

Fatal Methadone Toxicity: Potential Role of CYP3A4 Genetic Polymorphism

Lauren L. Richards-Waugh^{1*}, Donald A. Primerano², Yulia Dementieva³, James C. Kraner⁴ and Gary O. Rankin⁵

¹Forensic Science Department, Marshall University, Huntington, WV, USA, ²Department of Biochemistry and Microbiology, Marshall University, Huntington, WV, USA, ³Department of Mathematics, Emmanuel College, Boston, MA, USA, ⁴Office of the Chief Medical Examiner, Charleston, WV, USA, and ⁵Department of Pharmacology, Physiology and Toxicology, Marshall University, Huntington, WV, USA

*Author to whom correspondence should be addressed. Email: richards18@marshall.edu

Methadone is difficult to administer as a therapeutic agent because of a wide range of interindividual pharmacokinetics, likely due to genetic variability of the CYP450 enzymes responsible for metabolism to its principal metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). CYP3A4 is one of the primary CYP450 isoforms responsible for the metabolism of methadone to EDDP in humans. The purpose of this study was to evaluate the role of CYP3A4 genetic polymorphisms in accidental methadone fatalities. A study cohort consisting of 136 methadone-only and 92 combined methadone/benzodiazepine fatalities was selected from cases investigated at the West Virginia and Kentucky Offices of the Chief Medical Examiner. Seven single nucleotide polymorphisms (SNPs) were genotyped within the CYP3A4 gene. Observed allelic and genotypic frequencies were compared with expected frequencies obtained from The National Center for Biotechnology Information dbSNP database. SNPs rs2242480 and rs2740574 demonstrated an apparent enrichment within the methadone-only overdose fatalities compared with the control group and the general population. This enrichment was not apparent in the methadone/benzodiazepine cases for these two SNPs. Our findings indicate that there may be two or more SNPs on the CYP3A4 gene that cause or contribute to the methadone poor metabolizer phenotype.

Introduction

Methadone has become more widely prescribed for pain control in the USA since 1997 when new clinical guidelines for pain management were introduced (1). Indications for prescribing methadone now include pain relief (for severe, malignant and postoperative pain), detoxification of narcotic addiction and temporary maintenance treatment of narcotic addiction (2, 3). Methadone is an efficacious method of treatment for heroin addiction because of high bioavailability, long elimination half-life, lack of detrimental behavior modification and the availability of the antagonist naloxone as an antidote (4, 5). However, selection of the appropriate methadone dose is difficult because a given dose results in a wide range of interindividual pharmacokinetics, increasing the likelihood of an adverse drug reaction (ADR) (6).

Methadone is a synthetic μ -opioid receptor (MOR) agonist (3) administered as a racemic mixture of (*R*)- and (*S*)-methadone (7). Activation of the MOR leads to analgesia, respiratory depression and physiological dependence (2). The two enantiomers of methadone exhibit different pharmacodynamic properties. The (*R*)-enantiomer is responsible for most, if not all, of the opioid effect (7, 8) with a 10-fold higher affinity for the MOR than (*S*)-methadone (9). A fixed oral dose of methadone can produce a range of blood concentrations, demonstrating large variations

in response to treatment (10). The oral bioavailability of methadone ranges between 41 and 95% (11). The time required to reach maximum plasma concentration varies from 0.5 to 6.0 h (11, 12) with detection in the blood possible 15–45 min after ingestion (10). Duration of analgesia ranges from 4 to 6 h following a single oral dose of methadone (6). Therefore, the relationship between dose, plasma concentrations and effects are not clearly defined (13, 14).

Much of the described interindividual variability in response to methadone can be explained by polymorphisms in a variety of proteins, including the CYP450 enzymes responsible for methadone metabolism (11). The principal metabolite of methadone is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is formed during first-pass metabolism through an *N*-demethylation reaction (9, 11, 12, 15, 16) followed by spontaneous cyclization (17). CYP involvement in methadone metabolism is stereoselective. CYP3A4 is thought to metabolize both enantiomers (9, 18), but is the primary CYP isozyme mediating *R*-methadone metabolism. A 60–85% decrease in EDDP formation following chemical or monoclonal antibody inhibition of the CYP3A4 was observed in human liver microsomes (19). CYP2D6 also metabolizes the *R*-enantiomer (20), but the contribution by this isozyme is minor (19). The (*S*)-methadone enantiomer is mostly metabolized by CYP2B6 (19, 21, 22). Drugs with agonist activity at the μ - or δ -opioid receptors can cause respiratory depression (23), which is the most common mechanism by which methadone causes death (24). Thus, CYP variants could cause or contribute to the poor methadone metabolizer phenotype leading to an increased likelihood for fatal methadone toxicity.

The role of CYP2D6 polymorphisms in both fatal and nonfatal methadone overdoses has been studied in mixed drug toxicity and/or small sample cohorts. For example, Shiran *et al.* (25) examined 52 patients undergoing methadone maintenance treatment with 40 (77%) of the patients taking additional medications. The authors determined that CYP2D6*4 was associated with lower CYP2D6 activity, but this analysis was based on methadone dose rather than plasma concentration with only 34 of those patients actually genotyped for CYP2D6 (25). Wong *et al.* (6) examined 23 deaths in which methadone was contributory and observed a nonsignificant increase in poor metabolizers compared with the general population. Only one fatality included in the study population had methadone as the sole drug detected (6).

