CHARACTERIZATION OF THE HUMAN CYT19 GENE PRODUCT: AN ARSENIC METHYLTRANSFERASE

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Chronic arsenic exposure poses a threat to millions of people throughout the world due to arsenic in drinking water; however the mechanisms underlying arsenic carcinogenicity and individual susceptibility are unknown. Methylation has been considered the primary detoxification pathway of inorganic arsenic in many species but there is evidence that methylation may increase arsenic toxicity. It has been shown that methylated arsenicals that contain As^{III} are more cytotoxic and genotoxic than either arsenate or arsenite. Rat liver *S*-adenosyl-*L*-methionine: $arsenic^{III}$-methyltransferase has been identified and is homologous to human cyt19, but there are species specific differences in arsenic biotransformation and toxicity. Additionally, there is considerable variation among humans in the rate of methylation of inorganic arsenic leading to measurable differences in toxicity. Therefore, it is important to better understand the enzymes that catalyze the methylation of arsenic in humans. In this study, we PCR amplified and cloned cyt19, a putative arsenic methyltransferase from human HepG2

hepatoma cells. The PCR product was ligated into an *E. coli* pET expression vector with a polyhistidine tag at the amino-terminal residue. The recombinant human cyt19 was successfully expressed in BL21 (DE3) and purified using a nickel-nitrilotriacetic acid metal-affinity chromatography. The recombinant protein catalyzes the methylation of arsenite as well as monomethylarsonic acid (MMA). The specific activity of arsenite methylation was 597 pmol/mg protein/min in a reaction mixture containing 5mM GSH, 1 mM DTT, 1 mM $MgCl_2$, 100 µM *S*-adenosyl-*L*-methionine, 50µM sodium m-arsenite, and 5 µg of *S*-adenosyl-*L*-methionine: arsenic methyltransferase in 100mM tris/100mM sodium phosphate buffer pH 7.4 at 37 °C for 30 minutes. The results suggest that the human cyt19 gene, in fact, is translated to an *S*-adenosyl-*L*-methionine: arsenic methyltransferase which methylates both arsenite and MMA.

Studies have shown that humans exposed to arsenic excrete variable amounts of methylated arsenicals in the urine which may be due to differences in arsenic methyltransferase activity. While polymorphisms in the coding region of cyt19 may account for some of the observed variation in arsenic methylation, other mechanisms are likely to be involved. In this study we identified an alternative splice variant of the human cyt19 (cyt19ΔE2), in which exon 2 is removed creating a bicistronic transcript that is unlikely to produce an active protein. This variant was expressed in 7 out of 7 male Caucasian human liver samples tested and in HepG2 cells. The human cyt19 appears to be alternatively spliced in many individuals and may play a role in the observed variation in arsenic methylation seen in individuals.

CHAPTER 1
INTRODUCTION

Sources of Arsenic

Natural Sources

Arsenic (As) is a member of the nitrogen group in the periodic table and is classified as a metalloid. This metalloid is a naturally occurring element and is the 20th most abundant element in the earth's crust [1]. Arsenic is found in the environment as sulfides, and complex sulfides of iron, nickel, and cobalt. The natural weathering of rocks and soils containing various forms of arsenic contribute to its levels in the environment. Arsenic is present in the atmosphere, aquatic environments, soils & sediments, and in organisms. This metalloid is found naturally in rocks, geothermal wells, minerals, and metal ores such as copper and lead. Arsenic is present in the environment in both organic and inorganic forms and exists in four valence states, -3, 0 (elemental), +3 (trivalent), and +5 (pentavalent arsenic), however it exists mainly in the latter two valence states. Many marine plant and animal species have naturally high levels of As, but in organic forms that appear to cause little toxicity. The main species of arsenic in marine animals is the arsenosugar, arsenobetaine [2]. In general, organic forms of arsenic are less toxic than inorganic forms of arsenic and the pentavalent inorganic forms are less toxic than trivalent inorganic arsenic compounds.

Anthropogenic Sources

Anthropogenic sources of arsenic stem from its use in pesticides and wood preservatives as well as mining and smelting wastes. In the U.S., 2,200 tons of arsenic

was produced in 1985 [3]. Since 1985, the domestic production of arsenic in the US has ceased. However, arsenic is still used domestically and therefore is imported from countries such as China, Japan and Mexico. In 2003, the United States was world's largest consumer of arsenic, demanding 21,600 metric tons [4] . Arsenic has also been used in the production of dessicants and as growth stimulants for plants and animals. Organoarsenicals have been shown to have both therapeutic and growth promoting properties in poultry and swine. Arsanilic acid and its sodium salts, such as 4-nitrophenylarsonic acid are added to pet feed [5]. Most of this arsenic passes through the animal and becomes part of the waste stream resulting from animal production. In addition, arsenic has been used for therapeutic purposes and of course as a poison. Arsenic has been used to treat syphilis, tropical diseases such as trypanosomiasis (African sleeping sickness), yaws, amoebic dysentery and recently as an anticancer. Paul Ehrlich developed an organic arsenical, arsphenamine, also known as Salvarsan, which was used to treat syphilis [6]. Arsphenamine was also believed to be effective in treating trypanosomiasis [6]. Arsenic trioxide has been shown to be highly effective in the treatment of various cancers especially of acute promyelocytic leukaemia [7].

Exposure and Health Effects

Exposure

Arsenic is present throughout our environment, in the air we breathe, the water we drink, and the food we eat. Water contributes more to iAs exposure than food or air. On average Americans are exposed to 50 μg per day of arsenic of which 10 μg is in the inorganic form [8]. People are exposed to higher than average arsenic due to living or working around higher exposure sources. For example living or working near a hazardous waste site can lead to exposure via the air, ingestion, or the food chain.

Arsenic is present in 47% of all sites on the National Priorities List (NPL) sites or Superfund sites making it second only to lead as the most common contaminant of concern [9]. Occupational settings such as workers who use or produce arsenic compounds: vineyards, ceramics, glass-making, smelting, pharmaceuticals, refining of metallic ores, pesticide manufacturing & application, wood preservation, or semiconductor manufacturing, have the potential to be exposed to higher than average arsenic levels [1].

Currently, people in Taiwan, Mexico, western United States, western South America, China, West Bengal and Bangladesh are exposed to high levels of arsenic due to anthropogenic and/or natural contamination of potable water. It has been estimated that 200 million people worldwide are at risk from health effects associated with this exposure [10]. The two most affected areas in the world are Bangladesh and West Bengal, India; it has been estimated that around 122 million people in these areas are exposed to groundwater arsenic concentrations above the World Health Organization maximum permissible limit of 50µg/L [11]. The study showed that in West Bengal, 26.4% (n=10,991 tube wells) of the water samples had arsenic ranging in 100-299 µg/L. In the U.S. the arsenic maximum contaminant level (MCL) was decreased to 10µg/L by the US Environmental Protection Agency (EPA) in January 2001. Frost et al. [12] identified 33 counties in 11 states in the western United States with mean arsenic concentrations of 10µg/L or greater. In addition, from 1950-1990 there were over 60 million people in the US exposed to arsenic contaminated water exceeding 10µg/L [12].

Health Effects

Ingestion of arsenic is a widespread human health problem. There are different symptoms associated with acute and chronic arsenic exposure. Acute exposure to arsenic

can result in acute paralytic syndrome, acute GI syndrome, and even death. Oral exposure above 60 ppm in food or water can result in death [3]. Chronic arsenic exposure can result in skin disorders such as hyper and hypo pigmentation, and hyperkeratosis [3]. It was found that even at 0.005-0.01mg/L of arsenic in water there is an increase in the prevalence of skin lesions [13]. In addition, chronic iAs exposure can affect the circulatory and nervous systems leading to diseases such as Blackfoot disease. Major organs such as the liver, kidneys, lung, bladder, and heart can be affected as a result of arsenic cytotoxicity. There is an increase of cancer, death from cancer, and diabetes mellitus associated with chronic arsenic exposure. Epidemiological studies show that there is dose-response relationship between exposure to iAs and skin cancer [14]. A study in Taiwan[15] and Japan[16] demonstrated a significant association between long-term arsenic exposure in drinking water with lung and bladder cancer. In northern Chile an increase in mortality from bladder, lung, kidney, and skin cancer is associated with As exposure; bladder and lung cancer showing highest increase in mortality [17]. A follow up study in Taiwan compared the incidence of diabetes mellitus in an arsenic exposed population to two control areas showed an association between As exposure and diabetes mellitus [18].

Mechanisms of Toxicity

The mechanism of arsenic toxicity is dependent on oxidation state. Trivalent arsenicals, including methylated arsenicals produce toxicity by enzyme inhibition by interactions with sulfhydryl groups in proteins [19] and the generation of reactive oxygen species (ROS). For example, in vitro studies have shown that MMA^{III} and arsenite are capable of inhibiting pyruvate dehydrogenase (PDH) activity in hamster kidney and purified porcine heart PDH resulting in the subsequent blockage of adeonosine

triphosphate (ATP) production because of the disruption of the citric acid due to the depletion of cellular citrate [20]. Inorganic arsenicals, arsenite and arsenate, have been shown to induce ROS and reactive nitrogen species (RNS) which result in DNA and protein oxidative damage [21]. Another study demonstrated that MMA^{III} and DMA^{III} induced ROS result in DNA damage [22]. Arsenate has been shown to interfere with ATP production by substituting for phosphate leading to production of an unstable ADP-arsenate complex which spontaneously hydrolyzes [23]. This process leads to a depletion of cellular energy due to this futile cycle.

Although there is strong evidence of the carcinogenicity of arsenic in humans, the mechanism by which tumors are produced is unknown. Studies of arsenic carcinogenesis have been hampered because there are very few animal models in which arsenic induces carcinogenesis [3]. DMA concentrations of 50 and 200 ppm have been shown to be carcinogenic in F344 rats urinary bladder [24]. Recent work has demonstrated the promotion effects of inorganic arsenicals and methylated arsenicals. Inorganic arsenic (42.5 and 85 ppm)has been shown to be a transplacental carcinogen in mice [25]. Organic arsenicals, such as MMA, DMA, and TMAO have been shown to act as promoters in carcinogenesis of several rat organs [26]. However, some of these studies have received much criticism due to the high arsenical exposure levels used ranging from about 50 to 400 ppm and the use of several initiators such as diethylnitrosamine, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, *N*-methyl-*N*-nitrosourea, dihydroxy-di-*N*-propylnitrosamine and *N*-*N*'-dimethylhydrazine, prior to arsenic exposure.

Several possibilities for mechanisms of arsenic induced malignancies have been hypothesized such as chromosomal abnormality, oxidative stress, and the promotion of

tumorigenesis. Zhao et al. [27], have shown that chronic low level arsenic (0-0.5μM) exposure will result in the malignant transformation of epithelial cells associated with DNA hypomethylation due to depletion of SAM and aberrant gene expression. An in vivo long-term arsenic exposure study to mice demonstrated that arsenic in potable water can induce aberrant gene expression, global DNA hypomethylation, and hypomethylation of the gene for the estrogen receptor-α resulting in enhanced transcription, which cumulatively could lead to arsenic hepatocarcinogenesis [28]. Arsenate was shown to have a dose-dependent transcriptional induction of several different signal transduction pathways, including the dose-response induction of several promoters and/or response elements responsive to oxidative damage and DNA damage [29] which may help understand the mechanisms of carcinogenicity for arsenic. Binet et al. [30] has shown that arsenic induced apoptosis via reactive oxygen species (ROS) production occurs but, the ROS is not produced from nicotinamide adenine dinucleotide phosphate dehydrogenase activation. Trivalent methylated arsenicals have been shown to indirectly cause DNA damage by ROS. One study showed that DMA^{III} promotes tumorigenesis and gentoxicity via dimethylated arsenic peroxides [31]

Arsenic Biotransformation

Reduction of Pentavalent Arsenicals

The biotransformation of iAs alternates between the reduction of arsenate (iAs^{V}) to arsenite (iAs^{III}) followed by oxidative methylation. The hypothesized scheme of iAs methylation involves oxidative methylation and reduction[32]:

$$As^{v}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + CH_3^{+} \rightarrow CH_3As^{v}O_3^{2-} + 2e \rightarrow CH_3As^{III}O_2^{2-} + CH_3^{+} \rightarrow (CH_3)_2As^{v}O_2^{-} + 2e \rightarrow (CH_3)_2As^{III}O$$

Arsenic reduction must occur first before it can be methylated. Several enzymes have been shown capable to reduce arsenic. Purine nucleoside phosphorylase (PNP) has the ability to reduce iAs^{V} to iAs^{III} in the presence of a dithiol and a purine nucleoside (guanosine or inosine) in vitro [33, 34]. However, studies performed by Németi et al. 2003 showed that PNP does not play a role in iAs^{V} reduction in vivo [35]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the presence of glutathione (GSH) and NAD has the ability to reduce iAs^{V} to iAs^{III} in human red blood cells and rat liver cytosol [36, 37]. A human arsenate reductase was discovered capable of reducing arsenate but not methylarsonic acid (MMA^{V}) [38]. Zakharyan et al 1999 presented an enzyme from rabbit liver capable of reducing MMA^{V}, arsenate, and dimethylarsinic acid (DMA^{V}) in the presence of GSH; this enzyme was also present in human liver [39]. MMA^{V} reductase was sequenced and 92% of the sequences were identical to human glutathione-S-transferase Omega class (hGSTO-1) [40]. This hGSTO-1 catalyzes the reduction of iAs^{V}, MMA^{V}, and DMA^{V} [39, 41]. There is evidence that pentavalent arsenicals can be reduced nonenzymatically. Glutathione (GSH) has been shown to reduce pentavalent arsenicals [42, 43].

