.0		65.8		27.5

*** One Mauritian male was withdrawn from calculations due to genetic determination of Indonesian origin. (See blue arrow - Diagram 1 Mauritian Study Animals) CYP 450 metabolism was markedly different for this animal. CYP Isoform metabolism rates were (pmol/min/mg): 1A2 (0.9), 2C8/9 (8.1), 2D6 (16.4), 2E1 (110.9), 3A4 (3.7), 3A4 (1.7)**

RESULTS

Table 2 shows p-values of mean comparisons of Mauritian males and females versus Chinese and Cambodian sourced cynomolgus. As expected, males show differences in metabolism versus females for nearly every CYP 450 isoform. However, Mauritian cynomolgus females appeared to have lower overall variability in metabolic phenotype and showed significantly different metabolic phenotype for several CYP450 isoforms compared to Chinese and Cambodian sourced cynomolgus. One Chinese female (see in diagram 1) appeared as an outlier and when removed from the evaluation, resulted in changes in the statistical significance between evaluated groups for several probes due to a reduced standard deviation.

Table 2. Metabolic Phenotype Comparisons of Mauritian Cynomolgus versus Chinese and Cambodian Derived Cynomolgus

Mauritian - Males						
	1A2	2C8/9	2D6	2E1	3A4	3A4
	Paraxanthine	4-OH Diclofenac	Dextrorphan	6-OH Chlorzoxazone	6-OH Testosterone	Methoxymorphinan
Mauritian-Females n=3	0.652	**0.011**	**0.002**	**0.002**	**0.001**	**0.001**
Chinese-Females n=4	*0.060*	*0.094*	**0.010**	**0.046**	**0.048**	**0.012**
Cambodian-Females n=4	*0.087*	**0.047**	**0.001**	**0.006**	**0.004**	**0.003**
Mauritian - Females						
	1A2	2C8/9	2D6	2E1	3A4	3A4
	Paraxanthine	4-OH Diclofenac	Dextrorphan	6-OH Chlorzoxazone	6-OH Testosterone	Methoxymorphinan
Chinese-Females n=4	0.284	0.106	0.236	0.210	0.191	0.129
Chinese-Females n=3*	0.173	*0.051*	**0.036**	**0.016**	*0.068*	**0.028**
Cambodian-Females n=4	0.254	**0.007**	0.314	**0.014**	**0.005**	**0.007**

Reported values are p-values of mean comparisons of header cynomolgus (ex. Mauritius males) versus left hand column cynomolgus (ex. Chinese females)

*An outlier Chinese female was removed (See blue arrow Diagram 1 Chinese Study Animals) showing significant differences between female Mauritius cynomolgus and female Chinese cynomolgus which were obscured by high variability of Chinese female cynomolgus

BOLD = p values which are less than 0.05, two tailed t-test homogeneity of variance not assumed

Italics = borderline significant (p<0.1)

CONCLUSIONS

Cynomolgus macaques used in pharmaceutical testing may originate from a variety of geographic locations. Markers of genetic variability were developed that allow identification of geographic origin. Geographical variations in metabolic phenotype were explored using these markers as a guide. Significant differences in metabolic phenotype were observed based on geographic origin. As expected, Mauritius sourced populations display less heterogeneous metabolic phenotype and differ significantly from cynomolgus procured from Chinese and Cambodia sources. Another expected finding was sexual dimorphism in metabolism between male and female cynomolgus from the same as well as different geographic origins.

Comparisons to human pooled human microsomes (data not shown) showed enhanced metabolism of pooled cynomolgus macaque microsomes versus humans except for diclofenac metabolism (CYP2C8/9) (McGraw et al. 2011). However, inter-species variability in cynomolgus macaque phenotype based on geographical procurement has significant implications on cynomolgus to human extrapolation. Intra-species variability of cynomolgus macaques due to differences in phenotypic metabolism of geographical variants may obscure cynomolgus to human extrapolations.

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