CYP3A4 plays a role in the metabolism of 40–60% of all drugs ingested (26, 27). The CYP3A enzymes are the most abundant of the CYP450 isozymes (28), comprising ~40% of the hepatic CYP450 content (29, 30). CYP3A4 activity varies up to 40-fold

and is affected by a multitude of factors including health status, environment (smoking, diet and co-medication), hormonal profile and genetics (26, 30, 31). Genetic variability is hypothesized to account for anywhere from 60 to 90% of the interindividual differences in hepatic CYP3A activity (32–34). Studies have indicated that CYP3A5 plays a very limited role in methadone pharmacokinetics (35, 36). Although several reports have hypothesized that CYP3A4 polymorphism may play a role in interindividual variability of methadone disposition and clinical effects, there are few studies addressing the role of CYP3A4 polymorphism in methadone toxicity (17, 30). Single nucleotide polymorphism (SNP) genotyping of the CYP3A4 gene could help predict the drug-metabolizing phenotype of this enzyme (33). This study was designed to assist in bridging the gap in our understanding of the potential role of CYP3A4 variants in fatal methadone intoxications by testing for enrichment of CYP3A4 genotypes in these cases. Because intronic variants can perturb splicing and/or binding by microRNAs and exonic variants can affect enzymatic activity, we tested associations between CYP3A4 intronic and exonic SNPs and methadone toxicity.

Materials and methods

Chemicals and enzymes

TaqMan Universal PCR Master Mix No AmpErase® UNG, MicroAmp Optical Adhesive Film, MicroAmp Optical 96-well Reaction Plates and TaqMan SNP Genotyping Assays were purchased from Life Technologies™ (Foster City, CA). QiaAmp DNA Micro extraction kits were purchased from Qiagen (Valencia, CA). Absolute ethanol was purchased from Aaper (Shelbyville, KY). Whatman Protein Saver Cards were purchased from Fisher Scientific (Pittsburgh, PA). Diethylpyrocarbonate (DEPC)-treated water that was free of endodeoxyribonucleases, exodeoxyribonucleases, ribonucleases and phosphatases was purchased from Ambion (Austin, TX). All other reagents were purchased from Sigma and were the highest grade available (St Louis, MO).

Preparation of DNA samples

Genomic DNA was extracted from blood stain cards prepared from blood collected during autopsy at the West Virginia (WV) and Kentucky (KY) Offices of the Chief Medical Examiner (OCMEs) using the QiaAmp DNA Micro extraction kit following the manufacturer's protocol for dried blood spots. The protocol was modified to increase DNA yield by performing the extractions in triplicate. The three replicates were concentrated onto one MinElute column to improve DNA yield. Extracted DNA was quantitated using a NanoDrop 1000 instrument and diluted to a concentration of 1.78 ng/μL with DEPC-treated water (20 ng/11.25 μL) with a final volume of 200 μL for use in the SNP genotyping assays. Extracted DNA and 1.78 ng/μL stock solutions were stored at 4°C.

SNP selection

Seven different SNPs on the CYP3A4 gene were genotyped. Four SNPs (rs2246709, rs3735451, rs4646437 and rs2242480) were selected to maximize coverage of the CYP3A4 gene; all of

these SNPs were intronic and had a minor allele frequency (MAF) of at least 5%. Two SNPs (rs4987161 and rs4986910) were selected because they were exonic and considered polymorphic (MAF = 2%) in the Caucasian population. The seventh CYP3A4 SNP (rs2740574, CYP3A4*1B) is located in the 5'UTR region (–392A>G) and is associated with elevated trough levels of methadone in heterozygotes (35).

SNP genotyping

The following Life Technologies™ TaqMan SNP Genotyping kits were used to interrogate CYP3A4 genotypes: (1) C__1845287_10 (rs2246709), (2) C__25473812_10 (rs3735451), (3) C__32306227_10 (rs4646437), (4) C__26201900_30 (rs2242480), (5) C__27859822_10 (rs4987161) and (6) C__27535825_20 (rs4986910); a custom assay was developed by ABI for rs2740574. Real-time polymerase chain reaction (RT-PCR) and allelic discrimination analyses were performed according to the manufacturer protocols for a 25-μL reaction volume. The temperature program for the RT-PCR was 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 90 s. SNP genotypes were determined using the manual allele call option in the ABI7000 Sequence Detection software.

Study design and methods of analysis

All study cases were selected from fatalities investigated by the WV and KY OCMEs. Toxicology reports generated by the WV and KY OCMEs between 2003 and 2009 in which the presence of methadone was confirmed were selected for review. We selected cases in which methadone or methadone and benzodiazepines (diazepam and/or alprazolam) were the only drug(s) detected at time of death. An initial 245 cases (161 from WV and 84 from KY) were selected based on the toxicity criteria with 17 excluded due to a non-overdose cause of death (i.e., car accident, gunshot wound, etc.) or a non-Caucasian ethnicity. The remaining 228 cases were then divided into three groups: Group A (cause of death attributed to methadone intoxication), Group B (cause of death ascribed to a combined methadone and benzodiazepine toxicity) and Group C (144 cases) consisted of a combination of the cases included in Groups A (84 cases) and B (60 cases).

A control group (Group D), consisting of 297 cases, was selected from WV OCME fatalities wherein amphetamines, barbiturates, benzodiazepines, buprenorphine, cocaine, methadone, propoxyphene, opiates, oxycodone and tricyclic antidepressants were not detected by immunoassay screening. Cases that screened positive for cannabinoids and/or ethanol were not excluded in this group given the lack of involvement of CYP3A4 in their metabolism. These substances are also not inducers or inhibitors of CYP3A4. Cases in which race was non-Caucasian were excluded due to racial and ethnic diversities in CYP450 allele frequencies (37).