Methylation of Trivalent Arsenicals

Following the first reduction step, arsenite is enzymatically oxidatively methylated to MMA^{V}. In this reaction, a high energy methyl group from S-adenosyl-L-methione (SAM) is transferred to a trivalent arsenical in an oxidative process that produces a pentavalent methylated arsenical. The resulting monomethylarsonic acid (MMA) can be reduced a second time and methylated again to form dimethylarsinic acid (DMA). In West Bengal, MMA^{III} and DMA^{III} were detected in the urine of exposed humans in 48% and 72% respectively out of the 428 subjects [44]. In some animals, including humans, a

third methylation can occur resulting in formation of trimethylarsine oxide (TMAO). It was shown that a single dose of arsenic trioxide in hamsters resulted in a very small amount of TMAO production in the liver[45]. Yoshida et al 1997 demonstrated that rats excrete TMAO in their urine after a single oral administration of DMA [46]. Urine excretion of TMAO in man has been observed after ingestion of an arsenosugar and DMA [47, 48].

Arsenic methyltransferases (AS3MT) have been isolated from many mammalian species. An AS3MT has been purified 2000-fold from rabbit liver by DEAE chromatography to a single band [49]. The rabbit liver AS3MT was capable of performing both methylation steps. The Golden Syrian hamster liver was used to purify AS3MT and was shown to have similar activities as the rabbit AS3MT [50]. Rat liver S-adenosyl-L-methionine:arsenic^{III}-methyltransferase has been identified and is homologous to human cyt19 [51]. The rat arsenic methyltransferase has been shown to perform both mono and dimethylation of arsenic. Arsenic methyltransferase activity has been determined in mice and primates, including humans [52-55].

Variation in Arsenic Methylation

There is significant variation in the arsenic methylation rate and arsenic metabolite production among mammalian species. The variability of arsenic methylation is apparent in the amounts of methylated arsenic metabolites seen in the urine of exposed mammals such as the rat, rabbit, hamster, dog, and mouse. For example, mice quickly excrete about 90% of the dose in two days of which 80% is DMA [56]. The rat efficiently methylates arsenic, but it accumulates DMA in red blood cells resulting in subsequent lower DMA excretion levels making it a poor model for human metabolic studies [57]. Healy et al. [58], purified arsenic methyltransferases from livers of rabbit, hamster, and

rhesus monkey and found different rates of methylation which may affect arsenic elimination and toxicity.

However, these mammals with arsenic methylation capacity have something in common which distinguishes them from humans. On average, humans exposed to arsenic excrete more MMA in the urine than other mammals , specifically 10-30% iAs, 10-20% MMA, and 60-80% DMA [59]. This suggests that non-human mammals are more efficient at catalyzing the second methylation step which produces MMA. This may relate to their lower susceptibility to iAs carcinogenesis following exposure versus man. Additionally, there is significant variation in human susceptibility to As induced toxicity, which may be related to differences in arsenic biotransformation between individuals. Epidemiological studies have shown differences in the amount of MMA and DMA excreted in the urine of exposed populations which may be associated with differences in arsenic methyltransferase activity. Several studies on the urinary excretion of arsenic metabolites in native Andean people and mixed ethnicities in northeastern Argentina exposed to arsenic in potable water revealed low excretion of MMA [60-63]. One study revealed higher than normal MMA in urine, on average 27%, in people exposed to arsenic in drinking water on the northeast coast of Taiwan [64].

Only a few polymorphisms have been found in the coding region of cyt19 to date. The Met287Thr mutation has been reported on three different occasions [65-67]. In two of these studies, the methylation activity of this allozyme was determined and showed to have a higher methylation capacity than the wild-type [65, 67]. However, the activity of this allozyme was determined either from a cytosol preparation or by analysis of cells and culture media of exposed human hepatocytes. This type of analysis does not take into

account the possible different expression levels of the arsenic methyltransferase. In fact one study shows that the allozyme Met287Thr was expressed at higher levels than the wild-type arsenic methyltransferase [67], making unclear as to whether the increase in activity results from the mutation or the increased expression levels. There are differences in arsenic methylation capacities among individuals, which cannot be supported alone by polymorphisms within the cyt19 coding region. All of the single nucleotide polymorphisms (SNPs) in cyt19 available as seen in the NCBI SNP database on January 12, 2006 are within the intron or untranslated region (UTR). In addition, two separate studies which examined the frequency of polymorphisms within cyt19 found one nonsynonymous SNPs (nsSNPs) out of 58 SNPs [66] and the other study found 3 nsSPNPs out of 26 SNPs [67], the remainder of the SNPs occurring in introns or UTRs.

There is another mechanism which may help explain the differences seen in arsenic methylation, alternative splicing. Alternative splicing is frequently used to regulate gene expression and to generate tissue-specific mRNA and protein isoforms [68, 69]. Introns contain sequence elements in which splicesome assembly occurs [70]. Mutations within these sequence elements could alter the constitutive splicing of a gene which may affect the methylation capacity within and among different population groups.

Role of Methylation in Arsenic Toxicity

Arsenic methylation has traditionally been thought to be a detoxification pathway. Pentavalent methylated arsenic metabolites are less reactive and are readily excreted in the urine compared to iAs [56]. Pentavalent methylated arsenicals have also been shown to be less cytotoxic and genotoxic compared to arsenite [71]. Mure et al. [72], demonstrated that arsenite induces delayed mutagenesis and transformation in human osteosarcoma cells but MMA^{III} showed no significant increase in mutagenesis or

transformation. While As methylation has been viewed as a detoxification pathway, recent studies have shown monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) to be more toxic than inorganic arsenicals. In vitro and in vivo studies have shown that MMA^{III} is more cytotoxic than As^{III}. Petrick et al [20], revealed that the order of toxicity in Chang human hepatocytes is as follows: MMA^{III} > arsenite > arsenate > MMA^{V} = DMA^{V}. In vivo studies performed in hamsters demonstrated that MMA^{III} is more lethal than arsenite [73]. In addition, Hirano et al. [74], has shown that monomethylarsonous acid diglutathione is more acutely toxic than other arsenicals. It is important to point out that not all mammals methylate arsenic such as the marmoset monkey, tamarin, chimpanzee, and the guinea pig [57, 75-78]. These mammals have not been shown to be more susceptible to acute arsenic intoxication. One study showed no correlation between the induction of micronuclei and the ability to methylate arsenic in the leukocytes of four mammalian species, humans, mice, rats, and guinea pigs [79]. Another factor that questions the role of methylation is the fact that arsenic is a known human carcinogen, but very few animals exist in which arsenic initiates carcinogenesis. The debate over whether As methylation is a detoxification or bioactivation pathway leads to confusion over the role of methylation in toxicity.

Specific Aims of Research

The proposed study will address the role of arsenic methylation in human toxicity by better understanding the kinetics of the human arsenic methyltransferase. The overall hypothesis is the following: Human arsenic methyltransferase, cyt19, activity is the determining factor in the rate of arsenic methylation and toxicity. To test this hypothesis, I examined two specific aims: 1) clone and characterize the human arsenic methyltransferase (cyt19) and 2) determine cyt19s role in toxicity and arsenic

methylation variability. Specific aim one was addressed by determining rate of arsenic methylation by an in vitro assay. The optimum conditions required for human cyt19 activity were determined such as pH optimum, substrate specificity and concentration, and thiol requirements. Specific aim two was addressed by examining the role of polymorphisms and splice variants on arsenic methylation variability. The determination of cyt19s role in toxicity was addressed by As toxicity in the presence and absence of methylation activity. In order to determine if cyt19 is the only arsenic methyltransferase in humans, the mRNA levels, protein concentration, and activity, in the presence and absence of siRNA knockdown was determined.

CHAPTER 2
MOLECULAR CLONING AND CHARACTERIZATION OF HUMAN CYT19, AN S-ADENOSYL-L-METHIONINE:AS-METHYLTRANSFERASE FROM HEPG2 CELLS

Introduction

Chronic arsenic exposure is a threat to millions of people throughout the world. Exposure to arsenic has been linked to various types of cancers such as skin cancer, lung cancer, and cancer of other internal organs [71]. Methylation has been considered the major route of biotransformation and excretion of inorganic arsenic (iAs) in many species including humans. The hypothesized scheme of iAs methylation involves reduction followed by oxidative methylation [80]:

$$As^{V}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + CH_3^{+} \rightarrow CH_3As^{V}O_3^{2-} + 2e \rightarrow CH_3As^{III}O_2^{2-} + CH_3^{+} \rightarrow$$

$$(CH_3)_2As^{V}O_2^{-} + 2e \rightarrow (CH_3)_2As^{III}O$$

While traditionally thought to be a detoxification pathway, recent studies have shown monomethylarsonous acid (MMA^{III}) and dimethylarsonous acid (DMA^{III}) to be more toxic than inorganic arsenicals [73]. In addition, Hirano et al.[74], has shown that monomethylarsonous acid diglutathione is more acutely toxic than other arsenicals. The debate over whether As methylation is a detoxification or bioactivation pathway leads to confusion over the role of methylation in toxicity. Rat liver S-adenosyl-L-methionine:arsenic^{III}-methyltransferase has been identified and is homologous to human cyt19 [51]. While this enzyme can be used as a model for human arsenic biotransformation, the rat is considered a poor model for metabolic studies due to its accumulation in red blood cells and subsequent lower DMA excretion levels [57]. There

are also other species specific differences in arsenic biotransformation and toxicity. Healy et al. (1999) [58], purified arsenic methyltransferases from livers of rabbit, hamster, and rhesus monkey and found different rates of methylation which may affect arsenic elimination and toxicity. Humans excrete greater amounts of monomethylarsonic acid (MMA^V) compared to most other mammals [81]. Additionally, there is considerable variation among humans in the rate of methylation of inorganic arsenic possibly leading to measurable differences in toxicity [82]. Therefore it is important to better understand the arsenic methylation capacity in human. To date, human cyt19 has been expressed, but it has not been fully characterized [52]. In this study, we cloned, expressed, and characterized cyt19, an arsenic methyltransferase from human HepG2 hepatoma cells.

Materials and Methods

Molecular Cloning

Two separate sequences available from Genbank (accession number AK057833 and AF226730) were used to design primers to amplify the open reading frame (ORF) of cyt19, an arsenic methyltransferase (Table 1). Total RNA was isolated from HepG2 cells using Trizol reagent (Invitrogen, Carlsbad, USA)). Total RNA was treated with DNAase (DNA-free kit, Ambion, Austin, USA), and reverse transcribed (RETROscript for RT-PCR, Ambion) using 2 µg of RNA. HepG2 cDNA was polymerase chain reaction (PCR)-amplified, the PCR product was ligated in pET100/D-TOPO (Invitrogen) and transformed into chemically competent *Escherichia coli* One Shot TOP10 chemically competent cells (Invitrogen). The PCR reaction consisted of 2.5 U of Pfu DNA polymerase, 0.4 µM each primer, 5µl of the RT reaction, 0.2 mM dNTP mix, 5µl of 10X PCR Buffer, and nuclease-free water to 50µl. The PCR conditions were as follows: an initial denaturation at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for

1min, annealing at 60°C for 1min, extension for 72°C for 2min 30s and a final extension at 72°C for 7min. The PCR products, the complete open reading frame of cyt19 (cyt19-WT), the mutated cyt19 (cyt19S81R), and the truncated cyt19 (cyt19$_t$) were then ligated and transformed. Ampicillin resistant colonies were analyzed by PCR and visualized by agarose gel electrophoresis. Once a correct clone was identified it was sent for sequencing to the DNA Sequencing Core Laboratory at the University of Florida. Each clone was sequenced several times and the consensus sequence determined.

RACE PCR

Rapid amplification of cDNA ends (RACE) was performed to determine which of the two separate sequences available in Genbank was actually expressed in HepG2 cells. The RACE-PCR was performed using the FirstChoice® RLM-RACE kit from Ambion. Primers were designed (Table 1) according to the instruction manual and the PCR reaction consisted of 1.25 U of Taq DNA polymerase, 0.4 μM each primer, 1μl of the RT reaction, 0.2 mM dNTP mix, 5μl of 10X PCR Buffer, and nuclease-free water to 50μl. The PCR conditions were as follows: an initial denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, extension for 72°C for 1min and a final extension at 72°C for 7min. The PCR product was ligated in pGEM®-T Easy Vector (Promega) and transformed into chemically competent *Escherichia coli* JM109 chemically competent cells (Promega).

Expression of Recombinant cyt19

The pET100/D-TOPO constructs (cyt19-WT, cyt19S81R, and cyt19$_t$) were transformed into BL21 Star (DE3) *E. coli* strain for expression (Invitrogen). First, 10ml of Luria-Bertani (LB) broth containing ampicillin (100μg/ml) and 1% glucose were inoculated with the transformed bacteria and the cultures were grown overnight. The

next day, 5ml of the overnight culture was used to inoculate 250ml of LB broth containing ampicillin and 1% glucose and grown to an OD_{600} of 0.5. Expression was induced by the addition of 1mM isopropyl-1-thio-β-D-galactoside. The culture was allowed to grow for one hour.The pET100/D-TOPO construct was then transformed into BL21 Star (DE3) *E. coli* strain for expression (Invitrogen).

The cells were harvested by centrifugation at 5,000g for 15 minutes at 4°C. The pellet was resuspended in binding buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0). The cells were lysed by addition of lysozyme to a final concentration of 1mg/ml and incubated on ice for 30 minutes followed by further incubation for 10 minutes at 4°C on a rocking platform. Triton X-100 was added to a final concentration of 1% and the incubation continued for another 10 minutes at 4°C with rocking. The cellular debris was removed by centrifugation of the lysate at 3000g for 30 minutes at 4°C The recombinant 6xHis-tagged protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography according to the manufacturer's instructions (QIAGEN, Valencia, USA).