SNP allelic and genotypic frequencies were determined for each group. Expected genotypes were derived from MAFs published in the National Center for Biotechnology Information (NCBI) dbSNP database for the Caucasian population. The observed genotypic frequencies were then compared with the expected genotypic frequencies using a Chi-squared analysis to determine if there were significant differences between this

study and general populations. The genotypic frequencies for each SNP were analyzed for Hardy–Weinberg equilibrium (HWE) and actual allelic frequencies. Expected genotypic frequencies were compared with the observed genotypic frequencies using Chi-squared analysis with 1 degree of freedom and a desired *P*-value of 0.05. Comparisons between observed and expected genotypic frequencies were not useful for the exonic SNPs (rs4987161 and rs4986910) and, therefore, were not included in the comparisons between genotypic frequencies.

Statistical calculations including mean, standard deviation, SEM, one-way ANOVA and Kruskal–Wallis (K–W) Rank test were performed for the methadone/EDDP ratios and/or methadone concentrations. K–W analyses were performed to determine if there were any significant differences between the methadone concentrations or methadone/EDDP ratios between the three genotypes. Calculations involving samples from WV were performed on methadone concentrations and methadone/EDDP ratios (the KY OCME did not determine EDDP concentrations). K–W analyses were not performed on groups without the minimum number of cases (2) for each SNP. A *P*-value of <0.05 for the K–W analysis was considered statistically significant.

Results

The study cohort consisted of 228 fatalities investigated by the WV and KY OCMEs in which the cause of death was at least partially ascribed to methadone intoxication. Of the 228 cases analyzed, 133 involved methadone-only overdoses while the remaining 95 fatalities were deemed to have succumbed to combined methadone/benzodiazepine (diazepam and/or alprazolam) overdoses. The demographic information pertaining to the WV cases is outlined in Table I. The number of cases from KY is also included in Table I; however, age and gender data could not be obtained for the cases from KY because the lab was in the process of permanent closure during the time of sample collection. MAFs and genotype frequencies for SNPs rs2246709, rs3735451, rs4646437, rs2242480 and rs2740574 were calculated for Groups A, B and C using the data obtained from the allelic discrimination analysis and are provided in Table II.

MAFs in Groups A, B and C

Expected MAFs were obtained from the NCBI dbSNP database for the Caucasian population. An observed MAF greater than expected could be an indication that a SNP is enriched within the study cohort, which was the case for SNPs rs2242480 (CYP3A4_20239G>A) and rs2740574 (–392A>G) in Groups

A and C. MAFs less than expected were also observed in Group B for these two SNPs. SNPs rs3735451, rs4646437, rs2242480 and rs2740574 had the greatest MAFs in Group A and the lowest in Group B. MAFs for SNPs rs3735451 and rs4646437 were lower than the expected in all three groups. The expected MAF for SNP rs2740574 was 2.5%, resulting in an expected genotypic frequency of zero for individuals homozygous for the minor allele. The only cases that were homozygous for the minor allele were in Group A.

Comparison between observed and expected genotypic frequencies

A Chi-squared goodness-of-fit test was performed to determine if there were significant differences between the genotypic frequencies in the study cohorts compared with the general population. *P*-values ≤0.05 were considered to be statistically significant. The results of the Chi-squared goodness-of-fit tests are outlined in Table II. The genotypic frequencies for SNP rs2246709 were significantly different than those expected (*P* < 0.05 for Group B and *P* < 0.001 for Groups A and C). For SNP rs3735451, there was a difference between the observed and expected genotypic frequencies in Group B (*P* = 0.01). The significance is likely due to the increased number of homozygotes for the major allele and the decreased number of heterozygotes and homozygotes for the minor allele. Genotype frequencies for SNP rs4646437 were significantly different between observed and expected within Groups B and C but not Group A. SNP rs2242480 Group A and SNP rs2740574 Groups A and C also showed significant differences between the observed and expected genotypic frequencies. For SNP rs4646437, Group C was separated by gender and the resulting genotypic frequencies were compared with expected frequencies. Significant differences between the observed and expected genotypic frequencies were retained in the male but not the female study group (data not shown). Groups A and B contained too few females for comparison.

Hardy–Weinberg equilibrium

A Chi-squared goodness of fit test with 2 degrees of freedom was used to determine if the frequency distribution for each SNP within the study population was in HWE. A sample group in this study was in HWE with a Chi-squared value of <5.99 with two degrees of freedom, which resulted in a *P*-value >0.05. Because the study groups were not selected at random, lack of HWE could be an indication that a SNP may have a role in accidental methadone fatality. SNP rs4646437 was within HWE for Group A, but not for Groups B and C. Absence of HWE was also observed for SNP rs2740574 in Groups A and C, but this was not the case for Group B. Genotype frequencies in Groups A, B and C for SNPs rs2246709, rs3735451 and rs2242480 were all in HWE.

Control group comparisons

To determine if there were genotype frequency differences between the controls in Group D and the dbSNP-based frequencies or between Group D and Groups A–C, three SNPs were chosen for further analysis. SNPs rs4646437 and rs2740574 SNPs were genotyped within Group D, because these SNPs were not within

Table I
Breakdown of Study Groups and WV Case Demographics

	Group A	Group B	Group C	Group D
WV cases	84	60	144	258
KY cases	52	32	84	0
Total cases	136	92	228	258
Age range (years)	1.75–58	20–64	1.75–64	2–88
Mean age	34.3 ± 11.3	36.6 ± 11.0	34.7 ± 11.1	45.2 ± 18.6
Males (%)	66 (75)	47 (78)	109 (76)	196 (76)
Females (%)	22 (25)	13 (22)	35 (24)	62 (24)

Gender and age were not available for the KY cases.

Table II

MAFs, Observed and Expected Genotypic Frequencies for (A) rs2246709, (B) rs3735451, (C) rs4646437, (D) rs2242480 (E) rs2740574 and (F) Group D data^a

(A) rs2246709 (MAF = 35%)	Group A	Group B	Group C
Calculated MAF	24.3%	25.6%	24.8%
Homozygous major allele			
Expected	57.5	38.0	95.5
Observed	78	49	127
Mean methadone (mg/L)	0.53 ± 0.47	0.54 ± 0.49	0.55 ± 0.48
Heterozygotes			
Expected	61.9	41.0	102.8
Observed	50	36	86
Mean methadone (mg/L)	0.58 ± 0.05	0.53 ± 0.39	0.55 ± 0.46
Homozygous minor allele			
Expected	16.7	11.0	27.7
Observed	8	5	13
Mean methadone (mg/L)	0.80 ± 1.09	0.86 ± 0.73	0.82 ± 0.93
HWE			
Chi ²	1.2E-05	0.24	0.10
P-value	1.00	0.89	0.95
Observed versus expected	8.6E-04	0.03	2.8E-05
(B) rs3735451 (MAF = 15%)	Group A	Group B	Group C
Calculated MAF	14.7%	7.6%	11.8%
Homozygous major allele			
Expected	98.3	66.5	164.7
Observed	100	79	179
Mean methadone (mg/L)	0.57 ± 0.56	0.56 ± 0.51	0.57 ± 0.54
Heterozygotes			
Expected	34.7	23.5	58.1
Observed	32	12	44
Mean methadone (mg/L)	0.63 ± 0.44	0.42 ± 0.22	0.58 ± 0.40
Homozygous minor allele			
Expected	3.1	2.1	5.1
Observed	4	1	5
Mean methadone (mg/L)	0.59 ± 0.55	0.50	0.57 ± 0.48
HWE			
Chi ²	0.52	0.48	1.31
P-value	0.77	0.79	0.52
Observed versus expected	0.77	0.01	0.10
(C) rs4646437 (MAF = 13%)	Group A	Group B	Group C
Calculated MAF	11.0%	9.8%	10.5%
Homozygous major allele			
Expected	102.9	69.6	172.6
Observed	110	78	188
Mean methadone (mg/L)	0.62 ± 0.55	0.58 ± 0.51	0.60 ± 0.54
Heterozygotes			
Expected	30.8	20.8	51.6
Observed	22	10	32
Mean methadone (mg/L)	0.48 ± 0.45	0.32 ± 0.19	0.43 ± 0.39
Homozygous minor allele			
Expected	2.3	1.6	3.9
Observed	4	4	8
Mean methadone (mg/L)	0.49 ± 0.47	0.36 ± 0.21	0.43 ± 0.35
HWE			
Chi ²	4.20	13.58	14.81
P-value	0.12	1.1E-03	6.1E-04
Observed versus expected	0.12	5.3E-03	1.3E-03
(D) rs2242480 (MAF = 6%)	Group A	Group B	Group C
Calculated MAF	9.6%	5.6%	8.0%
Homozygous major allele			
Expected	120.2	79.5	198.8
Observed	112	80	192
Mean methadone (mg/L)	0.59 ± 0.55	0.57 ± 0.51	0.58 ± 0.53
Heterozygotes			
Expected	15.3	10.2	25.4
Observed	22	10	31
Mean methadone (mg/L)	0.59 ± 0.44	0.36 ± 0.17	0.52 ± 0.39
Homozygous minor allele			
Expected	0.5	0.3	0.8
Observed	2	0	2
Mean methadone (mg/L)	0.66		0.66
HWE			
Chi ²	0.56	0.31	0.35
P-value	0.75	0.86	0.84
Observed versus expected	0.02	0.85	0.20

Table II Continued

(E) rs2740574 (MAF = 2.5%)	Group A	Group B	Group C
Calculated MAF	3.7%	1.6%	2.9%
Homozygous major allele			
Expected	129.3	87.5	216.7
Observed	129	89	218
Mean methadone (mg/L)	0.59 ± 0.54	0.55 ± 0.49	0.58 ± 0.52
Heterozygotes			
Expected	6.6	4.5	11.1
Observed	4	3	7
Mean methadone (mg/L)	0.48 ± 0.34	0.31 ± 0.17	0.41 ± 0.28
Homozygous minor allele			
Expected	0.1	0.1	0.1
Observed	3	0	3
Mean methadone (mg/L)	0.56 ± 0.50		0.56 ± 0.50
HWE			
Chi ²	46.50	0.03	45.30
P-value	8.0E-11	0.99	1.5E-10
Observed versus expected	1.2E-22	0.75	1.7E-13
(F) Data for Group D db SNP id	rs4646437	rs2242480	rs2740574
Calculated MAF	10.9%	9.8%	3.7%
Homozygous major allele			
Expected	193.7	215.6	242.4
Observed	204	199	237
Heterozygotes			
Expected	57.9	27.5	12.4
Observed	48	42	17
Homozygous minor allele			
Expected	4.3	0.8	0.2
Observed	4	3	1
HWE			
Chi ²	0.36	0.21	1.27
P-value	0.83	0.90	0.53
Observed versus expected	0.32	9.0E-04	0.04

^aThe expected MAFs are in parentheses beside the db SNP id. The results of Chi-squared analyses for HWE and comparisons between observed and expected genotypic frequencies are also included.