Characterization

Activity of the expressed proteins was determined by the rate of arsenic methylation. All incubations were carried out at 37°C for 30 minutes in a final volume of 250 µl, unless otherwise noted. The reaction mixtures contained 5mM glutathione (GSH), 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 100 µM S-adenosyl-L-methionine (SAM), 13pM (^{3}H-methyl)-SAM (S.A.), 50µM sodium m-arsenite, and 5 µg of *S*-adenosyl-*L*-methionine: arsenic methyltransferase in 100mM tris/100mM sodium phosphate buffer pH 7.4. The pH optimum was determined using the above conditions

but at different pHs (6.0 – 11). The substrate specificity and optimum substrate concentrations were also determined by addition of various concentrations of sodium m-arsenite or MMA, ranging from 1μM to 200 μM or 10 μM to 1000 μM respectively. The requirements of SAM and reductants by cyt19 were determined by addition of various concentrations of SAM, and the reductants GSH and tris(2-carboxyethyl)-phosphine (TCEP). The methylation reactions were stopped by placing on ice. The standard extraction procedure described by Zakharyan et al. [49] was used to separate radioactive SAM from radioactive MMA and DMA. Briefly, the reaction mixture (250 μl) was treated with 10 μl of 40% KI, 20 μl of 1.5% potassium dichromate, 750 μl of concentrated HCl and 750 μl of chloroform. The mixture was then mixed on a vortex for 3 min followed by centrifugation at 1500g for 3 min. The upper aqueous phase contained SAM and was discarded. The lower organic phase was washed twice with 250 μl of water, 5 μl of 40% KI, and 750 μl of concentrated HCl. The mixture was mixed on a vortex and centrifuged and the upper aqueous phase was discarded each time. The methylated arsenicals contained in the organic phase were back extracted with 1 mL of water, vortexed for 3min and centrifuged at 1500g for 5 min. Half a milliliter from the final aqueous phase after back extraction was counted in a liquid scintillation counter. The activity was calculated from the dpm ^{3}H transferred from SAM to arsenic.

Confirmation of Methylated Arsenicals

Methylated arsenicals were separated from each other and contaminating species using the ion exchange method described previously by Zakharyan et al. (1995) [49]. A 10 mL glass pipette was filled to 2 mL with Bio-Rad AG 50W-X4 cation exchange resin (100-200 μM mesh). The column was equilibrated by addition of 0.5N HCl (30 mL), followed by water until the pH of the effluent was 5.5, 0.5N NaOH (30 mL), water until

the pH of the effluent was 5.5, 0.5N HCl (30 mL), and 0.05N HCl (50 mL). After equilibrating the column, 0.5 mL of the final aqueous phase extract from above was applied to the column. The columns were eluted by 6 mL of 0.05M HCl to obtain MMA and 10 mL of 0.5M NaOH for DMA elution. One milliliter of the these fractions were counted in a liquid scintillation counter.

Results and Discussion

Both cyt19 and cyt19$_t$ transcripts were amplified by PCR from HepG2 cells and human liver samples. The sequencing results of cyt19$_t$ showed 4 point mutations, 3 transverions and 1 transition, (Figure 2-1A) resulting in 3 missense mutations (Figure 2-1B) compared to the Genbank sequence (accession AF226730). The wild-type cyt19 was also amplified, cloned, and sequenced from two different populations of HepG2 cells. One of the clones, designated cyt19-WT, was aligned to the Genbank sequence, accession AK057833, and showed a 100% homology (data not shown). The other clone designated cyt19S81R contained a nonsynonymous single nucleotide polymorphism (nsSNPs) when compared to the Genbank sequence, accession AK057833 (Figure 2-2A), which results in a change from serine to arginine at residue 81 in the peptide sequence (Figure 2-2B). This change occurs in the SAM-binding site, however SAM-dependent methyltransferases have poor conservation of SAM-binding residues. SAM-dependent methyltransferases contain 3 regions of sequence similarity (motif I, II, and III) which are thought to be important in SAM binding. The only highly conserved residues in the SAM-binding N-terminal region appear to be the glycine-rich sequence E/DXGXGXG found at residues 76 to 82 [83]. Therefore, the amino acid change may not have a significant effect on the activity of the recombinant protein.

The cyt19$_t$ clone and the cyt19-WT clone are identical except for the deletion of a nucleotide at position 997 resulting in a premature stop codon (Figure 2-3A). Further analysis of the protein sequences revealed 6 missense mutations including a cysteine to valine mutation and deletions of the final 37 amino acids from the C-terminus including 4 cysteines due to the deletion in the nucleotide sequence (Figure 2-3B). In other SAM-dependent methyltransferases, the C-terminus is important in substrate binding [83]. The cyt19$_t$ protein showed no arsenic methylation activity. This indicates that the cysteine rich C-terminus is important for As binding and critical for activity.

RACE-PCR was performed on both the 5' and 3' ends. The sequencing results revealed that the 5'end of the cDNA was identical to the ORF of both sequences available in Genbank (accession AK057833 and AF226730). The 5'-untranslated region (UTR) is different from the Genbank sequences containing 18 mutations (Figure 2-4A). The 3'RACE-PCR revealed that HepG2 cells expressed mRNA identical to the 3'end of the Genbank sequence, accession AK057833. In particular, the sequencing showed that HepG2 cells cyt19 mRNA does not have a nucleotide deletion resulting in a premature stop codon (Figure 2-4B).

The recombinant human cyt19s (cyt19-WT, cyt19$_t$, and cyt19S81R) were successfully expressed in BL21 (DE3) and purified to homogeneity using a nickel-nitrilotriacetic acid metal-affinity chromatography (Figure 5). The recombinant proteins, cyt19S81R and cyt19-WT, catalyze the transfer of a methyl group from SAM to As^{III} as well as MMA^{III}, which is consistent with previous studies (Figure 2-6) [51, 52]. However, the different arsenite methyltransferase activity profiles between cyt19-WT and cyt19S81R are apparent. Arsenite concentrations above 50 μM appears to have an

inhibitory effect on cyt19S81R activity, which is similar to what is seen in the rabbit [49]. This inhibitory effect is not seen in the cyt19-WT arsenite methylation activity. The apparent K_m and V_{max} of cyt19 As^{III} methyltransferase (AS3MT) activity for cyt19-WT and cyt19S81R are 251.6 μM, 3505 pmole/mg/min, 6.176 μM, and 804.9 pmole/mg/min respectively (Table 2). The K_m and V_{max} values of cyt19-WT MMA methylation is 164.6 μM and 926.8 pmole/mg/min (Table 2). The MMA methylation profile is very similar to that seen in the rabbit. These enzymes seem to saturate at MMA concentrations of 1000 μM [49].

The K_m values can be used to interpret the affinity of an enzyme for its substrate (a larger K_m implies a weak affinity). Other kinetic analysis of the human arsenic methyltransferase had very low K_m [67, 84] values compared to cyt19-WT but, the K_m value of cyt19S81R was very similar to the other kinetic analysis. However, these other studies did not use purified enzymes which may explain the difference, especially the difference seen with cyt19-WT. The kinetic analysis suggests that the cyt19S81R has a higher affinity for arsenite than cyt19-WT. However, cyt19-WT has a considerably higher V_{max} value compared to cyt19S81R. The V_{max} values of both cyt19-WT and cyt19S81R are considerably higher than that seen among other mammals such as the hamster, rabbit, and rhesus monkey [50]. Kinetic analysis of MMA methylation demonstrates that the V_{msx} and K_m values for cyt19-WT are much higher than the values seen in the hamster, rabbit, and rhesus monkey. The higher V_{max} values of arsenite methyltransferase compared to MMA methyltransferase in cyt19-WT may explain the higher MMA urine excretion levels seen in humans compared to other mammals. The rabbit, which excretes higher amounts of MMA than most other mammals, has a higher

MMA than arsenite methyltransferase K_m [50]. Possibly, arsenite is converted very quickly to MMA, allowing it to accumulate before the dimethylation resulting in the higher excretion of MMA seen in humans.

The optimum pH of As^{III} methylation for cyt19-WT and cyt19S81R was found to be about 8 and about 9 respectively (Figure 2-7). This is similar to previous results which show that at basic pHs, methylation activity of rat cyt19, and As^{III} methyltransferase & MMA^{III} methyltransferase activity from rabbit liver increase [49, 51]. This may be due to the deprotonation of cysteines at higher pHs, which increases the rate of binding between arsenic and cysteines in the substrate binding domain. The reductant requirements were examined and it was determined that GSH is not required for cyt19-WT to methylate arsenic (Figure 2-8). In addition, it was determined that cyt19S81R does not require GSH. Previous study suggests that the substrates for cyt19 are arsenic triglutathione and monomethylarsonic glutathione [52]. Our results demonstrate that only a strong reductant such as TCEP is necessary for methylation of arsenic by cyt19, however, the addition of GSH appears to increase the activity above the reductant alone. Finally, the effect that different SAM concentrations would have on activity was determined. It was found that above 500 μM, SAM began to have an inhibitory effect (Figure 2-9). This differs from what is seen in rat, where SAM concentrations above 50 μM have an inhibitory effect [51].

The ion exchange method confirmed that cyt19 indeed produces MMA when arsenite is the substrate (Figure 2-10A). When MMA^V is used as the substrate, both MMA and DMA are seen as products (Figure 2-10B). However, DMA is the major

metabolite. The MMA^V used as a substrate is not 100% pure and likely contains some iAs as contaminants. It is possible that the contaminating iAs is methylated to MMA.

In conclusion, we have shown that cyt19 is in fact an arsenic methyltransferase methylating both arsenite and MMA. Examination of the $cyt19_t$ activity, indicates that the cysteine rich C-terminus is important for As binding and critical for activity. The data suggests that a mutation within the SAM-binding site of cyt19 can drastically change the methylation capacity of the enzyme. The characterization and kinetic analysis may explain the higher MMA urine excretion levels and increased susceptibility seen in humans compared to other mammals. It appears that arsenite is converted very quickly to MMA, allowing it to accumulate before the dimethylation resulting in the higher excretion of MMA seen in humans. The apparently deficient dimethylation activity in humans compared to other mammals is supported by the kinetic analysis and suggests that methylation may actually be a detoxification pathway.

Table 2-1. Primers used in the PCR amplification of cyt19

	Primer Sequence
cyt19	Forward: CACCATGGCTGCACTTCGTGACGCTGAGATACAG
	Reverse: TTAGCAGCTTTTCTTTGTGCCACAGCAGCCTCC
$cyt19_t$	Forward: CACCATGGCTGCACTTCGTGACGCTGAGATACAG
	Reverse: TTAACTCCAAAGCAGAACAGCTCCAGATGT
5'RACE	Outer: TTTCAGCCACTTCCACCTGGCCTT
	Inner: CAGGGATCACCAGACCACAGCCAT
3'RACE	Outer: AGGACCAACCAAGAGATGCCAA
	Inner: GCCAGAAGAAATCAGGACACACAA

Figure 2-2. Kinetic analysis of the methylation activity of cyt19-WT and cyt19S81R.

		cyt19-WT	cyt19S81R
Arsenite methyltransferase activity	Km (μM)	83.0±10.9	6.2±0.9
	Vmax (pmole/mg/min)	1585±142	804.9±33.4
MMA methyltransferae activity	Km (μM)	164.6±41.2	43.7±13.3
	Vmax (pmole/mg/min)	926.8±75.4	365.1±25.7

A.

```
         301                                                  350
AF226730 ATAGACATGA CCAAAGGCCA GGTGGAAGTG GCTGAAAAGT ATCTTGACTA
cyt19t   ATAGACATGA CCAAAGGCCA GGTGGAAGTG GCTGAAAAGT ATCTTGACTA

         351                                                  400
AF226730 TCACATGGAA AAATATGGCT TCCAGGCATC TAATGTGACT TTTTTCCATG
cyt19t   TCACATGGAA AAATATGGCT TCCAGGCATC TAATGTGACT TTTATTCATG

         401                                                  450
AF226730 GCAACATTGA GAAGTTGGCA GAGGCTGGAA TCAAGAATGA GAGCCATGAT
cyt19t   GCTACATTGA GAAGTTGGGA GAGGCTGGAA TCAAGAATGA GAGCCATGAT

         451                                                  500
AF226730 ATTGTTGTAT CAAACTGTGT TATTAACCTT GTGCCTGATA AACAACAAGT
cyt19t   ATTGTTGTAT CAAACTGTGT TATTAACCTT GTGCCTGATA AACAACAAGT
```

B.

```
         1                                                     50
AF226730 MAALRDAEIQ KDVQTYYGQV LKRSADLQTN GCVTTARPVP KHIREALQNV
cyt19t   MAALRDAEIQ KDVQTYYGQV LKRSADLQTN GCVTTARPVP KHIREALQNV

         51                                                   100
AF226730 HEEVALRYYG CGLVIPEHLE NCWILDLGSG SGRDCYVLSQ LVGEKGHVTG
cyt19t   HEEVALRYYG CGLVIPEHLE NCWILDLGSG SGRDCYVLSQ LVGEKGHVTG

         101                                                  150
AF226730 IDMTKGQVEV AEKYLDYHME KYGFQASNVT FFHGNIEKLA EAGIKNESHD
cyt19t   IDMTKGQVEV AEKYLDYHME KYGFQASNVT FIHGYIEKLG EAGIKNESHD

         151                                                  200
AF226730 IVVSNCVINL VPDKQQVLQE AYRVLKHGGE LYFSDVYTSL ELPEEIRTHK
cyt19t   IVVSNCVINL VPDKQQVLQE AYRVLKHGGE LYFSDVYTSL ELPEEIRTHK
```

Figure 2-1. Sequence alignment of cyt19$_t$ and Genbank sequence (accession AF226730). (A) Nucleotide alignment of cyt19$_t$ and Genbank sequence. The four point mutation are in red. (B) Alignment of the deduced cyt19$_t$ amino acid sequence and Genbank sequence. The 3 resulting missense mutations are in red.

A.

```
          151                                                 200
AK057833  CACGAAGAAG TAGCCCTAAG ATATTATGGC TGTGGTCTGG TGATCCCTGA
cyt19S81R CACGAAGAAG TAGCCCTAAG ATATTATGGC TGTGGTCTGG TGATCCCTGA

          201                                                 250
AK057833  GCATCTAGAA AACTGCTGGA TTTTGGATCT GGGTAGTGGA AGTGGCAGAG
cyt19S81R GCATCTAGAA AACTGCTGGA TTTTGGATCT GGGTAGTGGA CGTGGCAGAG

          251                                                 300
AK057833  ATTGCTATGT ACTTAGCCAG CTGGTTGGTG AAAAAGGACA CGTGACTGGA
cyt19S81R ATTGCTATGT ACTTAGCCAG CTGGTTGGTG AAAAAGGACA CGTGACTGGA
```

B.

```
          1                                                    50
AK057833  MAALRDAEIQ KDVQTYYGQV LKRSADLQTN GCVTTARPVP KHIREALQNV
cyt19S81R MAALRDAEIQ KDVQTYYGQV LKRSADLQTN GCVTTARPVP KHIREALQNV

          51                                                  100
AK057833  HEEVALRYYG CGLVIPEHLE NCWILDLGSG SGRDCYVLSQ LVGEKGHVTG
cyt19S81R HEEVALRYYG CGLVIPEHLE NCWILDLGSG RGRDCYVLSQ LVGEKGHVTG

          101                                                 150
AK057833  IDMTKGQVEV AEKYLDYHME KYGFQASNVT FIHGYIEKLG EAGIKNESHD
cyt19S81R IDMTKGQVEV AEKYLDYHME KYGFQASNVT FIHGYIEKLG EAGIKNESHD
```

Figure 2-2. Sequence alignment of cyt19S81R and Genbank sequence (accession AK057833). (A) Nucleotide alignment of cyt19S81R and Genbank sequence. The tranversion is in red. (B) Alignment of deduced cyt19S81R amino acid sequence and Genbank sequence. The resulting nsSNP is in red. The SAM-binding N-terminal region site is underlined.

A.

```
          951                                                  1000
cyt19-WT  AGATTTTCTG ATCAGACCAA TTGGAGAGAA GTTGCCAACA TCTGGAGGCT
cyt19t    AGATTTTCTG ATCAGACCAA TTGGAGAGAA GTTGCCAACA TCTGGA.GCT

          1001                                                 1050
cyt19-WT  GTTCTGCTTT GGAGTTAAAG GATATAATCA CAGATCCATT TAAGCTTGCA
cyt19t    GTTCTGCTTT GGAGTTAA~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1051                                                 1100
cyt19-WT  GAAGAGTCTG ACAGTATGAA GTCCAGATGT GTCCCTGATG CTGCTGGAGG
cyt19t    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1101                       1128
cyt19-WT  CTGCTGTGGC ACAAAGAAAA GCTGCTAA
cyt19t    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
```

B.

```
          201                                                  250
cyt19-WT  VLWGECLGGA LYWKELAVLA QKIGFCPPRL VTANLITIQN KELERVIGDC
cyt19t    VLWGECLGGA LYWKELAVLA QKIGFCPPRL VTANLITIQN KELERVIGDC

          251                                                  300
cyt19-WT  RFVSATFRLF KHSKTGPTKR CQVIYNGGIT GHEKELMFDA NFTFKEGEIV
cyt19t    RFVSATFRLF KHSKTGPTKR CQVIYNGGIT GHEKELMFDA NFTFKEGEIV

          301                                                  350
cyt19-WT  EVDEETAAIL KNSRFAQDFL IRPIGEKLPT SGGCSALELK DIITDPFKLA
cyt19t    EVDEETAAIL KNSRFAQDFL IRPIGEKLPT SGAVLLWS*~ ~~~~~~~~~~

          351                        376
cyt19-WT  EESDSMKSRC VPDAAGGCCG TKKSC*
cyt19t    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
```

Figure 2-3. Sequence alignment of cyt19-WT and cyt19$_t$. (A) Nucleotide alignment of cyt19-WT and cyt19$_t$ sequence. The missense mutations are in red. The 5 cysteine residues which are not included in the cyt19$_t$ are highlighted in the cyt19-WT sequence.

A.

```
           1                                                    50
5'RACE     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
AK057833   ACAGGAGCTG GCTGCGGGAG CCCGCCGTCC TGAGTCGCAG GCCGAGGAGA
AF226730   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

           51                                                   100
5'RACE     ~~~~~~~~~~ ~~~~~~~~~~ ~~ACAGGAGC TGGCTGCGGG AGCCCGCCGT
AK057833   CAGTGAGTGC GCGCCCTGAG TCGCAGGCCG AGGAGACAGT GAGTGCGCGC
AF226730   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~GAGACAGT GAGTGCGCGC

           101                                                  150
5'RACE     CCTGAGTCGC AGGCCGAGGA GACAGTGAGT GCGCGCCCTG AGTCGCAGGC
AK057833   CCTGAGTCGC AGGCCGAGGA GACAGTGAGT GCGCGCCCTG AGTCGCAGGC
AF226730   CCTGAGTCGC AGGCCGAGGA GACAGTGAGT GCGCGCCCTG AGTCGCAGGC

           151                                                  200
5'RACE     CGAGGAGACA TGGCTGCACT TCGTGACGCT GAGATACAGA AGGACGTGCA
AK057833   CGAGGAGACA TGGCTGCACT TCGTGACGCT GAGATACAGA AGGACGTGCA
AF226730   CGAGGAGACA TGGCTGCACT TCGTGACGCT GAGATACAGA AGGACGTGCA
```

B.

```
           1151                                                 1200
3'RACE     CTGGAGGCTG TTCTGCTTTG GAGTTAAAGG ATATAATCAC AGATCCATTT
AK057833   CTGGAGGCTG TTCTGCTTTG GAGTTAAAGG ATATAATCAC AGATCCATTT
AF226730   CTGGA.GCTG TTCTGCTTTG GAGTTAAAGG ATATAATCAC AGATCCATTT

           1201                                                 1250
3'RACE     AAGCTTGCAG AAGAGTCTGA CAGTATGAAG TCCAGATGTG TCCCTGATGC
AK057833   AAGCTTGCAG AAGAGTCTGA CAGTATGAAG TCCAGATGTG TCCCTGATGC
AF226730   AAGCTTGCAG AAGAGTCTGA CAGTATGAAG TCCAGATGTG TCCCTGATGC

           1251                                                 1300
3'RACE     TGCTGGAGGC TGCTGTGGCA CAAAGAAAAG CTGCTAAATC TATAGCCAAC
AK057833   TGCTGGAGGC TGCTGTGGCA CAAAGAAAAG CTGCTAAATC TATAGCCAAC
AF226730   TGCTGGAGGC TGCTGTGGCA CAAAGAAAAG CTGCTAAATC TATAGCCAAC

           1301                                                 1350
3'RACE     CAGGGGACCA CAGTAGTGGG CAAGAGTGAT CTGCATGTTT TTTAACCTGC
AK057833   CAGGGGACCA CAGTAGTGGG CAAGAGTGAT CTGCATGTTT TTTAACCTGC
AF226730   CAGGGGACCA CAGTAGTGGG CAAGAGTGAT CTGCATGTTT TTTAACCTGC
```

Figure 2-4. Sequence alignments of the 5' & 3'RACE-PCR products and the Genbank sequences (accession AK057833 and AF226730). (A) Sequence alignment of the 5'RACE-PCR product against the Genbank sequences. The mutations in the 5'RACE product are in red. The start codon for all three sequences are highlighted. (B) Sequence alignment of the 3'RACE-PCR product and the Genbank sequences. The nucleotide deletion in the Genbank sequence, accession AF226730, is highlighted. The stop sites for the Genbank sequences are in red.

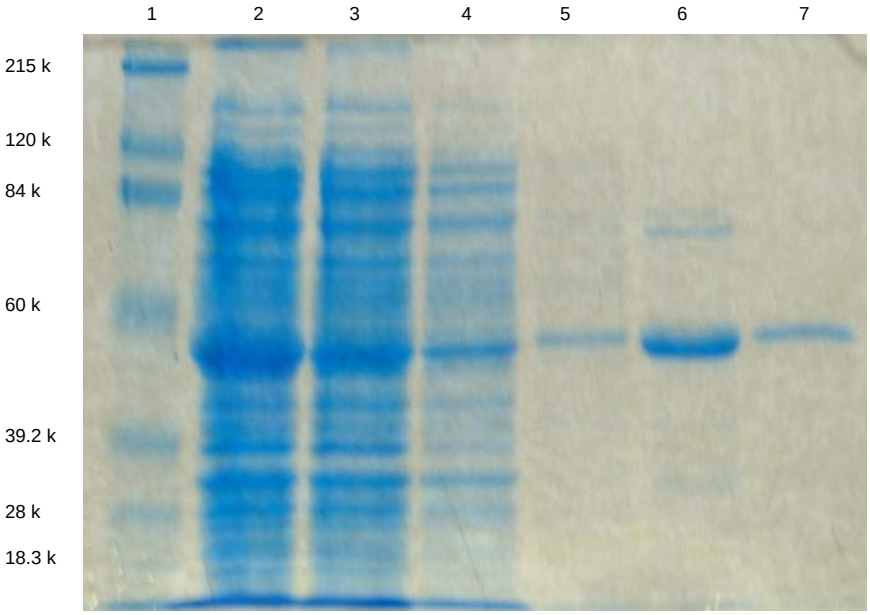
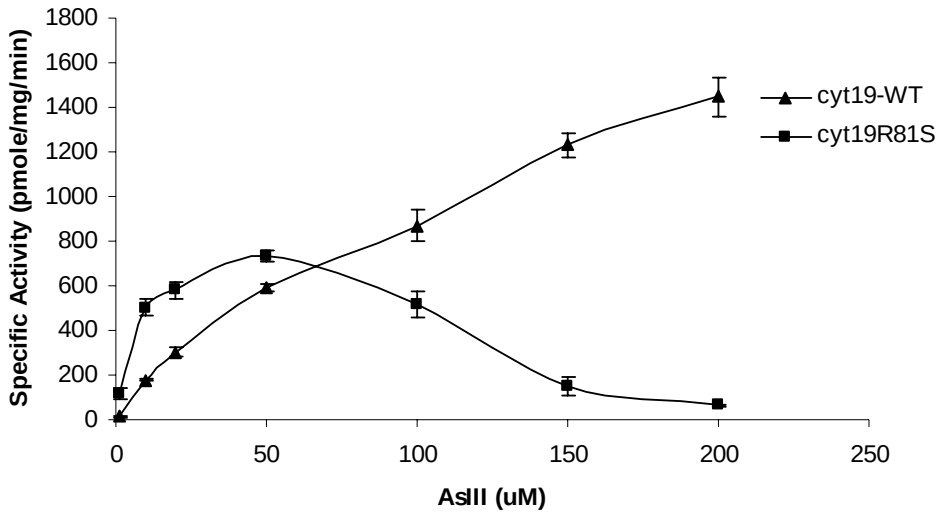


Figure 2-5. Purification of recombinant human cyt19. Fractions were electrophoresed on a 10% polyacrylamide gel and stained. Lane1, molecular weight markers; Lane2, cell lysate; Lane3, flowthrough, Lane4, Wash1, Lane5, Wash2, Lane6, Wash3, Lane7, Elution

A.



B.

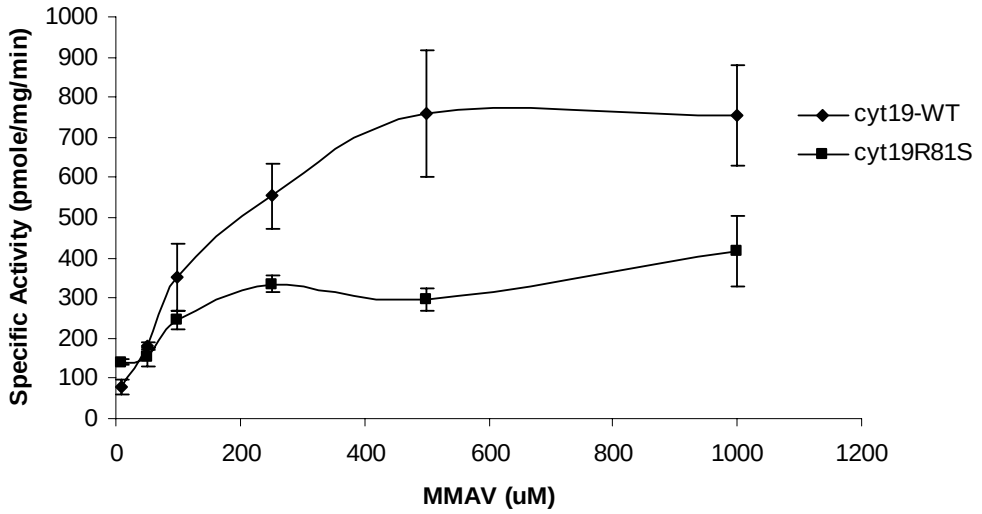


Figure 2-6. The effects of As^{III} & MMA^{V} concentrations. All incubations were carried out at 37°C for 30 min. in a final volume of 250 µl. A) Reaction mixtures contained 5 mM GSH, 1 mM DTT, 1 mM $MgCl_2$, 13 pM [^{3}H]SAM, 0.1 mM SAM, various [As^{III}], and 5 µg of cyt19, in 100 mM Tris/100 mM Na phosphate, pH 7.4 B) Same as B but with various [MMA^{v}].