HWE within these groups. SNP rs2242480 was genotyped within Group D because this SNP had significant findings within the cases due to methadone-only intoxication. The results of the Group D versus dbSNP comparisons are shown in Table F of Table II. The observed Group D genotypic frequencies for the rs4646437 SNP were not statistically different than the expected genotypic frequencies. However, the observed Group D genotypic frequencies for rs2242480 and rs2740574 were significantly different than expected frequencies ($P = 2.53 \times 10^{-5}$ and $P = 0.03$, respectively). In Group D, all three SNPs were within HWE with Chi-squared values < 5.99 and $P > 0.05$.

Genotype frequencies for Group D were then compared with Groups A–C using a Chi-squared goodness-of-fit test with 2 degrees of freedom. In comparisons for SNP rs4646437, Group D only showed one significant difference among Groups A, B and C ($P = 0.37$, 0.04 and 0.07 , respectively). There was a significant difference ($P < 0.05$) between Group A and Group D genotypic frequencies for SNP rs2740574, but not for Groups B and C ($P = 0.34$ and 0.07 , respectively). For SNP rs2242480, there were no significant differences between Group A ($P = 0.74$), Group B ($P = 0.12$) and Group C ($P = 0.31$).

To examine the hypothesis that methadone concentrations or methadone/EDDP ratios were correlated to CYP3A4 genotypes, we compared mean blood concentrations between SNP variants. For the vast majority of cases, blood was obtained from the subclavian vein or femoral vein. Heart blood was tested for several cases for which peripheral blood was not available. Methadone/EDDP

ratios were calculated within the WV cases. The KY OCME did not determine EDDP concentrations as part of their toxicology testing. SNP rs2246709 displayed a pattern of increasing mean methadone concentration among genotypes in Groups A: 0.54 ± 0.47 mg/L (major allele homozygotes), 0.58 ± 0.05 mg/L (heterozygotes) and 0.80 ± 1.09 mg/L (minor allele homozygotes). None of the SNPs showed a pattern of increasing methadone/EDDP ratios for any of the study groups. There were no SNPs that related significant differences in either methadone concentrations or methadone/EDDP ratios. Medical history and prescription history are of significance in evaluating methadone-related fatalities in relation to potential P450 polymorphisms. However, with a total of 228 deaths in the study, inclusion of medical and prescription history is beyond the scope of this report.

Discussion

The interindividual variability in methadone pharmacokinetics is predominantly caused by genetic polymorphism and co-medication (11). The effects of co-medication were reduced in this study by selection of individuals who died due to methadone toxicity with no other drugs, other than diazepam and/or alprazolam in their system at the time of death. SNPs rs2242480 and rs2740574 have an enrichment in Groups A and C, but not Group B with MAFs greater in the study population than the general population. Co-administration of benzodiazepines with methadone resulted in a loss of significance for the genotyping results for two SNPs that may be associated with the poor methadone metabolizer phenotype. The potential for drug interactions exists due to overall abundance and the broad substrate specificity of CYP3A4. CYP3A4 metabolizes numerous drug classes including antidepressants, antipsychotics, calcium channel blockers antiarrhythmics, antivirals and others. Thus, substrate competition for the isoform is recognized. Many drugs act as CYP3A4 inhibitors and a few also induce CYP3A4 expression. The loss of concordance between Group A and Group B genotype frequencies for these SNPs suggests the need for assessing cohorts with single drug overdoses when determining the potential role of SNPs in unexpected overdose and that a pharmacological interaction between the methadone and benzodiazepine(s) contributed to overdose rather than the sole effect of CYP3A4 polymorphisms.

SNP rs4646437 was not within HWE for Groups B and C. Groups A and C were not in HWE for SNP rs2740574. These two SNPs were within HWE in Group D. The deviation from HWE in Groups A–C could stem from nonrandom selection, which in this case could be selection of groups susceptible to methadone or methadone/benzodiazepine intoxication. This explanation is consistent with the hypothesis that Groups A–C are enriched for the minor allele of the genotyped SNP(s) or those in linkage disequilibrium with the SNPs tested and these alleles confer susceptibility to methadone overdose. SNP rs2740574 was also one of the two SNPs with an increased MAF in Groups A and C compared with the expected. While SNP rs2242480 also had an apparent enrichment in Groups A and C, it was in HWE for all Groups (A, B, C and D).

Gender could also contribute to the effect of certain SNPs. The lack of association between fatal methadone toxicity in females and SNP rs4646437 genotype in this study can possibly be attributed to a sex-dependent effect. Schirmer *et al.* (38) have

demonstrated that CYP3A4 expression and activity are increased in females carrying the minor allele (T) compared with males. An increase in CYP3A4 activity would lead to increased metabolism of methadone to inactive metabolites, which would explain the loss of association between SNP rs4646437 and fatal methadone intoxication in the female population.

For SNP rs2740574, Group B was the only group in HWE and the only group that did not have a significant difference between observed and expected genotypic frequencies. Chi-squared analysis suggests that the SNP was enriched in Groups A and C. However, this SNP was also enriched within Group D. This could be due to the fact that Groups A and C were comprised of fewer cases ($n = 133$ and 228 , respectively) as compared with Group D ($n = 269$). Alternatively, this SNP may be enriched within the general WV population. The MAF and genotypic frequencies indicate an enrichment of this SNP within individuals who succumbed to methadone toxicity, which suggests that this SNP may play a role in accidental fatal methadone toxicity.

The significant difference between the observed and expected genotypic frequencies for SNPs rs2242480 and rs2740574 in Group D was an unexpected finding. Group D consisted of 269 cases, which is much larger than similar studies examining the role of genetic polymorphism in methadone fatality; however, the results of this study were compared with a large population database. A sampling error may have occurred in which Group D was inadvertently selected to be enriched for the SNPs that were studied. The selected control group may not have been ideal because the cases selected were fatalities involving a wide range of circumstances (homicide, accidental deaths not due to drug toxicity, natural and undetermined). There may be other underlying pathologies that were not apparent that may affect their ability to metabolize methadone.