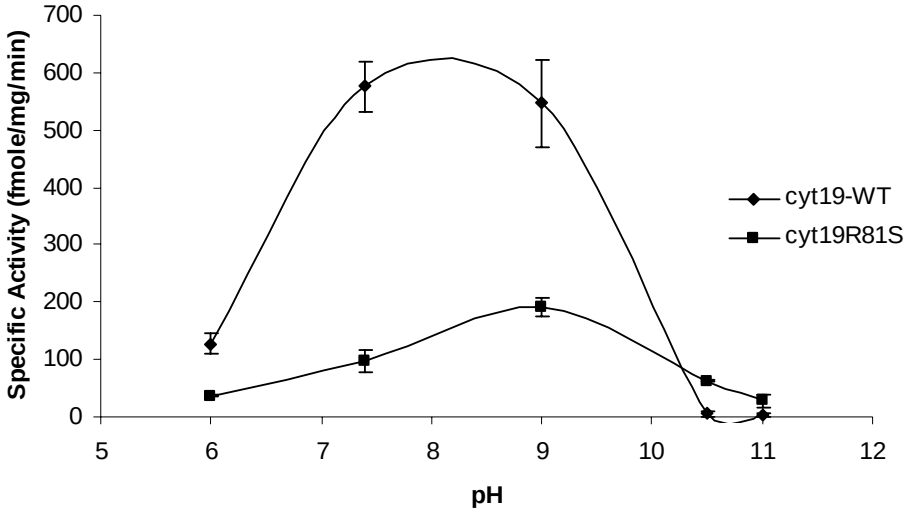


Figure 2-7. The effect of pH on activity. All incubations were carried out at 37°C for 30 min. in a final volume of 250 µl. Reaction mixtures contained 5 mM GSH, 1 mM DTT, 1 mM $MgCl_2$, 13 pM [^{3}H]SAM, 50 µM AsIII, and 5 µg of cyt19, in 100 mM Tris/100 mM Na phosphate of the appropriate pH.

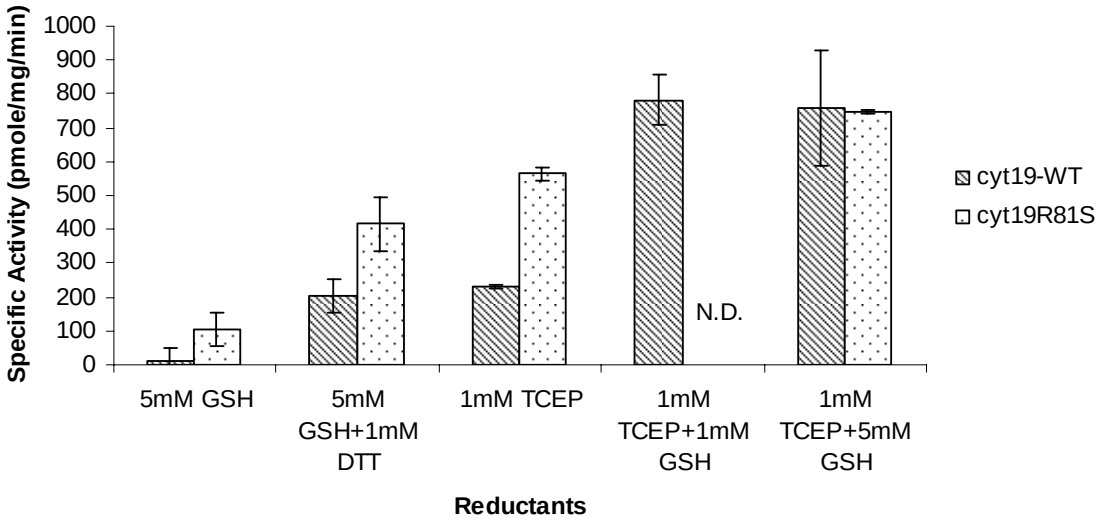


Figure 2-8. The effects of reductants on methylation activity. All incubations were carried out at 37°C for 30 min. in a final volume of 250 µl. Reaction mixtures contained 1 mM $MgCl_2$, 13 pM [^{3}H]SAM, 0.1 mM SAM, 50 µM AsIII, and 5 µg of cyt19-WT, in 100 mM Tris/100 mM Na phosphate, pH 7.4, with different reductants. The activity of cyt19R81S was not determined for 1 mM TCEP + 1 mM GSH.

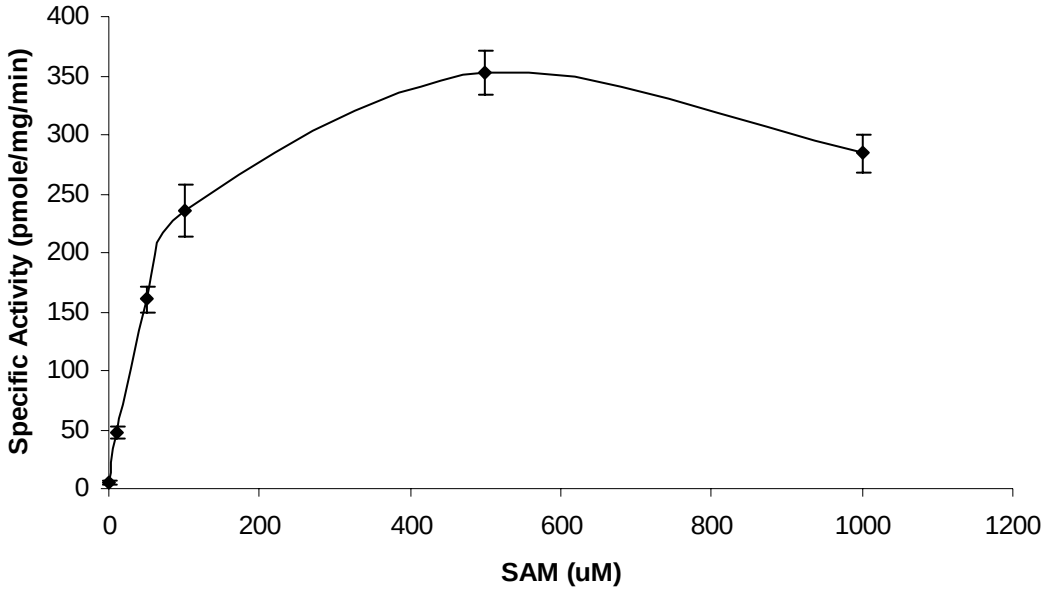
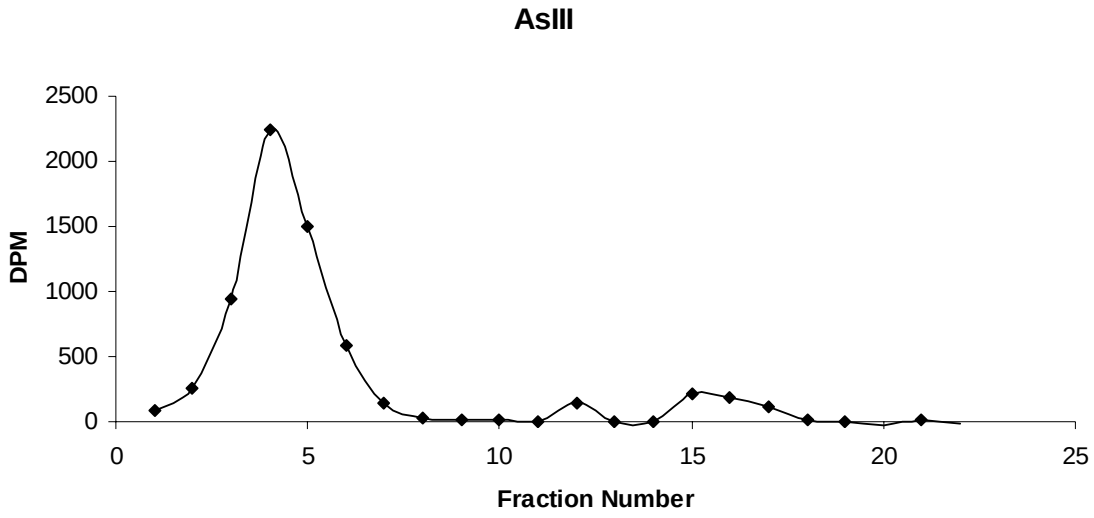


Figure 2-9. The effect of SAM concentration on activity. All incubations were carried out at 37°C for 30 min. in a final volume of 250 µl. Reaction mixtures contained 5 mM GSH, 1 mM DTT, 1 mM $MgCl^2$, 13 pM [^{3}H]SAM, 50 µM AsIII, and 5 µg of cyt19-WT, in 100 mM Tris/100 mM Na phosphate pH 7.4. with various [SAM].

A.



B.

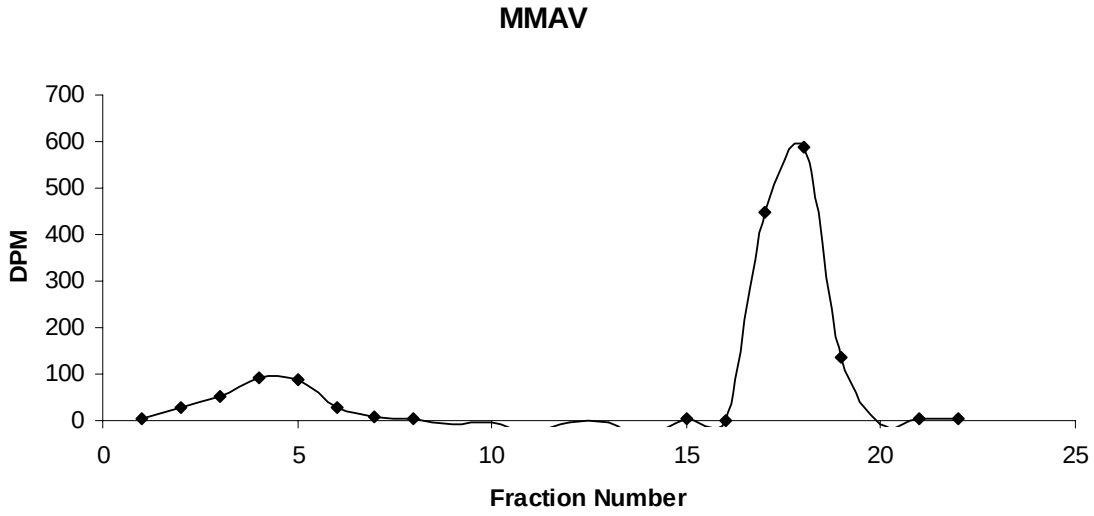


Figure 2-10. Arsenical metabolites formed after incubation with [^{3}H]SAM and cyt19 for 30 min at 37C. (A) Formation of MMA and DMA using As^{III} as a substrate. (B) Formation of MMA and DMA using MMA^{V} as the substrate.

CHAPTER 3
IDENTIFICATION OF A SPLICE VARIANT OF HUMAN CYT19 ARSENIC METHYLTRANSFERASE

Introduction

Arsenic (As) is a naturally occurring element and ranks 20th in abundance in the earth's crust [85]. Arsenic is present in the environment in both organic and inorganic forms and exists mainly in three valence states, -3, +3, and +5. Generally, inorganic arsenic (iAs) is the more toxic form and people are exposed to iAs primarily through food and potable water. In Taiwan, Mexico, western United States, western South America, China, and Bangladesh, people are exposed to high levels of arsenic due to anthropogenic and/or natural contamination of potable water [12, 62, 86]. In these areas, chronic exposure to arsenic is associated with various tumors occurring in skin, liver, lung, urinary bladder, and prostate [8, 87].

Once in the body, many mammals, including humans, methylate iAs to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) [49, 51, 54]. The biotransformation of iAs alternates between the reduction of arsenate (iAs^{V}) to arsenite (iAs^{III}) followed by oxidative methylation (Figure 1) [88, 89]. Because pentavalent methylated arsenicals are less toxic than inorganic arsenic, methylation has been considered a detoxification mechanism. Recent studies indicate that trivalent methylated arsenicals may be more acutely toxic and genotoxic than iAs suggesting that methylation may actually be a bioactivation of iAs [20, 73, 74, 90]. For this reason the role of methylation in acute and chronic arsenic toxicity remains unclear. There is significant

variation in human susceptibility to As induced toxicity, which may be related to differences in arsenic biotransformation between individuals [82]. Epidemiological studies have shown differences in the amount of MMA and DMA excreted in the urine of exposed populations which may be associated with genetic polymorphisms [62]. Cyt19 has been identified as a human S-adenosyl-L-methionine:arsenic methyltransferase [52, 55] however, only a few coding region polymorphisms have been detected which may alter the iAs methylation rate [65, 67]. In this study we identified an alternative splice variant of human cyt19, which contains an upstream open reading frame (uORF) followed by an internal start codon (AUG). This variant was expressed in 7 out of 7 human livers and represents another possible mechanism for regulating As methylation.

Materials and Methods

Molecular Cloning of cyt19 Splice Variants

Total RNA was isolated from HepG2 cells and human liver samples using Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, USA). Total RNA was treated with DNase I using the DNA-*free*™ kit from Ambion (Austin, TX) and cDNA was made using the RETROscript™ Kit for RT-PCR and 2 µg of RNA as template (Ambion). HepG2 cDNA was polymerase chain reaction (PCR)-amplified using the following primers: forward primer (5'-CACCATGGCTGCACTTCGTGACGCTGAGATACAG-3') and the reverse primer (5'-TTAACTCCAAAGCAGAACAGCTCCAGATGT-3'). The PCR reaction consisted of 2.5 U of Pfu DNA polymerase, 0.4 µM each primer, 5µl of the RT reaction, 0.2 mM dNTP mix, 5µl of 10X PCR Buffer, and nuclease-free water to 50µl. The PCR conditions were as follows: an initial denaturation at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min, extension for 72°C

for 2min 30s and a final extension at 72°C for 7min. The PCR products, designated cyt19 and cyt19ΔE2, were ligated into the pET100/D-TOPO vector (Invitrogen) and transformed into competent *Escherichia coli* (*E. coli*) One Shot TOP10 chemically competent cells (Invitrogen). Ampicillin resistant colonies were analyzed by PCR and visualized by agarose gel electrophoresis. Several clones containing inserts were sequenced by the DNA Sequencing Core Laboratory at the University of Florida.