Two homozygotes for SNP rs4986910 were found in our study population of 226 individuals (data not shown). Exonic SNPs rs4987161 and rs4986910 both had low MAFs of 2% in the Caucasian population, proving an association with a given phenotype, such as poor methadone metabolizer, difficult (39). All of the coding SNPs for CYP3A4 have MAFs $<5\%$ and no homozygotes have been reported in the literature (39–44). A larger study population may reveal even more homozygotes for the variant allele; however, determination of two homozygotes within this study cohort was significant.

The CYP3A4 gene has 18 known coding SNPs most of which are concentrated in exons 5, 6, 7, 11 and 12 (45). These SNPs are known to cause nonsynonymous changes in the amino acid sequence (31). The coding SNPs analyzed in this study, rs4987161 (CYP3A4*17) and rs4986910 (CYP3A4*3), were located in exons 7 and 12, respectively. Twenty different CYP3A4 variant proteins have been identified, with some (CYP3A4*6, *17 and *20) purportedly resulting in proteins with decreased enzyme activity (45). The CYP3A4*6 polymorphism causes a frameshift mutation resulting in a truncated protein due to a premature stop codon in exon 9. The frequencies of these variants are small enough that they are likely not causative for the interindividual variability in CYP3A4 activity, suggesting that the associated SNPs must be located elsewhere in the CYP3A4 gene (46).

The CYP3A4 gene is particularly difficult to genotype because most of the exonic SNPs have $<5\%$ MAF (47), which would

require a larger study group than was feasible for this investigation. CYP3A4*1B (rs2740574) causes decreased binding of nuclear proteins to the proximal promoter of CYP3A4 and is associated with a much lower enzymatic activity compared with CYP3A4*1A (48). It has also been associated with elevated trough concentrations of methadone in heterozygotes (36) and is polymorphic within the Caucasian population with a MAF of 2.5%. There was an apparent enrichment of this SNP within this study cohort as indicated by significant differences between observed and expected genotypic frequencies and lack of HWE in Groups A and C.

Intronic SNPs were chosen to help maximize coverage of the CYP3A4 gene. While not translated into the final protein, intronic SNPs may directly or indirectly have an impact on gene function. Furthermore, these SNPs could be in linkage disequilibrium with a coding SNP associated with the poor methadone metabolizer phenotype. Genetic polymorphisms have also been associated with regulation of microRNAs, an important class of regulators involved in a wide range of biological processes. The splicing of a gene can be altered by intronic SNPs, which can potentially cause large insertion or frameshift mutations that could result in a large impact on the protein if synthesized at all. Allelic RNA expression imbalance (AEI) was measured in 136 human liver autopsy samples by Wang *et al.* to search for common functional CYP3A4 polymorphisms. Several of the same SNPs genotyped in this study (rs2740574, rs2246709 and rs2242480) were found to be noncontributory to CYP3A4 variability in the liver (49).

Conclusion

Polymorphism of the CYP3A4 gene appears to correlate with the increased likelihood of accidental fatal methadone intoxication. SNPs rs2242480 and rs2740574 had altered genotype frequencies within the study populations. There were two homozygotes within the study population for the rs4986910 exonic SNP, which was a rare finding. The lack of HWE for two of the SNPs (rs4646437 and rs2740574) was also of interest because these SNPs were within HWE in a control population. The combination of these findings indicate that variant alleles in CYP3A4 may play a role in methadone metabolism, but the effect on methadone metabolism is most likely the result of a combination of SNPs and not a single CYP3A4 polymorphism. The rs2242480 and rs2740574 SNPs may be key in the apparent combination needed to cause an increased likelihood of methadone fatality. This study also demonstrated the need for methadone-only overdoses rather than combined drug overdoses when determining the potential role of genetic polymorphism in unexpected methadone fatalities. Many of the significant findings were isolated to Group A (methadone only intoxications). Given the contributions of CYP3A4 and other CYP450 enzymes involved in methadone metabolism, SNPs in CYP3A4 are acknowledged as only a part of the overall picture when interpreting the relevance of these findings. Additional research is needed to fully elucidate the role of the different isoforms and their polymorphisms in methadone pharmacokinetics and therapeutic outcome.

Acknowledgments

The authors thank Nabila Haikal, MD of the WV OCME, for assistance in the preparation of this manuscript and the Marshall University Genomics Core Facility for use of shared instruments.

Taha Ahmad offered technical assistance. Mike Ward of Premier Tox coordinated sample collection with the KY OCME.