Human Liver Samples

Human liver samples were obtained from Vitron (Tucson, AZ). All the tissues were from Caucasian males between the ages of 24 and 46. The tissues were preserved in Viaspan after death. The tissue samples were stored at -80°C until use. All procedures using human samples were approved by the Institutional Review Board at the University of Florida and all identifying information has been removed.

qPCR of cyt19 Splice Variants

Total RNA was isolated and cDNA synthesized as described above. Real-time quantitative PCR (qPCR) was carried out using a Bio-Rad iCycler with the following primers: cyt19 forward primer: (5'-TTCGTGACGCTGAGATACAGAAG-3'); reverse primer: (5'-TGGAGGTCTGCCGATCTCTT-3'); cyt19ΔE2 forward primer: (5'-GATACAGAAGGACGTGCAGATATTATG-3'); reverse primer: (5'-CCAGATCCAAAATCCAGCAGTT-3'). Each PCR reaction consisted of 12.5 µl iTaq SYBR Green Supermix with ROX (Bio-rad), 0.4 µM each primer, 5µl of the RT reaction, and RNase/DNase-free water to 25 µl. The PCR cycling conditions included an initial denaturation of 95°C for 3 min followed by cycling at 95°C for 15s, 60°C for 45s for 45 cycles. The constructs, pET100-cyt19 and pET100-cyt19ΔE2 were used to generate

calibration curves for quantification of cyt19 and cyt19ΔE2. A melting curve analysis was performed after every run to determine product uniformity.

Results

When the full open reading frame of cyt19 was amplified using the primers described in *Molecular Cloning of cyt19 splice variants* under Materials & Methods, two products were generated, 1132 bp (cyt19) and 1005 bp (cyt19ΔE2) products (Figure 3-2). Sequencing of these two products revealed that the 1132 bp product is the reference cyt19 while the 1005 bp product is a splice variant (Figure 3-3). The reference cyt19 mRNA is composed of 10 exons (Figure 3-4A) which encode a 375 amino acid protein with a theoretical molecular weight of 41.747 kDa (Figure 3-5). The product, cyt19ΔE2, is missing 128 bp due to a deletion of exon 2 which could result in a protein that is about 102 amino acids shorter and the creation of a short 24 amino acid peptide as a product from an upstream open reading frame (uORF) (Figure 3-3 & Figure 3-4B). This variant may encode a 273 amino acid polypeptide chain that is identical to the reference cyt19 but lacks the first 102 amino acids present in the reference (Figure 3-5). Analysis of the sequences surrounding the splice revealed that the splice occurs at conserved acceptor and donor sites (Figure 3-4C). The individual information (Ri) technique and Shapiro's method were used to compare the splice-sites strength of exon 3 and exon 2 of cyt19 [91]. Exon3, a constitutive exon has a stronger splice site compared to exon2, the alternative exon (Table 3-1).

The steady state mRNA levels of each transcript were determined in 7 human liver samples as well as in HepG2 cells by qPCR (Table 3-2). Expression of cyt19 mRNA in the human liver samples ranged from 7.04×10^{4} to 1.26×10^{6} copies per microgram of

RNA. The cyt19ΔE2 splice variant was detected in all 7 human liver samples tested. (Table 3-2). The amount of cyt19ΔE2 mRNA was much lower than cyt19 mRNA ranging from 1.89×10^{3} through 4.33×10^{3} copies per microgram of RNA. The HepG2 cells had an average of 5.14×10^{6} copies/μg RNA and 8.55×10^{4} copies/μg RNA of cyt19 and cyt19ΔE2 mRNA, respectively.

Discussion

In this study, we identified an alternative splice variant of cyt19, which contains an uORF. The variant mRNA contains a short ORF followed by an internal AUG codon beginning 106bp downstream from the uORF (Figure 3-3). While this alternative variant may encode a 273 amino acid protein it is unlikely that expression of the cyt19ΔE2 splice variant will result in production of an active protein. Studies have shown that SAM dependent methyltransferases share 3 regions of sequence similarity (motif I, II, and III) [92]. These motifs are found in the same order on the polypeptide chain and separated by similar intervals [92]**.** It has been suggested that these conserved regions are important in SAM binding [92]. Mutations of a conserved amino acid in rat guanidinoacetate methyltransferase near motif I have resulted in an inactive enzyme. In addition, mutations of motif II lead to reduced Kcat/Km values for substrates [93]. It is unlikely that the protein translated from cyt19ΔE2 would result in an active protein due to the removal of motif I (Figure 3-5).

Whether the mRNA actually is translated into protein is not clear because the internal AUG codon contains a relatively weak Kozak sequence suggesting that translation may not reinitiate at the internal start codon. The sequence (GCCA/GCCATGG) is a consensus Kozak sequence for the initiation of translation in

vertebrates [94, 95]. Deviation from the consensus sequence at position -3 and +4 would be considered a weak initiator codon. The cyt19ΔE2 transcript deviates from the consensus sequence at position +4; the variant contains an A instead of a G (Figure 3-3).

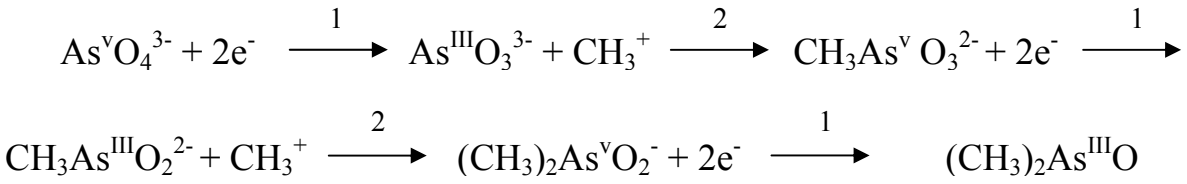
It is also possible that cyt19ΔE2 will not be translated but that this variant is a substrate for the nonsense mediated decay (NMD) pathway due to the premature stop codon. NMD is a pathway that recognizes and quickly degrades mRNAs containing premature translation termination codons (PTC) in eukaryotes [96]. While cyt19ΔE2 does contain a PTC, Zhang et al. identified a sequence motif which when present 3' of a nonsense codon promotes rapid decay of the mRNA transcript by the NMD pathway [97]. This sequence motif (TGYYGATGYYYYY) is not found in the cyt19ΔE2 mRNA transcript and it remains unclear if this variant will undergo degradation by the NMD pathway.

The cyt19ΔE2 variant was present in all seven human liver samples tested, suggesting that cyt19 mRNA exists both in the full length and alternatively spliced form in most individuals. The cyt19ΔE2 variant mRNA comprised 0.2 to 3.8% of the total cyt19 transcript. The liver samples had lower copy numbers per microgram of RNA of both reference and cyt19ΔE2 variants compared to HepG2 cells. It is possible that some degradation of cyt19 message occurred during collection and storage of the livers which reduced apparent copy number.

Many mammalian species methylate arsenic through an enzymatic reaction that is performed by cyt19. There are significant variations in the arsenic methylation capacity between species and within species including humans [58, 59, 62, 81]. The reason for this variation is unclear but has been attributed to cyt19 polymorphisms. However, only

a few polymorphisms have been found in the coding region of cyt19 to date [65, 67], while the vast majority of mutations are found within the introns and the 5' and 3' untranslated region (UTR). Introns contain the acceptor site, branchpoint, polypyrimidine tract, and the donor site, which are conserved sequences in which splicesome assembly occurs [70]. While mutations within these sequence elements could alter the constitutive splicing of a gene [98-100], there are differences in arsenic methylation capacities among individuals, which are unlikely to be supported solely by polymorphisms within the cyt19 coding region.

Alternative splicing is frequently used to regulate gene expression and to generate tissue-specific mRNA and protein isoforms [68, 69]. Thirty-five to 60% of human genes produce transcripts that are alternatively spliced, in addition 70-90% of these variants alter the resulting protein products [101, 102]. Further studies should analyze the mRNA expression levels of cyt19 splice variants in a larger number of fresh liver samples or primary hepatocytes and correlate it to arsenic methylation activity. In addition, work to determine if this transcript is a substrate for the NMD pathway or if a variant protein is expressed will help clarify the role of cyt19ΔE2 in human arsenic metabolism. Even though the splice variant comprises a relatively small fraction of the total cyt19 transcript in the livers tested it is possible that different population groups have varying amounts of the cyt19 splice variant. It is also likely that the level of cyt19ΔE2 in an individual will change over time as alternative splice selection can be controlled by many variables including developmental stage and xenobiotics [103, 104]. In conclusion, cyt19 appears to be alternatively spliced in many individuals and may play a role in the observed variation in arsenic methylation seen in individuals.



1 – Reduction step of As biotransformation
2 – Oxidative methylation step of As biotransformation

Figure 3-1. The hypothesized scheme of iAs methylation proposed by Cullen, McBride et al. 1984.

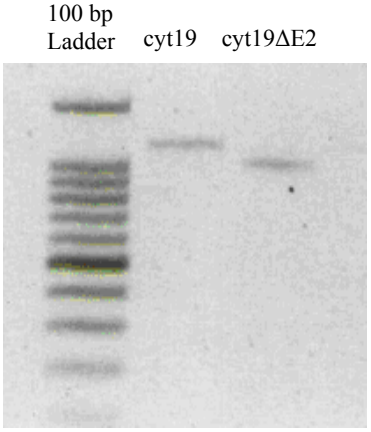


Figure 3-2. PCR products of cyt19 amplification. 1% Agarose DNA gel of cyt19 and cyt19ΔE2.

A.

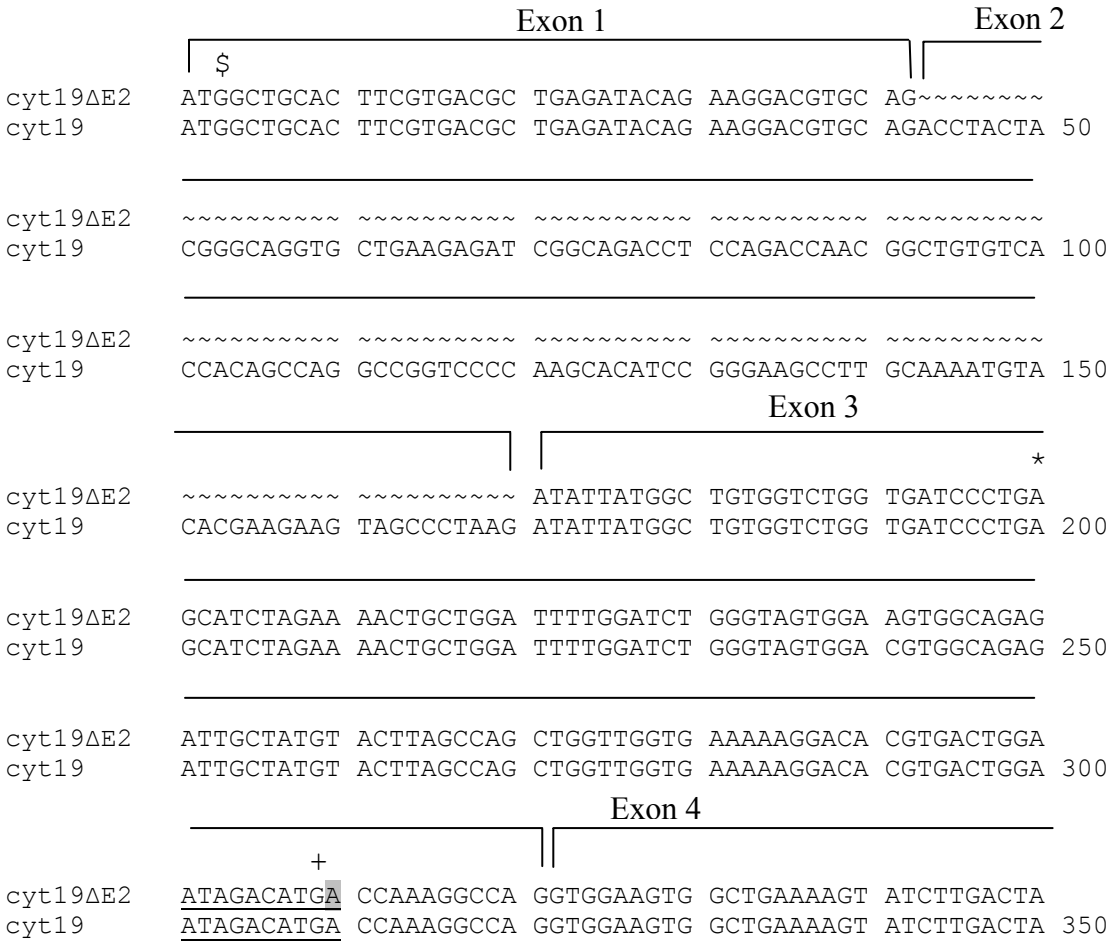


Figure 3-3. Alignment of the reference cyt19 nucleotide sequence and cyt19ΔE2. $ Represents the initial start codon. *Represents the putative PTC which results due to the removal of exon 2. + Represents the putative downstream start site. The kozak sequence is underlined. The deviation from the kozak sequence at position +4 is highlighted in grey.

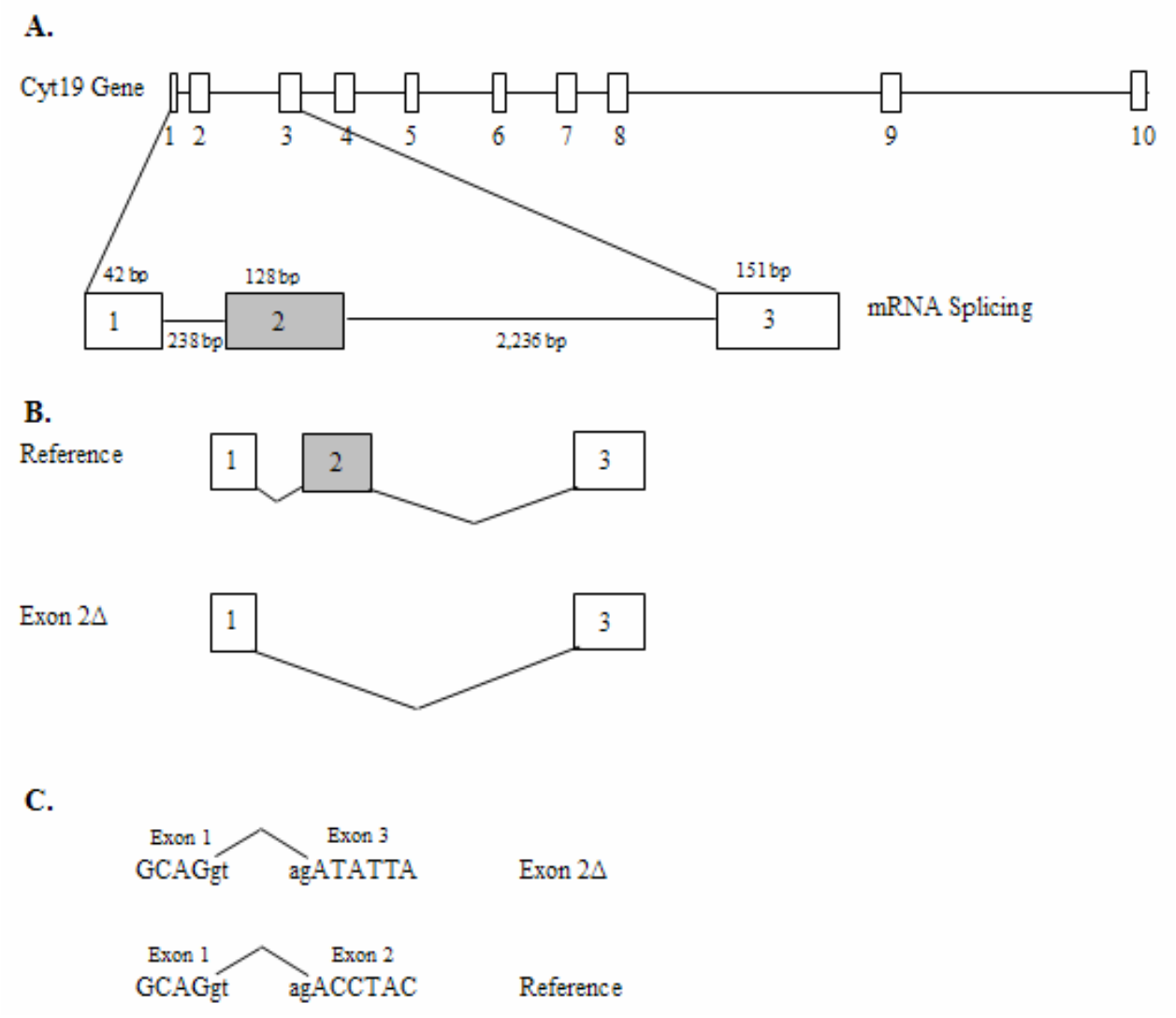


Figure 3-4. cyt19 isoforms. (A) Diagram representing the exonic regions of the wild-type cyt19 mRNA. The region in which the alternative splicing occurs is demonstrated in greater detail. (B) Schematic representation of the two alternative splice variants, the reference or wild-type sequence, and the deletion of exon 2. The shaded box represents the cassette exon. (C) The donor and acceptor site of the two alternative splice variants. The exonic nucleotides are capitalized while the acceptor and donor sites are in lower case letters.

```
           1                                                    50
cyt19      MAALRDAEIQ KDVQTYYGQV LKRSADLQTN GCVTTARPVP KHIREALQNV
cyt19ΔE2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

           51                                                   100
cyt19      HEEVALRYYG CGLVIPEHLE NCWILDLGSG SGRDCYVLSQ LVGEKGHVTG
cyt19ΔE2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

           101                                                  150
cyt19      IDMTKGQVEV AEKYLDYHME KYGFQASNVT FIHGYIEKLG EAGIKNESHD
cyt19ΔE2   ~~MTKGQVEV AEKYLDYHME KYGFQASNVT FIHGYIEKLG EAGIKNESHD

           151                                                  200
cyt19      IVVSNCVINL VPDKQQVLQE AYRVLKHGGE LYFSDVYTSL ELPEEIRTHK
cyt19ΔE2   IVVSNCVINL VPDKQQVLQE AYRVLKHGGE LYFSDVYTSL ELPEEIRTHK

           201                                                  250
cyt19      VLWGECLGGA LYWKELAVLA QKIGFCPPRL VTANLITIQN KELERVIGDC
cyt19ΔE2   VLWGECLGGA LYWKELAVLA QKIGFCPPRL VTANLITIQN KELERVIGDC

           251                                                  300
cyt19      RFVSATFRLF KHSKTGPTKR CQVIYNGGIT GHEKELMFDA NFTFKEGEIV
cyt19ΔE2   RFVSATFRLF KHSKTGPTKR CQVIYNGGIT GHEKELMFDA NFTFKEGEIV

           301                                                  350
cyt19      EVDEETAAIL KNSRFAQDFL IRPIGEKLPT SGGCSALELK DIITDPFKLA
cyt19ΔE2   EVDEETAAIL KNSRFAQDFL IRPIGEKLPT SGGCSALELK DIITDPFKLA

           351                       376
cyt19      EESDSMKSRC VPDAAGGCCG TKKSC*
cyt19ΔE2   EESDSMKSRC VPDAAGGCCG TKKSC*
```

Figure 3-5. Alignment of the reference cyt19 amino acid sequence and product of cyt19ΔE2 uORF. Consensus Motif I for SAM dependent methyltransferases is underlined.

Table 3-1. The individual information and Shapiro's score of cyt19 exon 2 and exon 3.

	Sequence	Splice site	Shapiro's method	Ri, bits
Exon 2[a]	GACGCTGGGTCAGA	Acceptor (-13 to +1)	61.5	-1.9
	AAGGTAGAGT	Donor (-3 to +7)	72.0	5.5
Exon 3	TTCCATTTCCCAGA	Acceptor (-13 to +1)	84.8	9.5
	CAGGTGAGGC	Donor (-3 to +7)	88.1	7.4

a. Exon 2 is the alternative exon of cyt19 and exon 3 is the constitutive exon.

Table 3-2. The amount of cyt19 and cyt19ΔE2 in different human liver samples and HepG2 cells. The percentage of cy19 ΔE2 in each sample.

Samples	cyt19 (copies/µg RNA)	cyt19ΔE2 (copies/µg RNA)	%cyt19ΔE2[a]
HL-541	$1.21\times10^{6}\pm0.49\times10^{6}$	$3.25\times10^{3}\pm1.14\times10^{3}$	0.27
HL-546	$1.22\times10^{5}\pm0.35\times10^{5}$	$3.55\times10^{3}\pm0.89\times10^{3}$	2.83
HL-611	$7.04\times10^{4}\pm3.91\times10^{4}$	$1.89\times10^{3}\pm1.45\times10^{3}$	2.61
HL-612	$7.89\times10^{5}\pm1.78\times10^{5}$	$3.63\times10^{3}\pm2.70\times10^{3}$	0.46
HL-656	$1.09\times10^{5}\pm0.44\times10^{5}$	$4.33\times10^{3}\pm0.69\times10^{3}$	3.84
HL-710	$8.40\times10^{5}\pm2.64\times10^{5}$	$3.95\times10^{3}\pm1.06\times10^{3}$	0.47
HL-714	$1.26\times10^{6}\pm0.42\times10^{6}$	$3.22\times10^{3}\pm1.23\times10^{3}$	0.26
HepG2 cells	$5.14\times10^{6}\pm1.08\times10^{6}$	$8.55\times10^{4}\pm6.42\times10^{4}$	1.64

a. The percentage of cyt19ΔE2 is calculated by dividing the copies/µg of RNA for each sample by the total copies of both transcripts for each sample (cyt19ΔE2/(cyt19 + cyt19ΔE2)*100).

CHAPTER 4
GENERAL CONCLUSIONS

The first study showed that cyt19 is an arsenic methyltransferase. In addition, we have demonstrated that this enzyme does both methylation steps involved in arsenic biotransformation seen in humans. We also showed that the C-terminus is critical in the activity of the protein. In other SAM-dependent methyltransferases, the C-terminus is important in substrate binding. This indicates that the cysteine rich C-terminus is important for As binding and critical for activity. Others have shown that mutations can change the methylation capacity of the protein. Here, we demonstrated that a single mutation can drastically change the activity of the protein even though we believed that the amino acid change would not have a significant effect on the activity of the recombinant protein. This change occurs in motif I of the SAM-binding site which might inhibit its ability to bind SAM therefore decreasing its activity. The mutation appears to cause a change in the substrate affinity of the proteins as well as cause different methylation profiles specifically for arsenite. However, the V_{max} values of both cyt19-WT and cyt19R81S are considerably higher than that seen among other mammals such as the hamster, rabbit, and rhesus monkey. The kinetic analysis of these proteins may explain the high levels of MMA excreted in human urine. Possibly, arsenite is converted very quickly to MMA, allowing it to accumulate before the dimethylation step resulting in the higher excretion of MMA seen in humans. The human arsenic methyltransferase did have some similarities with the other mammalian arsenic methyltransferases. These arsenic methyltransferases have been shown to increase in activity at basic pHs which

may be due to the deprotonation of cysteines at higher pHs, which increases the rate of binding between arsenic and cysteines in the substrate binding domain. Our results demonstrate that only a strong reductant is necessary for methylation of arsenic by cyt19, however, the addition of GSH appears to increase the activity above the reductant alone.

The second study introduced another possible explanation for the variability in arsenic methylation capacities among individuals, alternative splicing. Alternative splicing is frequently used to regulate gene expression and to generate tissue-specific mRNA and protein isoforms. Thirty-five to 60% of human genes produce transcripts that are alternatively spliced, in addition 70-90% of these variants alter the resulting protein products. In this study we identified an alternative splice variant of the human cyt19 (cyt19ΔE2), in which exon 2 is removed creating a bicistronic transcript. The cyt19ΔE2 variant was present in all seven human liver samples tested, suggesting that cyt19 mRNA exists both in the full length and in alternatively spliced forms in most individuals. It is unlikely that this variant would result in expression of an active protein. Studies have shown that SAM dependent methyltransferases share 3 regions of sequence similarity (motif I, II, and III). It has been suggested that these conserved regions are important in SAM binding. Therefore, it is unlikely that the protein translated from cyt19ΔE2 would result in an active protein due to the removal of exon 2 which cotains motif I. Whether the mRNA actually is translated into protein has not been determined. The majority of mutations discovered within the cyt19 gene occur within the intron or untranslated regions [66, 67]. In fact, only 4 mutations have been found within the coding region. Introns contain sequence elements in which splicesome assembly occurs [66, 67]. Mutations within these elements could alter the constitutive splicing of a gene.

Future studies should focus on isolating the human AS3MT from human livers. To this date, preparations from human livers, cytosolic or homogenates have shown no activity in arsenic methylation. Reasons for this may be due to inhibitory factors present in the liver preparation or the process of making the preparations may render the protein inactive. Yet another explanation may be due to the possibility that cyt19 is an inducible protein. Perhaps the average daily exposure does not cause high levels of expression of the protein, making it difficult to purify from liver samples. It may be beneficial to attempt protein purification from known higher than normal arsenic exposed populations. Perhaps populations exposed to higher than normal levels have reached a threshold of exposure resulting in higher expression levels of cyt19. Another possibility for purification of cyt19 from normally exposed populations may be by immunoprecipitation from human liver preparations using an antibody which is highly selective for cyt19.

Further studies should analyze the mRNA expression levels of cyt19 splice variants in a larger number of fresh liver samples or primary hepatocytes and correlate it to arsenic methylation activity. In addition, work to determine if this transcript is a substrate for the NMD pathway or if a variant protein is expressed will help clarify the role of cyt19ΔE2 in human arsenic metabolism. In addition, more investigations should look at mutations within the intron of cyt19 and determine if these alter splicing events. Another important study would be to establish a method to test exposed populations at both the mRNA and protein levels of cyt19. This study would help determine vulnerable individuals in high arsenic exposed populations. Finally, the ultimate question which still remains unanswered: is arsenic methylation a bioactivation or detoxification mechanism.

If time and funding were not a factor, I would enjoy working on solving these different unanswered questions which may help identifying possible vulnerable populations.

APPENDIX
ROLE OF CYT19 IN ACUTE ARSENIC TOXICITY. IS CYT19 THE ONLY HUMAN ARSENIC METHYLTRANSFERASE?

While my work has clearly demonstrated that cyt19 is a human arsenic methyltransferase, it is not clear that it is the only arsenic methyltransferase in humans. In order to address this question, it is necessary to: 1) alter the expression of cyt19 and 2) have the ability to measure cyt19 protein levels. The first challenge was addressed by the use of small interfering RNA (siRNA) to reduce the expression of cyt19. The second challenge was addressed by developing an antibody specific for human cyt19.