Funding

This project was supported in part by National Institutes of Health (grant numbers P20GM103434 and P20RR016477). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- Hall, A.J., Logan, J.E., Toblin, R.L., Kaplan, J.A., Kraner, J.C., Bixler, D. *et al.* (2008) Patterns of abuse among unintentional pharmaceutical overdose fatalities. *The Journal of the American Medical Association*, **300**, 2613–2620.
- Garrido, M.J., Troconiz, I.F. (1999) Methadone: a review of its pharmacokinetic/pharmacodynamic properties. *Journal of Pharmacological and Toxicological Methods*, **42**, 61–66.
- Gouldin, W.M., Kennedy, D.T., Small, R.E. (2000) Methadone: history and recommendations for use in analgesia. *American Pain Society Bulletin*, **10**, 5–6.
- Hendra, T.J., Gerrish, S.P., Forrest, A.R. (1996) Fatal methadone overdose. *British Medical Journal*, **313**, 481–482.
- Santiago, T.V., Edelman, N.H. (1985) Opioids and breathing. *Journal of Applied Physiology*, **59**, 1675–1685.
- Wong, S.H., Wagner, M.A., Jentzen, J.M., Schur, C., Bjerke, J., Gock, S.B. *et al.* (2003) Pharmacogenomics as an aspect of molecular autopsy for forensic pathology/toxicology: does genotyping CYP 2D6 serve as an adjunct for certifying methadone toxicity? *Journal of Forensic Sciences*, **48**, 1406–1415.
- Bertschy, G. (1995) Methadone maintenance treatment: an update. *European Archives of Psychiatry and Clinical Neuroscience*, **245**, 114–124.
- Mitchell, T.B., Dyer, K.R., Newcombe, D., Salter, A., Somogyi, A.A., Bochner, F. *et al.* (2004) Subjective and physiological responses among racemic-methadone maintenance patients in relation to relative (S)- vs. (R)-methadone exposure. *British Journal of Clinical Pharmacology*, **58**, 609–617.
- Foster, D.J., Somogyi, A.A., Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology*, **47**, 403–412.
- Eap, C.B., Buclin, T., Baumann, P. (2002) Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence. *Clinical Pharmacokinetics*, **41**, 1153–1193.
- Li, Y., Kantelip, J.P., Gerritsen-van Schieveen, P., Davani, S. (2008) Interindividual variability of methadone response: impact of genetic polymorphism. *Molecular Diagnosis and Therapy*, **12**, 109–124.
- Ferrari, A., Coccia, C.P., Bertolini, A., Sternieri, E. (2004) Methadone—metabolism, pharmacokinetics and interactions. *Pharmacological Research*, **50**, 551–559.
- Boulton, D.W., Arnaud, P., DeVane, C.L. (2001) Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology and Therapeutics*, **70**, 48–57.
- Leshner, A.I. (1997) Drug abuse and addiction treatment research: the next generation. *Archives of General Psychiatry*, **54**, 691–694.
- Moody, D.E., Alburges, M.E., Parker, R.J., Collins, J.M., Strong, J.M. (1997) The involvement of cytochrome P450 3A4 in the N-demethylation of L-alpha-acetylmethadol (LAAM), norLAAM, and methadone. *Drug Metabolism and Disposition*, **25**, 1347–1353.
- Nilsson, M.I., Anggard, E., Holmstrand, J., Gunne, L.M. (1982) Pharmacokinetics of methadone during maintenance treatment:

- adaptive changes during the induction phase. *European Journal of Clinical Pharmacology*, **22**, 343–349.
17. Iribarne, C., Berthou, F., Baird, S., Dreano, Y., Picart, D., Bail, J.P. *et al.* (1996) Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. *Chemical Research in Toxicology*, **9**, 365–373.
 18. Totah, R.A., Allen, K.E., Sheffels, P., Whittington, D., Kharasch, E.D. (2007) Enantiomeric metabolic interactions and stereoselective human methadone metabolism. *Journal of Pharmacology and Experimental Therapeutics*, **321**, 389–399.
 19. Chang, Y., Fang, W.B., Lin, S.N., Moody, D.E. (2011) Stereo-selective metabolism of methadone by human liver microsomes and cDNA-expressed cytochrome P450s: a reconciliation. *Basic & Clinical Pharmacology & Toxicology*, **108**, 55–62.
 20. Eap, C.B., Broly, F., Mino, A., Hammig, R., Deglon, J.J., Uehlinger, C. *et al.* (2001) Cytochrome P450 2D6 genotype and methadone steady-state concentrations. *Journal of Clinical Psychopharmacology*, **21**, 229–234.
 21. Eap, C.B., Crettol, S., Rougier, J.S., Schlapfer, J., Sintra Grilo, L., Deglon, J.J. *et al.* (2007) Stereoselective block of hERG channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. *Clinical Pharmacology and Therapeutics*, **81**, 719–728.
 22. Gerber, J.G., Rhodes, R.J., Gal, J. (2004) Stereoselective metabolism of methadone N-demethylation by cytochrome P4502B6 and 2C19. *Chirality*, **16**, 36–44.
 23. Shook, J.E., Watkins, W.D., Camporesi, E.M. (1990) Differential roles of opioid receptors in respiration, respiratory disease, and opiate-induced respiratory depression. *The American Review of Respiratory Disease*, **142**, 895–909.
 24. White, J.M., Irvine, R.J. (1999) Mechanisms of fatal opioid overdose. *Addiction*, **94**, 961–972.
 25. Shiran, M.R., Chowdry, J., Rostami-Hodjegan, A., Ellis, S.W., Lennard, M.S., Iqbal, M.Z. *et al.* (2003) A discordance between cytochrome P450 2D6 genotype and phenotype in patients undergoing methadone maintenance treatment. *British Journal of Clinical Pharmacology*, **56**, 220–224.
 26. Guengerich, F.P., Hosea, N.A., Parikh, A., Bell-Parikh, L.C., Johnson, W.W., Gillam, E.M. *et al.* (1998) Twenty years of biochemistry of human P450s: purification, expression, mechanism, and relevance to drugs. *Drug Metabolism and Disposition*, **26**, 1175–1178.
 27. Li, A.P., Kaminski, D.L., Rasmussen, A. (1995) Substrates of human hepatic cytochrome P450 3A4. *Toxicology*, **104**, 1–8.
 28. Jin, M., Gock, S.B., Jannetto, P.J., Jentzen, J.M., Wong, S.H. (2005) Pharmacogenomics as molecular autopsy for forensic toxicology: genotyping cytochrome P450 3A4*1B and 3A5*3 for 25 fentanyl cases. *Journal of Analytical Toxicology*, **29**, 590–598.
 29. Paine, M.F., Hart, H.L., Ludington, S.S., Haining, R.L., Rettie, A.E., Zeldin, D.C. (2006) The human intestinal cytochrome P450 “pie”. *Drug Metabolism and Disposition*, **34**, 880–886.
 30. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., Guengerich, F.P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414–423.
 31. Westlind, A., Lofberg, L., Tindberg, N., Andersson, T.B., Ingelman-Sundberg, M. (1999) Interindividual differences in hepatic expression of CYP3A4: relationship to genetic polymorphism in the 5'-upstream regulatory region. *Biochemical and Biophysical Research Communications*, **259**, 201–205.
 32. He, P., Court, M.H., Greenblatt, D.J., von Moltke, L.L. (2005) Genotype-phenotype associations of cytochrome P450 3A4 and 3A5 polymorphism with midazolam clearance in vivo. *Clinical Pharmacology and Therapeutics*, **77**, 373–387.
 33. Ozdemir, V., Kalow, W., Tang, B.K., Paterson, A.D., Walker, S.E., Endrenyi, L. *et al.* (2000) Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics*, **10**, 373–388.
 34. Schmider, J., Greenblatt, D.J., von Moltke, L.L., Shader, R.I. (1996) Relationship of in vitro data on drug metabolism to in vivo pharmacokinetics and drug interactions: implications for diazepam disposition in humans. *Journal of Clinical Psychopharmacology*, **16**, 267–272.
 35. Fonseca, F., de la Torre, R., Diaz, L., Pastor, A., Cuyas, E., Pizarro, N. *et al.* (2011) Contribution of cytochrome P450 and ABCB1 genetic variability on methadone pharmacokinetics, dose requirements, and response. *PLoS One*, **6**, e19527.
 36. Crettol, S., Deglon, J.J., Besson, J., Croquette-Krokar, M., Hammig, R., Gothuey, I. *et al.* (2006) ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment. *Clinical Pharmacology and Therapeutics*, **80**, 668–681.
 37. Evans, W.E., Relling, M.V. (1999) Pharmacogenomics: translating functional genomics into rational therapeutics. *Science*, **286**, 487–491.
 38. Schirmer, M., Rosenberger, A., Klein, K., Kulle, B., Toliat, M.R., Nurnberg, P. *et al.* (2007) Sex-dependent genetic markers of CYP3A4 expression and activity in human liver microsomes. *Pharmacogenomics*, **8**, 443–453.
 39. Sata, F., Sapone, A., Elizondo, G., Stocker, P., Miller, V.P., Zheng, W. *et al.* (2000) CYP3A4 allelic variants with amino acid substitutions in exons 7 and 12: evidence for an allelic variant with altered catalytic activity. *Clinical Pharmacology and Therapeutics*, **67**, 48–56.
 40. Dai, D., Tang, J., Rose, R., Hodgson, E., Bienstock, R.J., Mohrenweiser, H.W. *et al.* (2001) Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. *Journal of Pharmacology and Experimental Therapeutics*, **299**, 825–831.
 41. Eiselt, R., Domanski, T.L., Zibat, A., Mueller, R., Presecan-Siedel, E., Hustert, E. *et al.* (2001) Identification and functional characterization of eight CYP3A4 protein variants. *Pharmacogenetics*, **11**, 447–458.
 42. Garcia-Martin, E., Martinez, C., Pizarro, R.M., Garcia-Gamito, F.J., Gullsten, H., Raunio, H. (2002) CYP3A4 variant alleles in white individuals with low CYP3A4 enzyme activity. *Clinical Pharmacology and Therapeutics*, **71**, 196–204.
 43. Lamba, J.K., Lin, Y.S., Thummel, K., Daly, A., Watkins, P.B., Strom, S.J. (2002) Common allelic variants of cytochrome P4503A4 and their prevalence in different populations. *Pharmacogenetics*, **12**, 121–132.
 44. van Schaik, R.H., de Wildt, S.N., Brosens, R., van Fessem, M., van den Anker, J.N., Lindemans, J. (2001) The CYP3A4*3 allele: is it really rare? *Clinical Chemistry*, **47**, 1104–1106.
 45. Lamba, J.K., Lin, Y.S., Schuetz, E.G., Thummel, K.E. (2002) Genetic contribution to variable human CYP3A-mediated metabolism. *Advanced Drug Delivery Reviews*, **54**, 1271–1294.
 46. Hirota, T., Ieiri, I., Takane, H., Maegawa, S., Hosokawa, M., Kobayashi, K. *et al.* (2004) Allelic expression imbalance of the human CYP3A4 gene and individual phenotypic status. *Human Molecular Genetics*, **13**, 2959–2969.
 47. Abraham, J., Earl, H.M., Pharoah, P.D., Caldas, C. (2006) Pharmacogenetics of cancer chemotherapy. *Biochimica et Biophysica Acta*, **1776**, 168–183.
 48. Rodriguez-Antona, C., Sayi, J.G., Gustafsson, L.L., Bertilsson, L., Ingelman-Sundberg, M. (2005) Phenotype–genotype variability in the human CYP3A locus as assessed by the probe drug quinine and analyses of variant CYP3A4 alleles. *Biochemical and Biophysical Research Communications*, **338**, 299–305.
 49. Wang, D., Guo, Y., Wrighton, S.A., Cooke, G.E., Sadee, W. (2011) Intrinsic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics*, **11**, 274–286.