Small RNAs can theoretically be used to reduce the expression of any target gene. There are two main categories of small RNA, microRNA (miRNA) and siRNA. These small RNA have natural functions such as defense from viral and transposon invasion as well as gene regulation. Scientists have used this new technology for several reasons such as determination of gene function, validating drug targets, and treatment of diseases. Small RNAs have two mechanisms by which protein translation is inhibited. If the small RNA is 100% homologous to its mRNA target it results in degradation of the mRNA. However, if the small RNA is not 100% identical to its mRNA target it results in inhibition of translation without mRNA degradation [105].

Materials and Methods

Multiple siRNAs were designed and synthesized according to the *Silencer*™ siRNA Construction Kit (Ambion). Forty-five thousand HepG2 cells were plated on a 24 well plate in normal growth media overnight and transfected in duplicates with the

appropriate siRNA according to *Silencer*™ siRNA Trasnfection Kit. Total RNA was isolated 2 days after transfection. The most efficient siRNA was assessed by measuring the mRNA levels of cyt19 normalized to RNA polymerase II (RPII) by quantitative PCR (qPCR). Once the most efficient siRNA is determined, further optimization steps can be taken such as cell plating density, transfection agent, and siRNA amount. The siRNA pool was further optimized in a 96-well plate using 16,000 cells per well and assayed. The mRNA levels were measured by qPCR as above, and the viability was determined by the XTT assay. GAPDH was used as negative control.

An antibody to cyt19 was developed in rabbits by sending purified recombinant cyt19 to Cocalico. The antibody specificity was determined from western blots of purified protein and HepG2 cell extracts. To increase the specificity of the antibody, it was affinity purified using the AminoLink® Kit (Pierce) according the manufacturer's instructions. Briefly, the purified cyt19 protein was coupled to the gel followed by affinity purification.

Results and Discussion

cyt19 mRNA Knockdown by siRNA

Three different siRNAs were designed (Table 1). The efficiency of all three siRNAs as well as a pool of the three siRNAs was determined (Figure 1). The data demonstrates that the knockdown was successful with the pool of siRNAs, however further optimization is still required. It appears that the first three siRNAs had no effect on the cyt19 mRNA levels. The pool of all three siRNAs appears to knockdown cyt19 mRNA levels by about 26%, relative to control. The second attempt at cyt19 mRNA knockdown demonstrated that 16,000 cells per well in a 96-well plate resulted in a greater knockdown of cyt19 mRNA of about 40% compared to 45,000 cells used in the

first attempt (Figure 2). It appears that cyt19 mRNA levels might have been slightly reduced in the negative control. In addition, the siRNA transfection did not result in a decrease of viability relative to control (Figure 3). The data demonstrates that the optimal cell density and the transfection agent are important to optimize. Taken together the results suggest that the concentration of the siRNA requires further optimization or perhaps new siRNAs can be designed and tested.

Antibody Specificity and Purification

Crude antiserum was shown to be able to detect the antigen up to about 25 ng (Figure 4). The antibody was not able to detect cyt19 in any of the human liver cytosol preparations tested. In addition, the antibody appears not to be very specific (Figure 5). Once purified, the antibody was characterized and found to be able to detect the antigen up to about 40 ng (Figure 6). The specificity of the antibody was increased dramatically and is able to detect cyt19 in all tested human liver samples and in HepG2 cytosol tested (Figure 7). In order to determine if the antibody is in fact recognizing cyt19 in these cytosol preparations, the protein must be immunoprecipitated and sequenced.

Future Experiments

Further studies are needed in order to better understand the role of arsenic methylation in acute human arsenic exposures. However, two very important steps in answering this question have been addressed, knockdown of cyt19 and the ability to measure cyt19 protein levels through antibody specificity. We have to determine the correlation between mRNA levels and protein levels. It may be possible to knockdown the mRNA levels without affecting the protein levels if the protein has a long half-life. The opposite may be true as well, we may not see much affect in the mRNA levels but the siRNA may be able to suppress protein expression thereby reducing methylation.

Once we prove the protein levels are down, we can determine the effects of As exposure as well as determine if cyt19 is the only arsenic methyltransferase in humans.

Table A-1. Sequence of double stranded siRNA

siRNA 1: 5’-CAU UGA GAA GUU GGC AGA GUU-3’ 3’-UU GUA ACU CUU CAA CCG UCU C-5’
siRNA 2: 5’-UGU GAC UUU UUU CCA UGG CUU-3’ 3’-UU ACA CUC AAA AAA GGU ACC G-5’
siRNA3: 5’-GUU GGC AGA GGC UGG AAU CUU-3’ 3’-UU CAA CCG UCU CCG ACC UUA G-5’

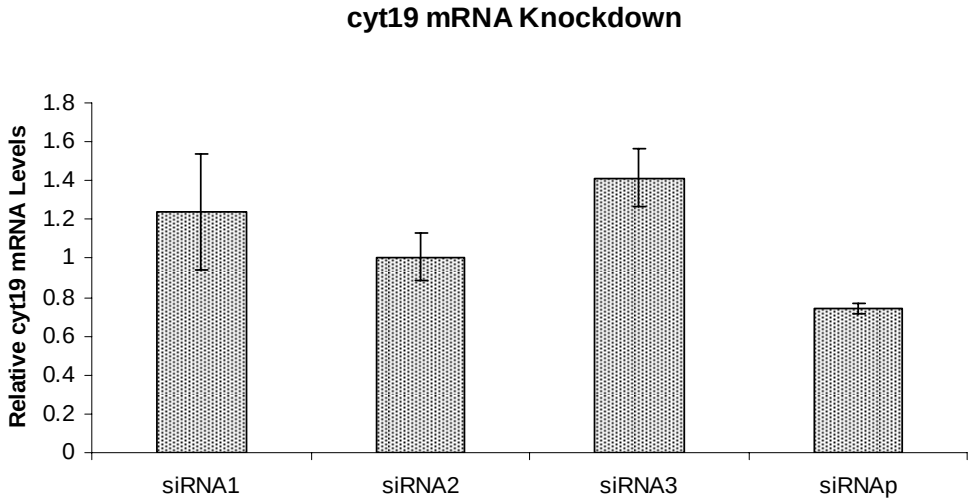


Figure A-1. Determination of the most efficient siRNA in the knockdown of cyt19 mRNA levels assayed by qPCR.

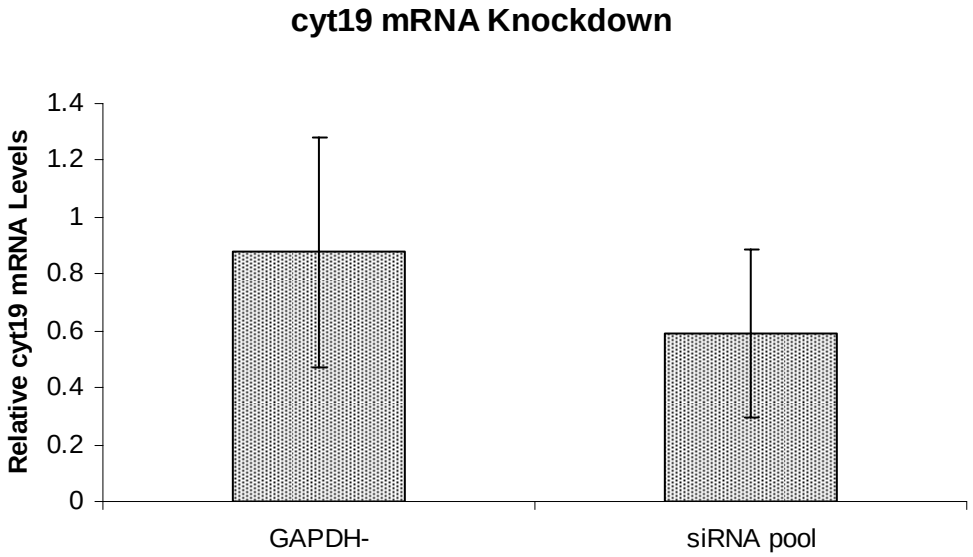


Figure A-2. The siRNA pool knockdown of cyt19 mRNA relative to control.

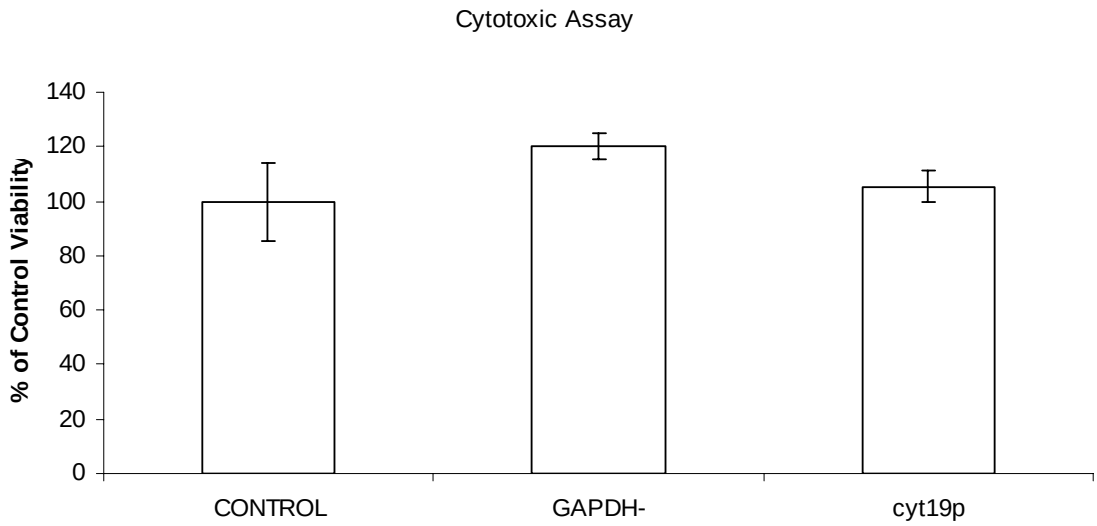


Figure A-3. Determination of cell viability after siRNA knockdown by the XTT assay.

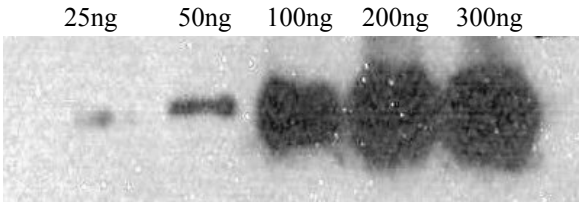


Figure A-4. Western blot of the specificity of the crude antisera to the antigen, purified cyt19 protein.

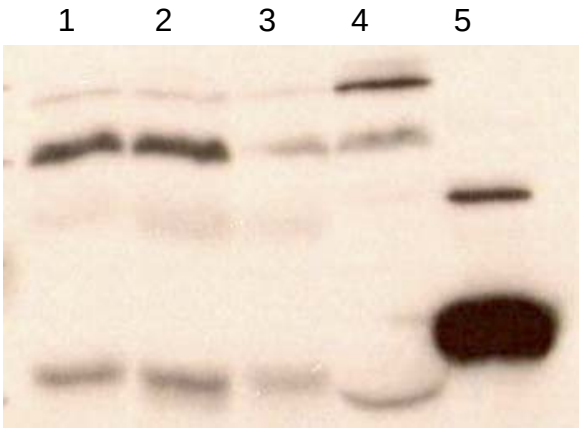


Figure A-5. Western blot of the specificity of the crude antisera to cyt19 in human liver cytosolic preparations. 1) HL-93-F6; 2) HL-93-F7; 3) HL-94-F4; 4) HL-97-21; 5) purified cyt19.

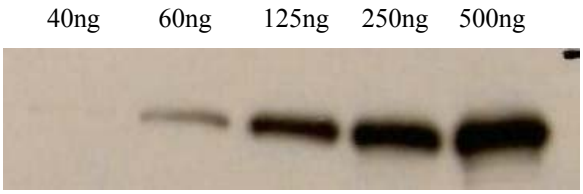


Figure A-6. Western blot of the specificity of the purified antibody to the antigen, purified cyt19 protein.

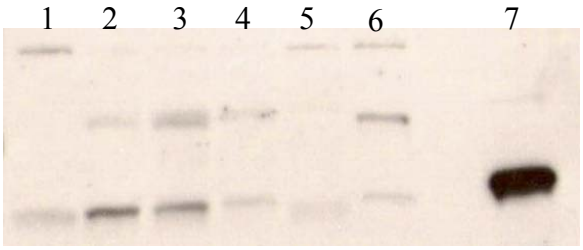


Figure A-7. Western blot of the specificity of the crude antisera to cyt19 in human liver cytosolic preparations. 1) HL-97-21; 2) HL-H-F6; 3) HL-93-F7; 4) HL-94-F4; 5) HL-714 6) HeG2 cytosol 7) purified cyt19.

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BIOGRAPHICAL SKETCH

Alex J. McNally is the youngest son of Eduaurdo and Ligia McNally, who immigrated from Managua, Nicaragua. He was born on April 6, 1980, in Miami, FL. He was raised in Hialeah, FL, where he attended North Miami Senior High and graduated with honors on May 1998. He then attended the University of Florida where he dualed major in animal science and microbiology and cell science. He further enhanced his undergraduate experience working as a student lab assistant at the University of Florida microbiology and cell science building. He worked with cloning arogenate dehydrogenase from Arabidopsis thaliana under the guidance of Dr. Carol Bonner and Dr. Nemat Keyhani. After earning his Bachelor of Science, on August 2003, Alex continued his graduate studies at the University of Florida, pursuing a Master of Science specializing in toxicology in May of 2004. He emphasized his graduate research on the characterization of cyt19, an arsenic methyltransferase, under the guidance of Dr. David S. Barber. He now plans to pursue a career in a research laboratory where he can enhance his knowledge and practical experience.